MOLECULAR MECHANISMS UNDERLYING CANNABIS ABUSE AND SCHIZOPHRENIA: FOCUS ON 5-HT2A RECEPTORS AND AKT/MTOR SIGNALING PATHWAY

Inés Ibarra Lecue

DOCTORAL THESIS

2020

eman ta zabal zazu

Universidad del País Vasco

Euskal Herriko Unibertsitatea

"I didn't want to just know names of things. I remember really wanting to know how it all worked."

Elizabeth Blackburn

AGRADECIMIENTOS / ACKNOWLEDGEMENTS

Ha llegado el momento de comenzar a escribir estas líneas. Y es que, aunque a veces parezca que no... todo llega. El trabajo que ha conformado lo que ahora es mi Tesis no habría sido posible sin la confianza y la ayuda que me han brindado muchas personas a lo largo de esta etapa. Tengo la impresión de que esta parte de las Tesis es, además de la más personal, la más leída... espero estar a la altura de las expectativas.

A estas alturas de la película... trataré de ser breve. No porque quiera evitar que el lomo de este libro siga engordando (que sí), ni porque me falte inspiración a estas alturas (que también); sino porque espero de todo corazón haber sido capaz de agradeceros en su momento a todos aquellos que, en uno u otro momento, y de uno u otro modo, me habéis apoyado a lo largo de todos estos años. Todo el mundo busca su nombre en estas líneas... y yo no soy quién para quitar a nadie esa ilusión, así que no innovaré en tan exquisita tradición.

Creo sinceramente que elegir embarcarse en una Tesis no es algo casual. En mi caso, no tengo dudas de que sin el apoyo de mis padres y de mis hermanos, ejemplo absoluto de dedicación y esfuerzo, constancia y sacrificio, además de firmes defensores de las cosas bien hechas, no estaría escribiendo estas líneas. A ellos les debo más que a nadie haber llegado hasta aquí. Gracias, os quiero infinito❤️.

Dra. Urigüen. Leyre. ¡Qué puedo decir! No creo que nadie me pudiera haber "engañado" a meterme en esto tan bien como lo hiciste tú. Mi opinión es que fue amor a primera vista (no sé qué opinas tú...). Siendo una desconocida, tú me cogiste del brazo y te plantaste donde Javier para decirle que yo merecía la pena. Sin que yo te hubiera demostrado nada. Creer en las personas como lo haces tú es una virtud que no se ve a menudo. Dejando de lado tu sobrado talento investigador, tu calidad humana hace que me sienta tremendamente afortunada de haberte tenido a mi lado🤗. Hemos formado un equipo estupendo. ¡Ojalá el positivismo de millones de Leyres por todos lados! Gracias por todo, de corazón.

Javier, te agradezco mucho tu confianza puesta en mí. Admiro tu esfuerzo incansable por tratar de hacer de todos nosotros unos científicos mejores, críticos y "sabidos". Gracias por haberme dado la oportunidad de trabajar en este magnífico equipo, del que espero haber estado a la altura... Muchas gracias por todo lo que me has enseñado, que créeme, es mucho.

Y este es tu turno Koldo. Tú también me viste con buenos ojos cuando mi única carta de presentación era una nota de expediente. (Mala). ¿Será porque soy de Bilbao? 🤫En cualquier caso, gracias por tus palmadas en la espalda en los momentos adecuados. También por tu franqueza cuando ha tocado. Te agradezco tu esfuerzo junto con Leyre en que me forme en aspectos "fuera de poyata": docencia, escritura, cursos... y la ya querida (¿podremos decir dominada?) divulgación. Siento que todo esto ha hecho de mí una científica más equilibrada y eso en parte es gracias a ti. Gracias por luchar por mí y por mi futuro como lo has hecho y sigues haciendo. Y por todos los: "si no fuera por estos momentos..." vividos. Me ha encantado ser tu "pequeño saltamontes".

Tengo que seguir contigo, Rebe. Porque lo mío contigo es ✨Absoluta Admiración✨, y te lo tengo que decir así de claro y así de fuerte. Diría que me identifico mucho contigo en muchas cosas... pero te mereces más justicia que la que te hace esa frase. Eres de lo mejorcito que me he topado en mi vida. Ok, dirás que no son muchos años, pero me atrevo a decir que personas como tú no se encuentran fácil (ojalá me equivocara). Y no es sólo porque hayas sido las manos detrás de muchos de los resultados de esta Tesis. No se te puede agradecer suficiente todo lo que has hecho y haces por mí y por todos en este laboratorio, todos los días. No quiero suscitar envidias en esta sección... así que confío en que te conformes con un GRACIAS, POR TODO. ¡Raulete! ¡No me olvido de ti! Muchísimas gracias por estar presente en todos los altibajos de esta etapa, por tu sentido del humor y optimismo incondicional. Y, por qué no decirlo, por el bendito whatsapp de malignos. ¡Por muchos momentos más de vernos las caras!

¡Evita! Tú me empezaste a enseñar lo que es trabajar en un laboratorio y lo que supone hacerlo en un grupo. Desde que me asignaste aquella mesa (aquella época en la que había hueco… qué tiempos aquellos ¿eh?), todo han sido buenas palabras y comprensión. Incluso cuando las aguas no han estado todo lo tranquilas que nos gustarían… siempre has tenido guardado un "mi puerta está siempre abierta" para mi. Gracias a ti todos esperamos que llegue junio y, con él, la más querida tradición Pharmateam, la Fiesta de las Flores. Gracias por acompañarme en este camino.

Empezando a dudar de la lógica que guarda el orden de estos agradecimientos… seguiré con el equipo "fuga de cerebros". Itzi, Patri, Aintzane… vosotras mirabais con asombro al moco que teníais en el *txokito* y esas gráficas que traía cuando me ponía a hacer los deberes de biocatálisis de la carrera. Poco a poco empecé a ocupar espacio en la poyata, y desde entonces, de vosotras sólo recuerdo consejos, apoyo y sonrisas. Ahora que me encuentro en una situación parecida a la que vivíais entonces vosotras, me doy cuenta del esfuerzo que supone a veces motivar a las nuevas generaciones; y os lo agradezco infinito. El equipo estupendo que formabais me convenció definitivamente a quedarme en el labo. Guardo especial cariño del viaje a Londres… y sus innumerables anécdotas. Patri, gracias a ti, y no al premio, guardo un recuerdo tremendamente tierno de mi viaje fugaz a Estocolmo. Por la sensación que supone "abrirnos en canal" de vez en cuando… ¡Gracias!

Repaso las puertas del departamento y me viene a la cabeza la única mente alejada (por suerte) de la vorágine de locura que es la ciencia, ¡Coradita! Tenemos mucha suerte de tener una secre tan sumamente eficiente. No hay asunto que no seas capaz de resolver en menos de 48h… ¡y siempre con una sonrisa de oreja a oreja! Es un placer tenerte cerca y compartir todos esos cafés contigo. Muchas gracias por sacarme una sonrisa en los momentos *chof*.

¡Ay, Igortxu! Tus vaciles me hacen quererte cada día más. Tú y yo sabemos que hubo conexión, no lo niegues (Román, *take it easy*). Me encanta la manera que tienes de demostrarme que me quieres (porque me quieres, ¿a que sí?), y contigo he vivido grandes momentos estelares – cuarteto Jorge-Igor-Irene-Inés en León… *the last survivors;* tu despedida de soltero vestido con aquel infame disfraz de pájaro…–, no te puedo explicar con palabras lo especial que me hiciste sentir aquella alubiada en tu baserri, con aquellas palabras en aquel "vamos a por pitis" que acabó en chupito y alguna que otra cosa más… Gracias, de verdad. Aunque te rías mientras me ves morir en el gym…

Ane, agradezco infinito tus palabras de cariño, tu disposición absoluta siempre que lo he necesitado y tu saber escuchar… no sabes la ayuda que brindas con tus palabras y gestos. Eres un ejemplo de fortaleza y valentía, ojalá pueda parecerme más a ti en un futuro. Espero que el tuyo te traiga todas las cosas buenas que tantísimo mereces.

Guada, ¿eres posiblemente una de las mentes más brillantes del laboratorio?, yo así lo creo. Tus opiniones y comentarios sobre ciencia siempre se me han quedado revoloteando la cabeza… supongo que eso es que me inspiras, ¿no? Tienes una personalidad desbordante, y un sentido del humor que envidio. Ay, ¡ojalá trabajar con gente como tú a mi lado siempre!. Muchas gracias por aquella lección de ChIP y por aconsejarme en las dudas que te he planteado en todo este tiempo. Amaia, ¡no sabes lo que se agradecen tus palabras de cariño y ánimo, siempre en el momento adecuado!, envidio tu empatía y deseo de todo corazón que consigas todos y cada uno de los objetivos que te plantees en la vida. Sigue luchando por la buena ciencia como lo haces, yo intentaré hacer lo mismo. Carol, muchísimas gracias por cuidar de mi en mis primeros pasos en el mundillo cannabinoide, empezando por el congreso en Sestri y el workshop en Pozzuoli, en el que me presentaste al Team Marsicano y cuidaste de mi como si fuese tu hermanita pequeña, y siguiendo por Canarias y demás SEIC… Gracias también por aquella boda de la que sólo tengo buenos recuerdos. Si en ciencia eres mi ejemplo a seguir, como persona vales aún más si cabe. Muchísimo ánimo en tus primeros pasos como mentora oficial, ¡estoy segura de que lo harás genial! Y sigo con Alfredo, el fichaje estelar emigrado a las Américas y aterrizado como noble Cajal. ¡Cuánto me has ayudado en tan poco tiempo! Me atrevo a decir que supones ya alguien fundamental en este labo, ¡y 1000% merecido! No sólo sabes un montón, sino que encima desprendes motivación y ganas de enseñar y transmitir. Alucino

con tu disposición, de la que espero haber aprendido en este tiempo. Siempre tienes un buen consejo o una buena broma en la boca. Tu nariz, aunque no sea fuente envidiable de células (no te rayes), fue la primera con la que pudimos contar. ¡Qué suerte tenemos de tenerte entre nosotros! Gracias por todo. Jorge, Don Risas y Bromas, gracias por los momentos estelares arriba mencionados, por lo bien que "quitas hierro al asunto" siempre y los buenos momentos *out-of-work* en general. ¡Te mereces todo lo bueno que te venga! Olga, muchas gracias por todos tus consejos cuando los he necesitado, por aquella inolvidable noche en León, y por aquella vuelta en blablacar que supuso días y días de risas.

Ay, chiquis, que os toca a vosotras... ¡mis Ladies del txokito! Empezando por ti, Blanca. Eres un ejemplo de tenacidad y constancia. Gracias por tus "parrafadas" inesperadas de whatsapp y por tener las palabras adecuadas cuando hacían falta. Cuando te regales la autoestima que te mereces, no habrá alma que evite que consigas todo cuanto te propongas. Te deseo lo mejor en tu nueva etapa en US. Iria, galleguiña mía, ¿quién me iba a decir que con el tiempo iba a conseguir la fuerza suficiente para soportar tus borderías? 😜 Te has convertido en alguien esencial para mi en esta etapa. Debajo de esas frases cortantes que tanto te caracterizan, se esconden un corazón enorme y un talento brutal. La fiel confidente de mis secretos... me siento muy afortunada de haberte conocido. ¡Porque el tiempo no nos separe! Itzi, ¡mi compi getxotarra! Muchas gracias por tu saber escuchar y por dejarte aconsejar como lo haces. Tus frikeces científicas hacen las delicias de todos los que te rodean. Sigue con esas ganas que tienes de aprender y de crecer como científica, y conseguirás lo que te propongas, no me cabe duda. Natalia, alias Greta. Gracias por esas charlas durante las comidas, por ser tan empática y por contagiarme tu pasión por la ciencia cuando a mi me ha faltado, ¡porque la pana nunca muera! Amaia (no tan) txiki, muchas gracias porque irradias motivación por aprender, y eso contagia. Vales muchísimo, así que perservera y verás que todo lo bueno llega solo. Teresa, nuestra mañica preferida. Muchísimas gracias por soportar mis/nuestros lloros en general, aunque no tengas ni media gana... y por supuesto, gracias por dejarme disfrutar de tus super25 y de ese finde lleno de risas y de gente espectacular. Marta, he echado mucho de menos tu sentido del humor en esta última etapa que se ha hecho tan cuesta arriba... gracias por tu apoyo cuando me ha hecho falta, y por esa manera tan optimista de ver la vida de la que espero haber aprendido algo. ¡Me alegro de tenerte ya de vuelta! Y esto va para todas: no puedo agradeceros suficiente lo que me habéis apoyado estos últimos meses. Gracias por las palabras de apoyo y comprensión en los momentos duros, por sacarme del zulo y por valorarme como lo hacéis. Con vuestras ganas y disposición absoluta, habéis supuesto el empujón que necesitaba para llegar al final viva☺️. ¡Os quiero mucho!

A las nuevas generaciones: Paula, Laura y Amaia. El Team neuroepitelio. Definitivamente, ¡Leyre tiene ojo para los fichajes! Sois un encanto y desprendéis ganas y más ganas de aprender. Gracias por dejaros enseñar y por vuestra disposición para absolutamente todo (buceo entre arcones, entre otras cosas). Paula, gracias por ayudarme durante aquellas interminables tardes midiendo proteínas a ritmo de reggaetón 🎶... Os dejo en buenas manos, ¡os irá genial! Inés, Jon y Oihane: aunque no hayamos coincidido mucho por el labo, os deseo todo lo mejor en esta nueva etapa, estoy segura de que lo haréis genial.

Y dando paso a los que ya no están... ¡Saruki! Aquel año de máster no habría sido lo mismo sin ti. Gracias por todas las juergas, por Barcelona y por hacerme un hueco siempre que voy para allí. ¡Tus aventuras nunca dejan de sorprenderme! 🙈 Gracias también a Virginia y a Kizkitza, por dejaros guiar y por vuestra entrega. Fue un placer trabajar codo con codo con vosotras.

Y, por supuesto, muchas gracias también a todas las personas del Departamento de Farmacología, en especial a los predocs: Mario, Elena, Sergio, Lorena, Unai. Por todos los *afterwork* y las sesiones de terapia 💣. Gracias también a Tere, por las charletas de tarde y obligarme a irme a casa tantas veces.

Irene, chati. Quizás te esperabas unas líneas en el sector "fugadas", pero tú y yo sabemos que te mereces párrafo aparte. Porque el destino quiso que nos diéramos una oportunidad como algo más que compis de labo, y por lo que ha valido la pena. Porque me acuerdo de ti todos los días y te echo de menos lo que no está escrito. Viviste conmigo la mejor etapa de mi vida y eso no lo olvidaré jamás,

y me soportaste en todos mis momentos *irascible-on*. Pero sobretodo, me has apoyado en los momentos duros que he vivido estos años. Me has enseñado tantas cosas... me vienen a la cabeza millones de momentos que hemos compartido y que darían para otra tesis, gracias por todos y cada uno de ellos. Siento no ser tan cariñosa y detallista como lo eres tú y no hacerte ver lo mucho que te echo de menos todo lo que debería. Espero seguir aprendiendo de ti siempre. *See you soon in London!*

Chris and Cathy, thank you so much for giving me the amazing opportunity to be part of your research group. You were totally supportive and pushed me to make the most of my stay in UCLA. I learned so much from you in a very short time, and because of you, I lived one of the best experience of my life, so thank you so much! I don't know if I will ever meet again a boss who lends me a surfboard... I will try my best though! Polly, thanks to you I was able to go to US, so I can't thank you enough. Amie, Lindsay, Josh, Alex, Steve, Suchi, you made my stay incredibly easy and fun, and you were always there for helping me with the experiments. I wish I was there to repeat that Korean BBQ...!

The perfect experience that was Los Angeles would not have been the same without you, Persian princes. Seena and Shahrad, I cannot explain how fortunate Irene and I were for getting to know you... it was a miracle! Although you were constantly making laugh about our *LA opposite* way of living (no car, no fitness, no organic food), we all know that we created a bond that is gonna last forever... thanks for crossing my way, and for appreciating that much my PhD work. Fardad, thanks for making me laugh that much with your silly things haha you'll be the cooler doctor in the world! French family: Vince, Louis, Raffoo, Hélène... thank you for all the good moments! I hope we'll see each other soon.

Muchas gracias también a Patricia, por acogerme tan bien aquellas dos semanas que pasé en el IMIM. En especial, gracias a Carla y a Cris, que hicieron de mi mini-estancia una experiencia increíblemente enriquecedora, en lo profesional y en lo personal.

Y ahora sí, toca agradecer a todas esas personas que, sin ser parte del trabajo, me han ayudado a lo largo de estos años hasta llegar a donde estoy. A mis clásicas: Melen, Mery, Paula... gracias porque cuando nos vemos, todo sigue igual. Porque siempre me habéis apoyado y hecho desconectar en mis etapas más flojas... os quiero infinito. A todos mis primos y tíos, que tanto me valoráis y os preocupáis siempre por mí y por cómo me va. Me sacáis los colores cuando me decís que estáis orgullosos de mi... y soy yo la que está tremendamente orgullosa de teneros como familia. Si no fuera porque no os dejo, muchos os vendríais a verme defender la tesis... En general, a todos los amigos y familia (Anita, ¡aquí estás tú!) que habéis estado dispuestos desde el momento cero a donar sangre para que la niña haga sus experimentos de la tesis. No os puedo nombrar a todos porque sois un montón... pero os lo agradezco de todo corazón.

No puedo agradecer suficiente a quien más ha tenido que soportarme en los peores momentos de esta etapa... Chati, ese eres tú. Me has enseñado a ser fuerte como nunca creí que podía serlo. De ti he aprendido a relativizar mis "males", a valorarme sin sentirme culpable y a dar lo mejor de mí, pase lo que pase. No podría contar las veces que has tenido que sufrir mis frustraciones... Estoy segura que no habría llegado aquí sin ti, así que te lo debo todo... gracias siempre.

Y termino estos agradecimientos como empecé, con mi familia. Papi, mami, Yeyi, Bo, Hay... ¡lo he conseguido! Sólo viendo vuestras caras cuando me felicitáis por ello, sé que ha merecido la pena. Gracias por soportarme, por haber estado ahí incondicionalmente, a las duras y a las maduras. Os lo debo todo. Extiendo estas líneas a mi familia 2, Txelis, Bego, Javichín, Aneto, gracias por vuestro apoyo y vuestro cariño durante todos estos años.

Esta Tesis es tan vuestra como mía.

Gracias, de todo corazón.

A mis Hermanos,

de sangre y “ficticios”.

This Doctoral Thesis has been carried out thanks to the financial support of several entities and subsidized projects:

- Departamento de Educación del Gobierno Vasco. Ayuda predoctoral de Formación de Personal Investigador no Doctor (2016 - 2020)
- Instituto de Salud Carlos III, Centro de Investigación Biomédica en Red de Salud Mental, CIBERSAM
- Ministerio de Economía, Industria y Competitividad (PI13/01529)
- Departamento de Educación del Gobierno Vasco (IT616/13)

This Doctoral Thesis has resulted in the publication of the following scientific articles:

1. Diez-Alarcia R, Ibarra-Lecue I, Lopez-Cardona ÁP, Meana JJ, Gutierrez-Adán A, Callado LF, Agirregoitia E, Urigüen L. Biased Agonism of Three Different Cannabinoid Receptor Agonists in Mouse Brain Cortex. *Front Pharmacol.* 2016, 7: 415.

2. Ibarra-Lecue I, Mollinedo-Gajate I, Meana JJ, Callado LF, Diez-Alarcia R, Urigüen L. Chronic cannabis promotes pro-hallucinogenic signaling of 5-HT2A receptors through Akt/mTOR pathway. *Neuropsychopharmacology.* 2018, 43(10): 2028-2035.

3. Ibarra-Lecue I, Diez-Alarcia R, Morentin B, Meana JJ, Callado LF, Urigüen L. Ribosomal protein S6 hypofunction in postmortem human brain links mTORC1-dependent signaling and schizophrenia. *Front Pharmacol.* 2020, 11: 344.

4. Ibarra-Lecue I, Pilar-Cuéllar F, Muguruza C, Florensa-Zanuy E, Díaz Á, Urigüen L, Castro E, Pazos A, Callado LF. The endocannabinoid system in mental disorders: Evidence from human brain studies. *Biochem Pharmacol.* 2018, 157: 97-107.

1., **2.** and **3.** Manuscripts are included in **Results** section; **4.** Manuscript is included in **Annex** section.

The specific contributions of the co-authors for the scientific articles were (II-L underlined):

1. LFC, JJM, and LU designed the study. ÁPL-C, AG-A and EA stablished and maintained mutant mice colonies. RD-A performed SPA experiments. II-L performed western blot assays. JJM, LFC, RD-A, and LU analyzed and interpreted the results. RD-A and LU drafted the manuscript. All the contributors revised critically and gave their approval to the final version of the manuscript.

2. LU, RD-A and LFC designed experiments. Chronic treatments were performed by II-L. Behavioral experiments were performed by II-L and IM-G with assistance from LU. Biochemical assays were performed by II-L and RD-A. II-L, LU, and JJM wrote the manuscript. All the contributors revised critically and gave their approval to the final version of the manuscript.

3. LU and LFC designed experiments. BM, LFC, and JJM obtained and classified postmortem human brain samples. Western blot assays were performed by II-L and RD-A. LU and II-L analyzed data and wrote the manuscript. All the contributors revised critically and gave their approval to the final version of the manuscript.

4. AP and LFC conceived the study. FP-C, EF-Z, ÁD and EC reviewed literature regarding emotional homeostasis, depression, and anxiety related disorders. II-L, CM and LU reviewed literature regarding schizophrenia. LU, EC, AP and LFC wrote the introduction and conclusions of the manuscript. All the contributors revised critically and gave their approval to the final version of the manuscript.

ABBREVIATION LIST

1-AG:	1-arachidonoylglycerol
2-AG:	2-arachidonoylglycerol
4E-BP:	Eukaryotic translation initiation factor 4E-binding protein
5-HT:	5-hydroxytryptamine / serotonin
5-HT1AR:	Serotonin 1A receptor
5-HT2AR:	Serotonin 2A receptor
5-HIAA:	5-hydroxyindoleacetic acid
AA:	Arachidonic acid
ABHD6:	α/β-hydrolase domain 6 / monoacylglycerol lipase
ABHD12:	α/β-hydrolase domain 12 / lysophosphatidylserine lipase
ADP:	Adenosine diphosphate
AEA:	N-arachidonoylethanolamine / anandamide
AMPAR:	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP-F:	Antipsychotic-free
AP-T:	Antipsychotic-treated
APA:	American Psychiatric Association
Arc:	Activity-regulated cytoskeleton-associated protein
ARF1:	Adenosine diphosphate-ribosylation factor 1
BC:	Before Christ
BDII:	Binocular depth inversion illusion
BOLD:	Blood-oxygen-level-dependent imaging
Ca^{2+}:	Calcium
cAMP:	Cyclic adenosine monophosphate
CB1R:	Cannabinoid type 1 receptor
CB2R:	Cannabinoid type 2 receptor
CBD:	Cannabidiol
CBG:	Cannabigerol

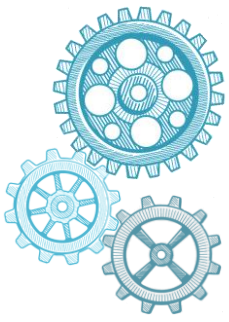
CBN:	Cannabinol
CNS:	Central nervous system
CNV:	Copy number variation
COX-2:	Cyclooxygenase -2
D2R:	Dopamine D2 receptor
DA:	Dopamine
DAG:	Diacylglycerol
DAGL:	Diacylglycerol lipase
DLPFC:	Dorsolateral prefrontal cortex
DOI:	2,5-dimethoxy-4-iodoamphetamine
DSM:	Diagnostic and Statistical Manual of Mental Disorders
DTI:	Diffusion tensor imaging
ECA:	Epidemiologic Catchment Area
eIF-4E:	Eukaryotic translation initiation factor 4E
ERK:	Extracellular signal–regulated kinase
FEP:	Fist episode psychosis
FAAH:	Fatty acid amide hydrolase
fMRI:	Functional magnetic resonance imaging
FMRP:	Fragile X mental retardation protein
GABA:	γ-aminobutyric acid
GAD:	Glutamate decarboxylase enzyme
GIRK:	G protein-coupled inwardly-rectifying potassium channel
GPCR:	G protein-coupled receptor
GPR55:	G protein-coupled receptor 55
GWAS:	Genome-wide association study
hiPSC:	Human induced pluripotent stem cells
i.p.:	Intraperitoneally
ICD:	International Statistical Classification of Diseases and Related Health Problems

INIA:	Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria
IP3:	Inositol trisphosphate
K^+:	Potassium
KO:	Knock-out
LSD:	Lysergic acid diethylamide
MAM:	Methylazoxymethanol acetate
MAO:	Monoamine oxidase
MAO-B:	Monoamine oxidase B
MAPK:	Mitogen-activated protein kinase
MGL:	Monoacylglycerol lipase
MHC:	Major histocompatibility complex
mTOR:	Mechanistic target of rapamycin
mTORC1:	mTOR complex 1
mTORC2:	mTOR complex 2
MRI:	Magnetic resonance imaging
mRNA:	Messenger ribonucleic acid
NAAA:	N-acylethanolamine acid amide hydrolase
NAPE:	N- acylphosphatidylethanolamine
NAPE-PLD:	N- acylphosphatidylethanolamine-specific phospholipase D
NCAM1:	Neural cell adhesion molecule 1
NGS:	Next generation sequencing
NMDAR:	N-methyl-d-aspartate receptor
NO:	Nitric oxide
OEA:	Oleoylethanolamine
OR:	Odds ratio
PBMC:	Peripheral blood mononuclear cell
PCP:	Phencyclidine
PDK1:	Phosphoinositide-dependent protein kinase 1
PEA:	Palmitoylethanolamide

PET:	Positron-emission tomography
PFC:	Prefrontal cortex
PI3K:	Phosphoinositide 3-kinase
PIP2:	Phosphatidylinosito-4,5-bisphosphate
PIP3:	Phosphatidylinositol-3,4,5-trisphosphate
PKA:	Protein kinase A
PKC:	Protein kinase C
PLA2:	Phospholipase A2
PLC:	Phospholipase C
PLD:	Phospholipase D
PMI:	*Postmortem* interval
Poly(I:C):	Polyinosinic:polycytidylic acid
PPARγ:	Peroxisome proliferator-activated receptor γ
PPI:	Prepulse inhibition
PRAS40:	Proline-rich Akt substrate of 40 kDa
PSD:	Postsynaptic density
PSD-95:	Postsynaptic density protein 95
RhoA:	Ras homolog family member A
RT:	Room temperature
rpS6:	Ribosomal protein S6
S6K:	p70 ribosomal protein S6 kinase
S6K2:	Ribosomal protein S6 kinase 2
SDS-PAGE:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEA:	Stearoyl ethanolamide
SERT:	Serotonin transporter
SNP:	Single-nucleotide polymorphism
SPA:	Scintillation proximity assay
SPECT:	Single-photon emission computed tomography
SZ:	Schizophrenia subjects

TBS:	Tris-buffered saline
THC:	Δ^9-tetrahydrocannabinol
THCV:	Tetrahydrocannabivarin
TRPV1:	Transient receptor potential voltage channel 1
TSC1/2:	Tuberous sclerosis complex
UNODC:	United Nations Office on Drugs and Crime
US:	United States
USA:	United States of America
WHO:	World Health Organization

INDEX



INTRODUCTION

1. SCHIZOPHRENIA

1.1. GENERAL ASPECTS

Schizophrenia is a chronic and disabling mental disorder that is characterized by a decreased ability to perceive reality. The illness profoundly disrupts individual ability to think clearly, manage emotions, make decisions and interact with other people. It is regarded as the most severe and disabling psychiatric disorder, affecting more than 20 million people worldwide (Charlson et al., 2018).

Lifetime prevalence of schizophrenia is estimated between 0.3-0.7% of the population worldwide (McGrath et al., 2008) and it is associated with significant health, social, and economic concerns (Whiteford et al., 2013), such as premature mortality, disproportionately high financial costs in terms of health care, loss of productivity and social service needs. These, among other factors, make schizophrenia one of the top 20 leading causes of disease-related disability worldwide, being also the seventh most costly medical illness in our society (Freedman, 2003; James et al., 2018).

The average age of onset is in the late teens to the early twenties for men, and the late twenties to early thirties for women (Üçok et al., 2012; Immonen et al., 2017).

The characteristic symptoms of schizophrenia fall into three dimensions:

- Positive symptoms refer to hallucinations, delusions, disorganized speech or disordered thinking. This cluster of symptoms is also referred as psychosis.

- Negative symptoms refer to absent or diminished abilities, such as anhedonia (inability to experience pleasure from positive stimuli), alogia (decrease in verbal output or expressiveness), affective flattening (lack of facial and emotional expression), or avolition (reduction of self-initiated and purposeful acts, difficulties following through with commitments).

- Cognitive symptoms include memory problems, difficulties with focus, attention and making decisions, and deficits in working memory.

Psychotic symptoms are usually episodic over time, and their emergence or worsening often requires temporary hospitalization. Meanwhile, negative and cognitive symptoms tend to be more stable over time, and contribute significantly to functional impairment. Moreover, psychotic symptoms of schizophrenia are treatment responsive, while current pharmacological treatment is practically ineffective on negative and cognitive symptoms.

Clinical diagnosis of schizophrenia is based on criteria of the Diagnostic and Statistical Manual of Mental Disorders (DSM) from the American Psychiatric Association (APA) or of the International Statistical Classification of Diseases and Related Health Problems (ICD) from the World Health Organization (WHO).

The latter version of the DSM, that is the DSM-V, was published on 2013. Diagnostic criteria of the DSM includes that the presence of two or more of the characteristic symptoms (hallucinations, delusions, disorganized speech, disorganized/catatonic behavior or negative symptoms) should persist for at least a month. One of them must also be one of the three first ones (hallucinations, delusions or disorganized speech, known as core positive symptoms). Moreover, at least one of the signs of the disorder must be present for a period of 6 months; and social, occupational or self-care dysfunction due to the disturbance must be evident. DSM-V included some innovations compared with the previous version, such as the removal of subtype classifications of schizophrenia (paranoid, disorganized, catatonic, undifferentiated, and residual). Similarly, the major criteria for schizophrenia diagnosis in the last version of ICD, that is ICD-10, published in 1992, are persistent hallucinations, delusions or thought disturbances during at least one month.

The disorder usually has a gradual, insidious onset that takes place over about 5 years, beginning with the emergence of negative symptoms followed shortly by cognitive and social impairment. In this first stage of the disorder, called the prodromal period, some first signs can appear (social isolation, unusual thoughts and suspicions, change of friends, academic failure, sleep alterations, irritability...) (see **Figure 1**). Yet these symptoms are common and

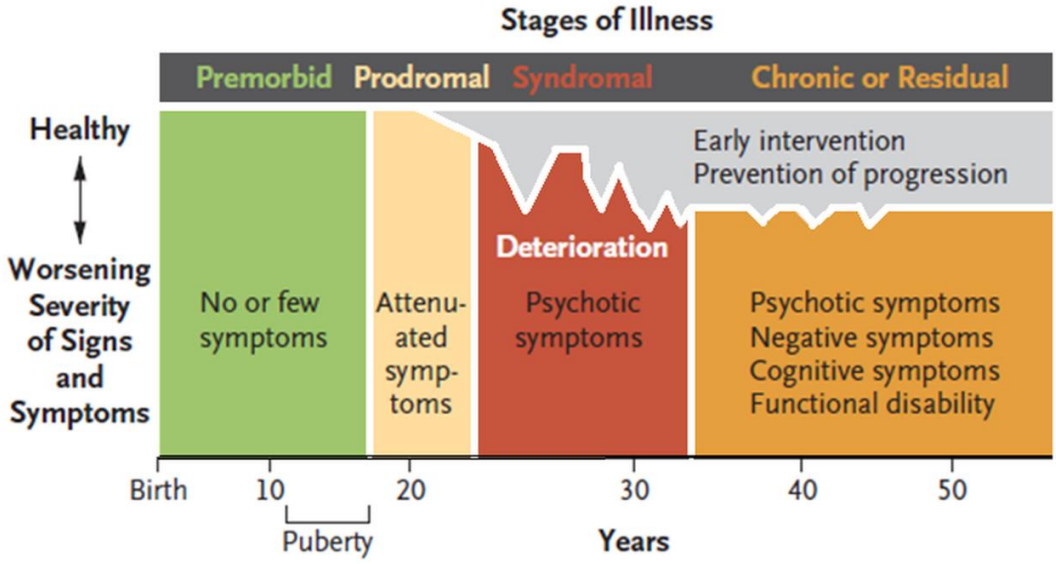


Figure 1. Stages of illness in schizophrenia. The syndromic stage begins with the first episode of psychosis and continues through the progressive stage. After the onset of the first episode of psychosis, decline in functioning leads to the chronic effects of the disease. Adapted from Lieberman & First, 2018.

nonspecific, they are not diagnostic, although neither are they typical of the mentally healthy state of the individual. This period is then followed by the emergence of psychotic symptoms and first psychiatric contact (Häfner et al., 1999). This debut in positive symptoms is known as First Episode Psychosis (FEP).

1.2. AETIOLOGY AND PATHOGENESIS

Emil Kraepelin (1850–1929) proposed that psychiatric disorders including the so-called Dementia praecox (now known as schizophrenia), were ''natural disease units''. Since then, accurately assign patients to these natural disease units, often based on course and outcome, became doubtful. However, he has remained as the founder of current psychiatric research, as we use categorical diagnoses to study the genetics, pathology, and treatment of psychiatric disorders.

What causes schizophrenia – aetiology – and how it develops – pathogenesis - are questions that have occupied the minds of every psychiatric researcher for over a century. These crucial aspects have stimulated successive cycles of proof and disproof, and excited much controversy. However, a wealth of evidence has accumulated in the recent decades, leading to a virtual universal acceptance of schizophrenia as a complex neurodevelopmental disease (Selemon and Zecevic, 2015).

One of the few features that are certainly accepted about schizophrenia – as well as for any other psychiatric disorder – is that it cannot be attributed solely to one factor. Instead, both genetic and environmental factors are known to take part in the onset and development of the illness (van Os et al., 2010; Sullivan et al., 2012). Each of them has to combine necessarily with others in order to develop the disease, and individually accounts to a minimum extent of the disorder variance. Nevertheless, none of them is either necessary or sufficient in schizophrenia.

Current knowledge about schizophrenia has pointed to genetics as the undoubtedly main factor in the aetiology of the disease. Recent studies estimate a proportion of variance explained by additive genetic effects around 80% (Hilker et al., 2018; Sullivan et al., 2003). This variance is explained by thousands of genetic loci with minimal prevalence differences from healthy people, most of which have not been discovered yet.

Despite this genetic association, the identification of specific molecular or structural variation has not been easy. Lack of clear *postmortem* differences in the brains of subjects with schizophrenia was likely one of the most puzzling scenario back in time. Modification of diagnostic criteria along the years has also hampered the progress in the knowledge of the basis of the disease. Nowadays, however, the development of more sensitive techniques is

demonstrating subtle but perceivable variances that offer more clues to the disorder pathophysiology.

Neuroimaging research in twins and first-degree relatives of patients has shown some heritable traits that underlie the illness. These include grey matter volume reductions in hippocampus, cortex (mainly frontal and, specifically, dorsolateral prefrontal), caudate nucleus or thalamus (Boos et al., 2007; Goldman et al., 2008), as well as ventricular enlargement (Kempton et al., 2010), and abnormalities in late brain maturation, with a disrupted trajectory of volume change with age (van Haren et al., 2008). A 18 years longitudinal study has also shown a progressive brain grey and white matter decrease, as well as cerebrospinal fluid increase in schizophrenia, being more severe during the early stages and correlating mainly with cognitive impairment (Andreasen et al., 2011). Ventricles enlargement and gray matter reduction seen to continue over time, probably contributing to the pathogenesis of the chronic disease (Olabi et al., 2011).

Functional brain imaging studies point to a prefrontal and temporal defective connectivity (Mwansisya et al., 2017). Moreover, some *postmortem* histological studies have reported cortical cytoarchitecture abnormalities, suggesting a defective neuronal migration during early developmental stage that could lead to an abnormal neuronal connectivity and circuitry postulated to underlie the illness (Akbarian et al., 1993). Reduced spine densities (Glantz and Lewis, 2000), smaller dendritic arbors and reduced neuropil on the pyramidal cells of the prefrontal cortex (PFC) (Selemon and Zecevic, 2015) also point to an aberrant pruning in schizophrenia (McGlashan, 2000). Apart from brain morphologic studies, neurophysiological studies have consistently disclosed alterations such as abnormal auditory P300, P50 amplitudes or prepulse inhibition (PPI) deficits (Keshavan et al., 2008).

According to the neurodevelopmental hypothesis (see **Figure 2**), this defective neural circuitry is then vulnerable to dysfunction when unmasked by certain developmental processes, and the exposure to stressors or drugs as the individual moves through the age of risk (Selemon and Zecevic, 2015).

Moreover, several neurotransmitter systems and circuits within the brain seem to be affected in patients with schizophrenia. These observations have been replicated along the years and have led to several hypotheses of schizophrenia etiopathogenesis, which will be presented in the **1.2.3** section.

This overall view offers only some clues about this complex disease. Knowing the molecular aetiological mechanism of schizophrenia is critical for drug discovery, as well as for stratification of the patients and the improving of pharmacological treatment.

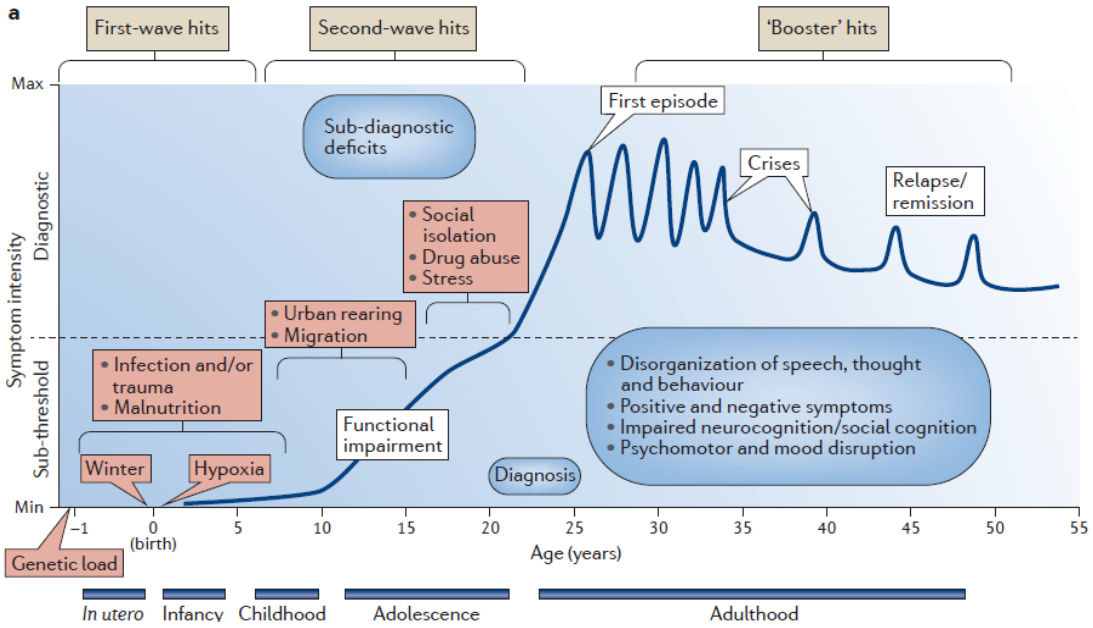


Figure 2. Scheme of the onset and progression of schizophrenia in relation to risk factors. Taken from Millan et al., 2016.

1.2.1. Genetics

The genetic component of schizophrenia was historically supposed due to its tendency to run in families, and schizophrenia disease has been shown to present higher heritability than other psychiatric diseases (Sullivan et al., 2012). The studies in families, twin and adoption carried out in the 80s and 90s provided increasing proofs that genetics plays a major aetiological role, and became the main foundation for the search of genetic risk factors (Frangos et al., 1985; Kety, 1987; Onstad et al., 1991; Tienari, 1991; Kendler and Diehl, 1993).

Classical twin studies are based on a simple theory: differences between a disease incidence in monozygotic twins are attributed to the environment, while differences between dizygotic twins to both hereditary and environmental factors. The first case reports related to schizophrenia in twins date from the 30s (Luxenburger, 1928; Rosanoff et al., 1934). However, twin studies in schizophrenia are recognized to be pioneered by Dr. Irving Gottesman. He evidenced that the risk for schizophrenia and related disorders are similar for the offspring of both the unaffected and the affected monozygotic twins. This fact supported the notions that carrying a heritable genetic vulnerability for schizophrenia is not sufficient for expressing the disease, and that non-shared environment contributes to the multifactorial aetiology of schizophrenia (Gottesman and Shields, 1972; Gottesman, 1989). Studies with adoptees, which investigated similarities with their biological and adoptive parents, as well as studies with adoptees' relatives also pointed to a major heritable genetic effect, discarding shared environmental effect explanation for the familiarity (Heston, 1966; Kety, 1994).

Large-scale studies estimate a concordance rate - the probability that a second twin will develop a disorder if the first examined twin has the disorder - of schizophrenia for monozygotic twins of around 40%. Thus, heritability - the proportion of variance explained by additive genetic effects – has been estimated in around 80% (Sullivan et al., 2003; Hilker et al., 2018), indicating that inherited genetics have a substantial influence on the risk for the disorder.

The first large-scale common single-nucleotide polymorphisms (SNPs) association study in schizophrenia was published in 2006 (Mah et al., 2006). Since then, large genome-wide association studies (GWAS) of thousands of SNPs, demonstrated that schizophrenia is significantly associated with a substantial number of common variants of small effect size (also known as 'common disease-common variant' model). Meanwhile, genome-wide copy number variations (CNV) studies have also demonstrated that some rare, highly penetrating CNVs – 22q11.2, 1q21.1 and 15q13.3 deletions, to name some – can play an important role in schizophrenia susceptibility (also known as 'common disease-rare variant' model) (Bassett et al., 2010).

Later GWAS (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Li et al., 2017), large-scale analyses of CNV (Marshall et al., 2017), and Next Generation Sequencing (NGS) studies (Fromer et al., 2014; Singh et al., 2016) suggest the implication of a number of genes involved in several neural functions, such as synaptic plasticity, neurogenesis and glutamatergic signaling.

The most recent GWAS in schizophrenia performed a meta-analysis of around 41,000 cases and 65,000 controls (Pardiñas et al., 2018) and identified 145 risk loci, adding 50 new loci to the largest report. The study showed an association of brain-relevant functional gene sets involved in synaptic networks, neurogenesis and cortical development, glutamate ion channels or abnormal long-term potentiation. Some of the most replicated gene associations involve postsynaptic density (PSD) proteins, activity-regulated cytoskeleton-associated protein (Arc), N-methyl-D-aspartate receptor (NMDAR), Fragile X mental retardation protein (FMRP) targets, voltage-gated calcium (Ca^{2+}) channels and neural cell adhesion molecule 1 (NCAM1).

This hypothesis-free approach has also provided surprising candidate mechanisms involving immunological pathways and major histocompatibility complex (MHC), neuronal Ca^{2+} signaling or miR-137 targets. Last studies using potent novel bioinformatics tools to integrate multi-omics and GWAS information to fine-tune data are providing high-confidence risk genes and advancing our understanding of schizophrenia aetiology (Wang et al., 2019).

Recently, the first genetic study with African population was carried out (Gulsuner et al., 2020). This novel work found that damaging gene variants – mutations that disrupt proteins – were more prevalent in subjects with schizophrenia and were concentrated in genes highly expressed in the brain and involved in the functioning of synapses.

Unfortunately, while scientific community has advanced in gaining insight into the genetic architecture of schizophrenia, it is estimated that a considerable proportion of heritability (about two thirds) is yet to be explained (Gershon et al., 2011; Foley et al., 2017). These studies are now opening the door to the possibility of clinical diagnostic subdivides of schizophrenia based on both the genotype and the phenotype.

1.2.2. Environmental factors

As stated before, environment unequivocally plays a role in the aetiopathogenesis of schizophrenia. Over the past decades, substantial evidence implicates environmental factors in the development of the disease. The general belief and more certainly proven fact is that some environmental factors can trigger the disease in people who already have a genetic predisposition, also known as 'stress-vulnerability' model. This genetic predisposition thus operates by making individuals selectively vulnerable for environmental risks (gene–environment interaction, or GxE). An extensive review on the environmental influence on schizophrenia was carried out some years ago (van Os et al., 2010), and a recent study has demonstrated the additive interaction between genetic risk for schizophrenia and several environmental exposures (Guloksuz et al., 2019). The environmental factors that have been associated most frequently with schizophrenia are the following:

Urbanicity

Urbanicity refers to the presence of conditions that are particular to urban areas or present more largely than in nonurban areas at a given time. Numerous studies have consistently reported an increased incidence of schizophrenia in urban areas across countries and cultures, especially when focusing on urban upbringing and showing a dose-response relationship, leading to an odds ratio (OR) of about 2 (Pedersen, 2001; Sundquist et al., 2004), and suggesting that the association may be causal (Krabbendam, 2005). Urbanicity seems to act synergistically with genetic liability (van Os et al., 2004) and, while studies addressing urbanicity lack of description consensus, it has been proposed that the social aspects of urbanicity may account for the major extent of the risk (March et al., 2008).

Migration and minority group position

Well-established evidence shows that some visible immigrant minority groups have a higher risk of developing psychotic disorders than the native-born individuals (Dykxhoorn et al., 2019). Moreover, refugees have an even higher risk of schizophrenia and other non-affective

psychotic disorders than non-refugee migrants from similar regions of origin (Hollander et al., 2016). The experience of traumatic incidents in this particular subgroup of immigrants is thought to be underlying this phenomenon (Abbott, 2016). Moreover, the increased risk of schizophrenia and related disorders among immigrants clearly persists into the second generation (Bourque et al., 2011). It has been proposed that the higher psychosis risk for certain migrant or ethnic minority groups is due to the chronic social adversity and discrimination (Morgan et al., 2010). This chronic social adversity results in a state of social 'defeat' (chronic experience of an inferior position or social exclusion) (Selten and Cantor-Graae, 2005), as it also applies to internal migrants and ethnic minorities without migration history, and not when living in a high own-group ethnic density area during puberty. These facts suggest that it is not the ethnic group *per se* that increases the risk, but rather the degree to which one stands out in relation to the wider social environment and find himself in a stressful outsider position (van der Ven and Selten, 2018).

The association between both urbanicity and migration may indicate a common environmental influence of chronic social disadvantage and isolation (McGrath et al., 2004) on schizophrenia etiopathogenesis, although further work is needed for the identification of specific mechanisms underlying the proxy risk factors.

Prenatal stressful environmental exposures and obstetric complications

A wide variety of environmental exposures in the mother during the first trimester of pregnancy (from the death of a first degree relative to earthquakes) has been reported to increase the risk for adult psychotic outcomes (Khashan et al., 2008; Procopio, 2008; Guo et al., 2019). Moreover, it has been also suggested that prenatal and perinatal events — including maternal viral infections, prenatal nutritional deficiency, rh incompatibility, or pregnancy and birth complications —increase the risk for psychosis. A meta-analysis of the prospective population-based studies (Cannon et al., 2002) revealed that some obstetrics complications, when pooled, might account for a relatively small proportion of incidence of schizophrenia (effect sizes around 2). However, history of obstetric complications was found to be associated with increased risk of transition to schizophrenia (OR around 6) in "at risk" individuals (Kotlicka-Antczak et al., 2018), pointing again to a gen-environment interaction. Studies addressing specifically prenatal infections and schizophrenia have moved from ecologic designs based on epidemics in populations, to investigations based on reliable biomarkers in individual pregnancies, and have targeted specific infections – influenza, *Toxoplasma gondii* – as schizophrenia risk factors (Brown and Derkits, 2010).

Early childhood trauma and adversity

A meta-analysis including prospective cohort studies, cross-sectional and case-control studies revealed that childhood adversities are associated with an increased risk for

developing psychosis (OR around 2.8). When adversities were subdivided, emotional abuse was the one that increased most the risk (OR around 3.4) (Varese et al., 2012). It has been also reported that childhood abuse increases the risk for psychotic symptoms in adulthood in a dose–response fashion (increasing from 2 to 48 times more likely in people who had experienced child abuse of increasing severity) (Janssen et al., 2004). An extensive review concluded that childhood abuse and neglect are related to some symptoms of schizophrenia, specifically hallucinations (Read et al., 2005). Moreover, childhood trauma has been related with less insight and poorer outcome in schizophrenia patients (Pignon et al., 2019). Interestingly, a study has shown a large shared effect of adversities including sexual, physical, emotional abuse, physical and emotional neglect, separation and institutionalization on the risk of psychosis, suggesting a common mechanism underlying traumatization (Trauelsen et al., 2015).

Cannabis use

Various lines of evidence point to associations between cannabis use and psychosis and suggest that early chronic exposure to cannabis is associated with a higher risk for psychotic outcomes, including schizophrenia in later life. This association is still a subject of study nowadays (Gage, 2019) and, as it supposes the main topic of this Doctoral Thesis, it will be thoroughly addressed in an independent section (Section **3**).

1.2.3. Neurotransmission systems

A major proportion of the studies in schizophrenia has been directed towards understanding the involvement of the different neurotransmitter systems in the pathology. Indeed, it was mainly through neuropharmacological observations using psychoactive drugs that upheld the formulation of hypotheses on the pathology of schizophrenia (Steeds et al., 2015). These hypotheses have implicated different neurotransmission systems, such as the dopaminergic, serotonergic, glutamatergic, or γ-aminobutyric acid (GABA)ergic systems in the pathology of schizophrenia, (see **Figure 3**).

Dopamine

The dopaminergic hypothesis has been the most enduring one to explain the aetiology of schizophrenia. This should not be surprising, as since the discovery of chlorpromazine on the 50s, all the antipsychotic drugs available on the market target this system. This hypothesis is also the most thoroughly studied, and perhaps the one most commonly accepted.

Few years after the introduction of chlorpromazine, which is considered the first antipsychotic, DA receptor blockade was presumed to be the basis of the antipsychotic effects of this drug and the recently discovered at that time haloperidol (Carlsson and

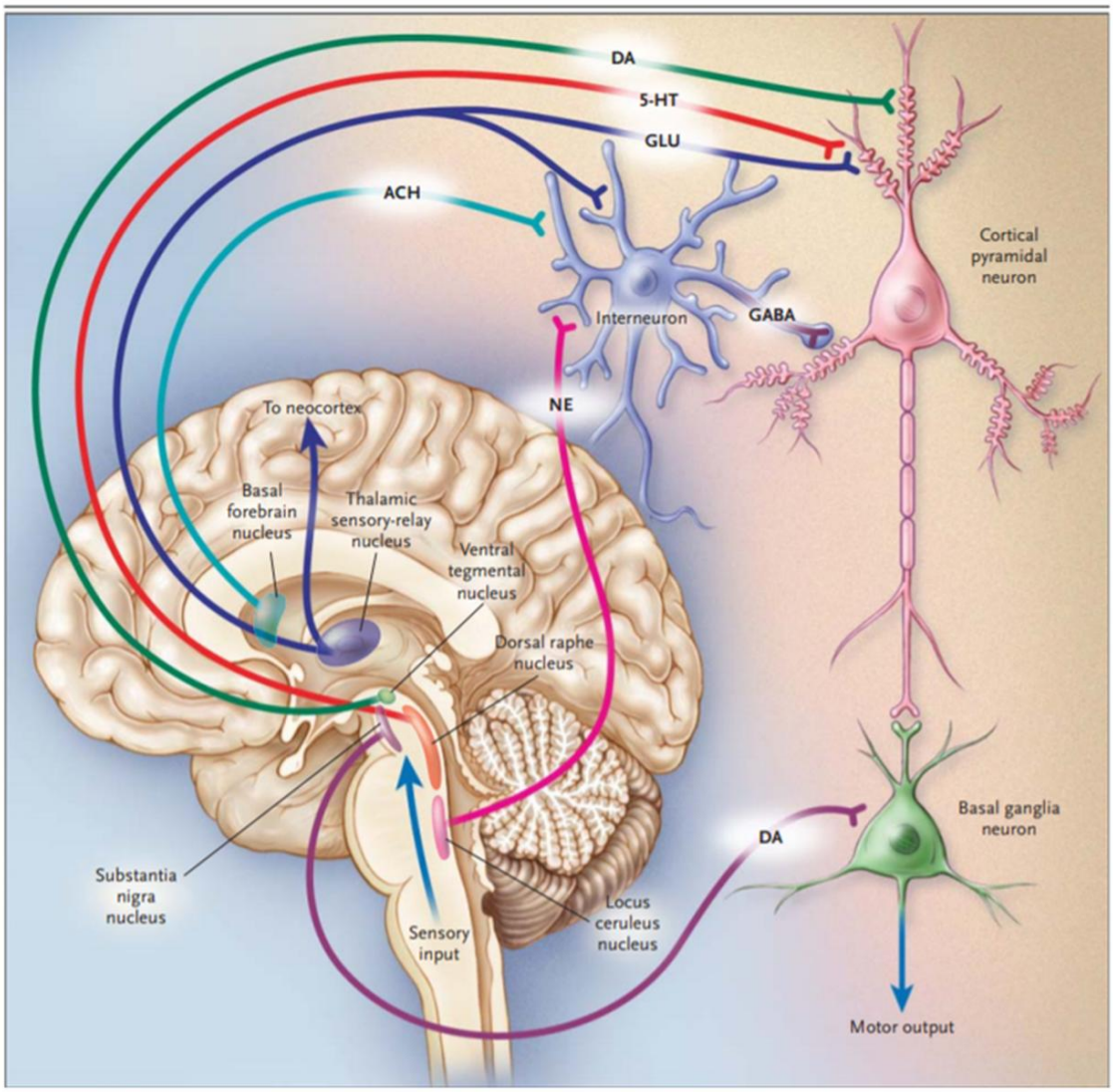


Figure 3. Neurotransmitter systems that have been involved in schizophrenia. Taken from Freedman, 2003.

Lindqvist, 1963). The "antipsychotic" DA receptor (Seeman and Lee, 1975; Burt et al., 1976), now known as the dopamine D2 receptor (D2R), was confirmed to be the primary site of action for all antipsychotics, and their clinical potency was found to be highly correlated with their affinity for the receptor (Creese et al., 1976; Seeman et al., 1976). Furthermore, stimulant drugs, such as cocaine and amphetamine, which increase synaptic DA, can induce and aggravate psychotic symptoms (Bell, 1965; Angrist et al., 1985). These studies, among others, led to the classic DA hypothesis that maintained that schizophrenia was a result of excessive DA activity.

A possible explanation on this classic DA hypothesis argues that mesolimbic DA system assigns importance, or salience, to stimuli or ideas and that in schizophrenia, excess DA leads to the assignment of salience to irrelevant or insignificant thoughts or events, creating a psychotic state (Kapur, 2003). However, the general hyperdopaminergic hypothesis was not

fully supported since some patients were treatment resistant. Furthermore, treatment was only partly effective or no effective at all, on negative and cognitive symptoms of the disease. This hypothesis was then believed to be too much simplistic for explaining such a complex disease with that many set of symptoms.

With the development of more sensible techniques and more specific drugs, a compelling number of studies (Ingvar and Franzén, 1974; Bunzow et al., 1988; Monsma et al., 1989, 1990; Sokoloff et al., 1990) led to the reformulation of the theory. It was then postulated that positive symptoms of schizophrenia may result from an excess of DA neurotransmission in mesolimbic and striatal brain regions, whereas dopaminergic neurotransmission deficits in prefrontal brain regions would be responsible for the negative symptoms and cognitive impairments (Davis et al., 1991). However, the increase in striatal D2R density in drug-free and naïve patients with schizophrenia was not consistent in the literature (Owen et al., 1978; Lee and Seeman, 1980; Ruiz et al., 1992). Thus, it was postulated that the supersensitivity to DA reported in patients with schizophrenia may arise from an increase in the proportion of D2R that are in a state of high sensitivity for DA, the so-called $D2^{High}$ (Seeman, 2011). Pharmacological tools for measuring $D2^{High}$ receptors are still ongoing (Subburaju et al., 2018), and this theory in schizophrenia is not clarified yet.

Still, enhanced functionality of D2R in striatal brain regions seems to contribute to positive symptoms of schizophrenia (Thompson et al., 2013), whereas negative and cognitive symptoms of the disorder may be partly attributed to either less DA or hypofunctionality of DA D1 receptor in the PFC (Toda and Abi-Dargham, 2007). Other DA receptors, such as D3 and D4, have been less studied, although it seems that they play a role in schizophrenia (Seeman et al., 1993) and its treatment (Sokoloff et al., 1990; Bitter et al., 2019).

Whereas DA alterations are directly associated with the manifestations of the symptoms, the DA theory of schizophrenia has several flaws. The role of DA in the brain is complex, and DA alterations are probably the endpoint of a number of events involving other transmitters such as serotonin, GABA and glutamate. Thus, investigation of the pathophysiology of schizophrenia has extended its field of inquiry beyond this system, to include other neurotransmitters.

Serotonin

The serotonergic hypothesis of schizophrenia arose from early observational studies with lysergic acid diethylamide (LSD). After commercialized in 1949 for research purposes, it was evidenced that LSD and other related compounds produced mental disturbances resembling those occurring at the onset of schizophrenia. Moreover, these compounds had some chemical similarities to serotonin (5-HT) (Woolley and Shaw, 1954), thus raising what it is

now known as the serotonergic hypothesis of schizophrenia. Some years later, in a study with several psychoactive agents, binding affinities for the so-called 5-HT2 receptor were found to correlate with human hallucinogenic potencies (Glennon et al., 1984). In the 90s and posterior years, it was demonstrated that LSD, psilocybin, 2,5-dimethoxy-4-iodoamphetamine (DOI) or mescaline agonism on serotonin 2A receptors (5-HT2AR) was responsible for their psychotic-like effects (Marek and Aghajanian, 1996; Vollenweider et al., 1998; Gonzalez-Maeso et al., 2003; González-Maeso and Sealfon, 2009).

In relation with the previous facts, atypical antipsychotic drugs were demonstrated to show higher affinity for 5-HT2 receptors than for D2 receptors (Meltzer et al., 1989a). Indeed, the atypical antipsychotic clozapine has been demonstrated to be the most effective in treatment-resistant patients with schizophrenia (Kane, 1988; Davis et al., 2003).

Despite these quite consistent reports associating 5-HT system and psychosis, studies in human brains from patients with schizophrenia are quite inconclusive and the findings have not been consistently replicated (Abi-Dargham, 2007). Moreover, patients that have received antipsychotic medications for many years are known to confound observations, hindering even more the discerning between aetiological alterations and the ones due to the course and the treatment of the disease (Vita et al., 2015). Overall, a comprehensive model of 5-HT transmission in schizophrenia has not yet emerged and additional research is needed in order to establish its role in symptomatology and treatment opportunity.

As mentioned above, the discovery of the high affinity of some antipsychotic drugs for 5-HT2AR made this target one of the most widely studied in schizophrenia. A more detailed review of 5-HT2AR physiology and its involvement in schizophrenia can be found in section **4.3**.

Glutamate

Like serotonergic theory, glutamate theory in schizophrenia also arose from causal observation of side effects. Phencyclidine (PCP) was legally manufactured and licitly used on humans as a short-acting analgesic and for general anesthesia. However, the regulation curtailed its use to animals because of post-operative side effects. First reported observation of its psychotomimetic effect, as well as of exacerbating thought disturbances in patients with schizophrenia, was in 1959 (Luby, 1959). Shortly after, together with ketamine, they were revealed to differ from other general anesthetics and, along with other related agents, they were classified as 'dissociative anesthetics' (Corssen and Domino, 1966).

Ketamine and PCP were classified as glutamate NMDAR non-competitive antagonists (Anis et al., 1983; Thomson et al., 1985). It was further described that at subanesthetic doses they mimic some positive, negative and cognitive symptoms of schizophrenia in healthy people

(Krystal, 1994). Thus, the glutamate hypothesis of schizophrenia postulated that NMDAR mediated dysfunction of neurotransmission might represent a primary deficit in the illness. Nowadays, it is well-established that administration of NMDAR antagonists can induce psychosis-like states resembling most of the symptoms seen in patients with schizophrenia (Newcomer, 1999; Stone et al., 2008). Nevertheless, studies regarding NMDAR subunits alterations in schizophrenia have shown discrepancies. In this way, decreased mRNA and protein expression of the obligatory GRIN1 subunit of this receptor in the cortex and hippocampus is probably the most consistent finding (Hu et al., 2015; Catts et al., 2016). However, the status of each NMDAR subunit in the brain of subjects with schizophrenia remains controversial.

An early report with patients with schizophrenia showed lower glutamate levels in cerebrospinal fluid, indicating that hypofunction of the glutamate neurotransmitter may contribute to the pathophysiology of this disease (Kim et al., 1980). Glutamate dysfunction has been mainly associated with cognitive disorders. However, double-blind randomized controlled trials with adjuvant treatment with glutamate positive modulators have failed to show procognitive effects in schizophrenia (Iwata et al., 2015).

Despite that first report, several others failed to replicate this finding (Perry, 1982), even reporting elevated glutamine/glutamate levels in left anterior cingulate cortex, thalamus and medial frontal cortex of drug-free individuals with FEP and adolescents at high-risk (Théberge et al., 2002; Tibbo et al., 2004). A recent meta-analysis of magnetic resonance studies revealed significant elevations in glutamate/glutamine in the basal ganglia, thalamus and medial temporal lobe in schizophrenia. While elevated medial frontal glutamate/glutamine levels were only evident in individuals at high risk for schizophrenia, the increase in the medial temporal lobe was seen in patients with chronic schizophrenia but not in the high-risk or FEP groups. Despite the functional significance of both metabolites, the findings suggested that some regional glutamatergic abnormalities could progress with the clinical course of the disorder or show differential responses to antipsychotic (Merritt et al., 2016).

Studies in animals and humans have led to some key findings on a mechanism that appears to unify the NMDA hypofuntion and the hyperglutamatergic hypothesis. While NMDAR blockade appears necessary to explain psychotomimetic effects of ketamine and PCP, ketamine administration at doses that impair cognitive functions increase glutamate efflux in the frontal cortex (Rowland et al., 2005; Stone et al., 2012; Abdallah et al., 2018), and blockade of cortical α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) reduce the cognitive effects (Moghaddam et al., 1997). This fact suggests that cognitive disruptions involving NMDAR hypofunction could be due to an increasing release of glutamate that thereby stimulates excessively postsynaptic AMPAR in pyramidal neurons

of the frontal cortex. Moreover, excessive cortical glutamate has been associated with failure to remission after first antipsychotic treatment (Egerton et al., 2012).

Glutamate hypothesis is indeed closely related to DA hypothesis. Interestingly, specific GABAergic interneurons in the PFC undergo dramatic changes in NMDAR/AMPAR ratio during the adolescent period (Wang and Gao, 2009). Blockade of NMDAR on these interneurons are thought to disinhibit glutamatergic neurons, increasing glutamate release and subsequently increasing DA neuron firing and release in projection targets such as the striatum and cortex (Moghaddam and Javitt, 2012; Kokkinou et al., 2018). Other findings include structural alterations of the proper glutamatergic pyramidal neurons in the layer III of the cortex or decreased mRNA expression of the AMPA subunit GRIA1 in the hippocampus (Meador-Woodruff, 2000; Hu et al., 2015).

Although both ketamine and PCP are interesting pharmacological tools for the study of schizophrenia, it has been reported that both can bind also to D2 and 5-HT2 receptors. Thus, these models may be reproducing a non-selective neurochemical perturbation, rather than an exclusive glutamatergic alteration (Kapur and Seeman, 2002).

γ-aminobutyric acid

Early studies on this neurotransmitter showed a reduction in thalamic and nucleus accumbens GABA content in *postmortem* tissue of subjects with schizophrenia (Perry et al., 1979; Spokes et al., 1980). Later on, other studies suggested a reduction of GABA reuptake sites in left temporal lobe, hippocampus and amygdala (Simpson et al., 1989), and decreased GABA content and glutamate decarboxylase enzyme (GAD) activity – the enzyme that catalyzes GABA formation - in frontal and temporal areas, as well as in the putamen (Sherman et al., 1991; Simpson et al., 1992).

GAD enzyme consists of two isoforms, GAD67 and GAD65, encoded by two different genes, GAD1 and GAD2. The most consistent findings regarding pathological changes in schizophrenia are reduced GAD67 messenger ribonucleic acid (mRNA) and reduced GAD67 mRNA positive neurons, which appear to involve specific populations of parvalbumin positive cells in the dorsolateral prefrontal cortex (DLPFC) (de Jonge et al., 2017). Interestingly, alternative splicing and epigenetic state of GAD1 appear to play a role in the developmental profile of GAD67 expression and deregulation in these processes in the PFC and hippocampus of patients, contributing to GABA dysfunction in schizophrenia (Tao et al., 2018).

A recent meta-analysis of GABA neuroimaging studies in schizophrenia failed to reveal consistent alterations in any brain region, despite there are not many studies, and most of them involve small patient samples (Egerton et al., 2017). Further studies accounting for

confounding effects of age, stage of illness, medications or other unknown factors will be useful to clarify the extent of implication of GABA alterations in schizophrenia pathophysiology.

1.3. ANTIPSYCHOTIC TREATMENT

Antipsychotic drugs are the basis of the pharmacotherapy in schizophrenia. In general terms, antipsychotic drugs are effective in reducing the severity of positive symptoms and preventing relapses (Leucht et al., 2012; Leucht and Davis, 2017; Huhn et al., 2019). However, the majority do not improve neither negative symptoms nor cognitive impairments (Miyamoto et al., 2012; Nielsen et al., 2015). Indeed, about 20% are resistant to standard antipsychotics (Kumar et al., 2016) and polypharmacy is commonly used in the treatment of schizophrenia. Up to 75% of patients with schizophrenia take at least two drugs, and about 25% of patients receive 5 drugs or more (Toto et al., 2019). Other pharmacological treatments apart from antipsychotic drugs used in the management of schizophrenia are antidepressants, mood stabilizers, and benzodiazepines. However, the evidence supporting the efficacy and safety of neither adjunctive medication nor combination therapy is limited (Stroup et al., 2019; Tiihonen et al., 2019).

Two generations of antipsychotic drugs have been developed and classified as typical or atypical antipsychotic drugs.

1.3.1. Typical antipsychotics

Chlorpromazine, haloperidol or fluphenazine are examples of the most commonly used first-generation or typical antipsychotic drugs. While all block dopamine D2R, high occupancy levels of this receptor are not associated with more effectiveness, but with more prevalence of extrapyramidal motor side effects and excess prolactin release (Kapur et al., 2000). A recent study has related these extrapyramidal effects with the association/dissociation kinetics over D2R (Sykes et al., 2017). Due to the relevance of these “on-target” side effects, discontinuation of therapy and therefore recurrence of psychosis often occur.

1.3.2. Atypical antipsychotics

Clozapine was the first compound that was described as an atypical antipsychotic. The main features that distinguish these antipsychotics are that (1) they show fewer extrapyramidal side effects at clinically effective doses, and that (2) they antagonize 5-HT2AR, and show less affinity to D2R. This fact leads to an enhanced 5-HT2AR/D2R affinity ratio, which is characteristic of this class of antipsychotics (Meltzer et al., 1989a). Other atypical antipsychotics commonly used are risperidone, olanzapine or quetiapine.

Nowadays, clozapine is the 'gold standard' of second-generation antipsychotics. It has practically no extrapyramidal side effects (Leucht et al., 2009, 2013). However, these antipsychotics produce more weight gain and insulin resistance (Newcomer, 2007; Leucht et al., 2013).

In general, second-generation antipsychotics are the mainstay of treatment of FEP in developed countries, although their superiority in improving overall symptoms and negative symptoms, decreasing relapses or increasing the quality of life, remain unclear for many of them (Leucht et al., 2009, 2013; Zhu et al., 2017).

Despite the efforts both at preclinical and clinical stages in schizophrenia research, no substantial improvements have emerged since the advent of antipsychotic medication, and up to 67% of patients with schizophrenia shows no symptomatic remission in the first 4-6 weeks of treatment, leading to more relapses (Samara et al., 2019). While a common tool in psychiatric practice is increasing the dose in non-responders, there is not evidence for this practice to be neither effective nor safe (Samara et al., 2018). These data shows the importance for the improvement of antipsychotic treatment.

1.4. COMORBIDITY IN SCHIZOPHRENIA

Comorbidity rates are very high in psychiatry, and most individuals who meet diagnostic criteria for one disorder meet diagnostic criteria for a second one, in some cases even for a third. Thus, when exclusion rules are bypassed, up to half of schizophrenia patients meet criteria for a co-occurring syndrome.

Given the evidence showing that symptoms are continuous rather than categorical and the overlap between psychiatric disorders, several empirical models of symptoms clustering tried to explain the comorbidity observed between closely related disorders. Clusters are usually categorized as internalizing (anxiety, depressive symptoms), externalizing (substance dependence, conduct disorder), psychotic/thought disorders (hallucinations, disorganized speech), pathological introversion... among others (Cloninger, 1987; Markon, 2010; Kotov, 2011). Recently, a general factor model was proposed, suggesting that there is one common liability to all forms of psychopathology and that covariation transcends these dimensions (Caspi et al., 2014; Caspi and Moffitt, 2018).

Irrespective of the theory, the truth is that there is an increased prevalence of a noteworthy amount of disorders among patients with schizophrenia compared with the general population, including anxiety disorders, depressive and substance abuse disorders, among others. These comorbidities occur at all phases of the course of illness and their presence is generally associated with increased severity of psychopathology and poorer outcomes, including more psychotic relapses, poor adherence to antipsychotic treatment, and greater

use of health services. Moreover, they often require specific treatment and management approaches (Buonocore et al., 2017).

Anxiety disorders are estimated to have a mean prevalence rate of 38% for at least one type (Achim et al., 2011). These disorders are known to occur spontaneously, intermittently, in direct response to psychotic symptoms or even as a side effect of antipsychotic medications (Aikawa et al., 2018).

Although schizophrenia is conceptualized as a non-affective psychotic syndrome, it is often associated with a variety of depressive states, that are intrinsic to the illness and import a poorer outcome, including more psychotic relapses (Conley et al., 2007). The lifetime prevalence have been determined to range from 7 to 75%, depending on the nature of the study, the diagnostic system and rating scales applied (Buckley et al., 2009). Although the heterogeneity is even bigger in depression symptoms than in anxiety, review of the studies addressing this issue indicate a modal frequency of around 25% (Siris, 2000). It has been suggested that depression and schizophrenia, as well as bipolar disorder, may share some early-life risk factors, as well as some aspects of aetiopathophysiology (Smoller et al., 2013; Witt et al., 2017). Moreover, evidence suggests that depression is linked to poorer outcomes in schizophrenia, such as worse quality of life, and suicide (Upthegrove et al., 2017).

Substance misuse is the most common co-occurring disorder in schizophrenia and worsens the course of illness. Data from the Epidemiologic Catchment Area (ECA) Study in US in the 90s described that patients with schizophrenia are 4.6 times more likely to have a concomitant substance use disorder (excluding nicotine and caffeine) than the rest of the population. With a lifetime prevalence of 47%, odds for alcohol disorders are up to 3 times as high, and for other drug disorders they are 6 times as high (Regier, 1990). The prevalence of smoking in schizophrenia is specially higher than among general population (88% versus 25-30%), even among psychiatric patients in general (52%) (Hughes et al., 1986). The specific comorbidity of cannabis use disorders in schizophrenia will be addressed in a separate section (section **3**). Despite the high co-occurring rates, patients with comorbid substance use disorders are often excluded from clinical trials, a fact that limits the generalization of results and ignores the potential effects of the intervention on substance use.

All of these comorbidities are not only common, but also important determinants of the patient's degree of suffering, disability, and even survival. Understanding this relationship is complicated, although the available evidence in genetics is providing an increasingly clearer picture of the boundaries of the schizophrenia spectrum (Cardno and Owen, 2014; Maier et al., 2018).

1.5. RESEARCH TOOLS FOR THE STUDY OF SCHIZOPHRENIA

One of the challenges of the research in schizophrenia is the development of better treatments that target the whole set of symptoms of the disease in a better way that the currently available ones do, i.e. causing less side effects.

Given the complex nature of schizophrenia, a great effort has been done to understand the deep roots of each symptom present in the illness. In this line, not only genetic, *postmortem* brain and neuroimaging studies, but also research in peripheral tissue, as well as the development of animal models have been essential for reaching the knowledge that we have about this complex disease. Each of these approaches has its own limitations and strengths, so as the combination of them are indispensable to address the most challenging questions regarding schizophrenia pathophysiology.

1.5.1. Human brain studies

From the most classical studies in *postmortem* brain tissue, to the most novel neuroimaging approaches, research in the human brain tissue is an irreplaceable tool as the biological substrate of psychiatric illness, including schizophrenia (Meana et al., 2014). Thus, these studies probably provide the most valuable evidence as to the nature of schizophrenia.

Studies in *postmortem* brain tissue usually involve genetic, mRNA and protein expression evaluation. Most recent meta-analysis studies show that these studies have been helpful to describe synaptic loss in regions such as hippocampus and frontal cortical regions (Osimo et al., 2019). Other recent significant findings involve a significant decrease of postsynaptic elements in the PFC (Berdenis van Berlekom et al., 2020), or an increase in the density of microglia and an overexpression of proinflammatory genes in postmortem brains of subjects with schizophrenia (van Kesteren et al., 2017). Moreover, positron-emission tomography (PET) and single-photon emission computed tomography (SPECT) imaging techniques have been important for describing alterations in receptors and transporters in several brain regions (Spies et al., 2015) in schizophrenia, as well as for demonstrating the mechanism of action of antipsychotic drugs (Mamo et al., 2007; Yilmaz et al., 2012).

Functional magnetic resonance imaging (fMRI) measures brain activity by detecting changes associated with oxygenated blood flow (hemodynamic response), which is directly correlated with neuronal activation. The fMRI uses the blood-oxygen-level dependent (BOLD) signal, either in response to a task, or showing subjects' baseline BOLD variance. Both resting-state fMRI and fMRI studies in response to cognitive tasks in FEP have shown similar and different brain regional changes, but the convergent functional abnormalities among the studies seem to be within the prefronto-temporal pathway, especially in the DLPFC, orbital frontal cortex and the left superior temporal gyrus (Mwansisya et al., 2017). Substantial intrinsic

dysconnectivity in insula, lateral-postcentral cortex, striatum, and thalamus (Brandl et al., 2019) and an aberrant reactivity of the limbic system to emotionally neutral stimuli have also been associated with schizophrenia (Dugré et al., 2019).

Diffusion tensor imaging (DTI) is a powerful magnetic resonance imaging (MRI) method for measuring the magnitude, the degree and the orientation of diffusion anisotropy, extensively used for estimating the integrity of white matter circuitry. In schizophrenia, white-matter dysconnectivity in the corpus callosum and left posterior cingulum fibers have been reported with this technique (Dong et al., 2017). Interestingly, deficits in white matter integrity are suggested to underlie cognitive function impairments in schizophrenia (Kochunov et al., 2017).

1.5.2. Peripheral tissue studies: focus on platelets

Peripheral tissues, especially peripheral blood mononuclear cells (PBMC) are extensively used in genetic markers association studies. Indeed, it has been the tissue used in every single GWAS carried out in schizophrenia. PBMC, along with saliva samples, are also used for epigenetic studies (Teroganova et al., 2016).

Platelets have also been extensively used for the study of several neuropsychiatric disorders, including autism, schizophrenia and depression (Asor and Ben-Shachar, 2012; Mazereeuw et al., 2013; Dietrich-Muszalska and Wachowicz, 2016). These small anucleated cells originate from megakaryocytes, and are thought to share embryologic origin, and some morphological and functional features with neurons. More specifically, they share many biochemical similarities with the neuronal monoamine system, particularly in the uptake, storage and metabolism of 5-HT (Stahl, 1977). Platelets possess 5-HT2AR and serotonin transporters (SERT), and contain the largest amount of 5-HT outside the CNS stored in specific dense granules (Mercado and Kilic, 2010). Interestingly, 5-HT2AR present in platelets has similar binding characteristics and engage the same signal transduction system than the central 5-HT2AR (Geaney et al., 1984; de Chaffoy de Courcelles et al., 1987), and studies of platelet secretion have indicated that it bears important similarities to neuronal exocytosis (Reed et al., 2000). Furthermore, they also contain mitochondria expressing monoamine oxidase-B (MAO-B) (the enzyme that deaminates 5-HT into 5-hydroxyindoleacetic acid (5-HIAA)), as well as α2 adrenoceptors (Barradas and Mikhailidis, 1993).

The major proportion of studies in platelets of subjects with schizophrenia and other neuropsychiatric diseases has been focus in serotonergic system, were abnormalities of different compounds of the system have been reported in relation with both the pathology and the treatment (Stahl et al., 1983; Ertugrul et al., 2007; Peitl et al., 2016). The first report showed a lower platelet monoamine oxidase (MAO) activity in subjects with schizophrenia

(Murphy and Wyatt, 1972). However, a subsequent meta-analysis of all the studies carried out in the next two decades concluded that this finding is not consistent and that it could be a result of the disease treatment (Marcolin and Davis, 1992). A more recent study reported lower 5-HT in platelets associated with more severe depressive symptoms in schizophrenia (Peitl et al., 2016).

Apart from studies regarding 5-HT system, other studies have pointed out that platelet $\alpha 2$ adrenoceptors functionality is altered in major depression and that antidepressant drugs modulate this receptor (Garcia-Sevilla et al., 1990; García-Sevilla et al., 2004). Other neurotransmitter systems, such as glutamate and dopamine, have also been studied in platelets, although to a lesser extent (Dean et al., 1996; Baier et al., 2009).

Altogether, the features mentioned above make platelets an interesting target for the study of receptors and mediated signal transduction mechanisms in the CNS. Moreover, the study of biochemical processes related to psychiatric disorders might be useful in order to find biological markers and unraveling pathophysiological processes relevant to the aetiology of schizophrenia(Asor and Ben-Shachar, 2012).

1.5.3. Animal models

Animal models of complex heterogeneous psychiatric disorders are very valuable preclinical tools to investigate the neurobiological basis of these diseases. They offer a more convenient platform to perform invasive monitoring of molecular changes that may underlie the symptoms, and to test novel therapeutic compounds not possible in patients for ethical reasons. The main advantage of animal models is the high experimental control, which is essential when analyzing the relative contributions of varying underlying factors to the symptoms of a disorder.

A useful and good animal model should fulfill three main validity criteria: construct, face and predictive criteria (explained in **Figure 4**). However, a major challenge with animal models used for the study of schizophrenia involves the modeling of uniquely human symptoms, e.g. delusions or auditory hallucination (Jones et al., 2011). In spite of that, the advancement in the knowledge of environmental and genetic risk factors, together with the development of behavioral assays with translational relevance to clinical manifestations, has improved our ability to create animal models for schizophrenia symptoms to a fare extent. These models are expected to improve clinical translation of novel therapies (Nestler and Hyman, 2010).

To date, several animal models for the study of schizophrenia have been developed so far. The most classical include pharmacological models, such as PCP and amphetamine administration (Jentsch and Roth, 1999; Featherstone et al., 2007). In addition, mutant

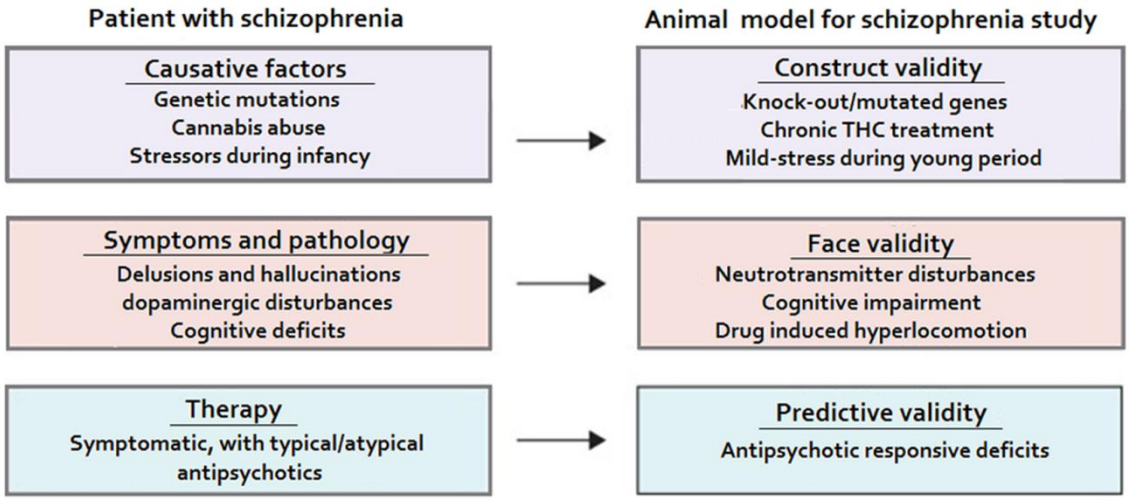


Figure 4. Criteria used to validate animal models for the study of schizophrenia. Inspired by Chesselet & Richter, 2011.

knock-out (KO) mice, such as disrupted in schizophrenia 1 (DISC1) KO or neuregulin1 KO, have been developed (O'Tuathaigh et al., 2008; Jaaro-Peled, 2009). Animal models mimicking maternal infection during gestation, e.g. polyinosinic:polycytidylic acid (poly(I:C)) (Zuckerman et al., 2003) or methylazoxymethanol acetate (MAM) (Moore et al., 2006) are gaining a lot of interest currently.

Lastly, the "two-hit" paradigm appeared as one of the most conceptual model, in which a combination of two causative factors – usually genetic + environmental – are used in order to reinforce construct validity (Uzuneser et al., 2019).

2. CANNABIS PLANT AND THE ENDOCANNABINOID SYSTEM

2.1. HISTORY AND GENERAL ASPECTS

Cannabis, also known as marijuana or hemp, is a genus consisted of a main species and two subspecies of flowering plants from the Division Magnoliophyta. The species is called *Cannabis sativa* L., and consists of two subspecies: *Cannabis sativa* L. ssp. *indica* and *Cannabis sativa* L. ssp. *sativa* (see **Figure 5**). There is a third one, *Cannabis ruderalis*, which is widely debated as to whether it is a subspecies or it may be included within *sativa* subspecies.

Rank	Scientific name	Common name
Kingdom	Plantae	Plants
Subkingdom	Tracheobionta	Vascular plants
Superdivision	Spermatophyta	Seed plants
Division	Magnoliophyta	Flowering plants
Class	Magnoliopsida	Dicotyledons
Subclass	Hamamelidae	
Order	Urticales	
Family	Cannabaceae	Hemp family
Genus	***Cannabis* L.**	Hemp, marijuana
Species	*Cannabis sativa* L.	
Subspecies	*Cannabis sativa* L. ssp. *indica*	
	Cannabis sativa L. ssp. *sativa*	

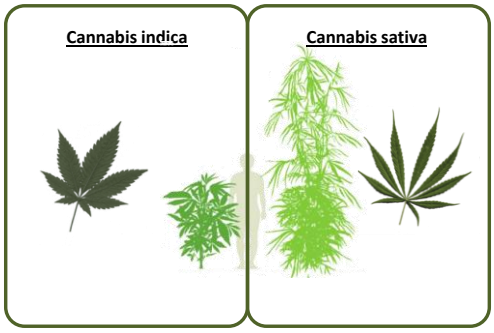


Figure 5. Left: Classification for Kingdom Plantae down to Subspecies sativa and indica. Right: Illustrations of the whole plant and the leaves of indica and sativa subspecies.

Cannabis genus is accepted to be indigenous to Central Asia, with some researchers also including northern South Asia in its origin. The large scale cultivation started in China for fiber and seed production, and soon after in India for resin production, although nowadays its cultivation has spread all over the world (ElSohly, 2007).

Cannabis usage dates back to at least the 3000 before Christ (BC) in written history, and possibly further back by archaeological evidence (Long et al., 2017). For millennia, the plant has been used for fiber and rope, as medicine, and for religious and recreational use. Indeed, the use of cannabis as a mind-altering drug has been documented in Eurasian and African prehistoric societies by archaeological finds (Merlin, 2003).

Nowadays, cannabis continues to be the most widely used illicit drug worldwide (see distribution in **Figure 6**). Data from 2017, coming from the United Nations, estimates a global number of cannabis users of around 192 million, that is around 2.5% of the world population. A global estimation based on available data from 130 countries suggests that, in 2016, 13.8 million people aged 15–16 years, equivalent to 5.6% of the population in that age range, used cannabis at least once in the previous 12 months (United Nations Office on Drugs and Crime (UNODC), 2017).

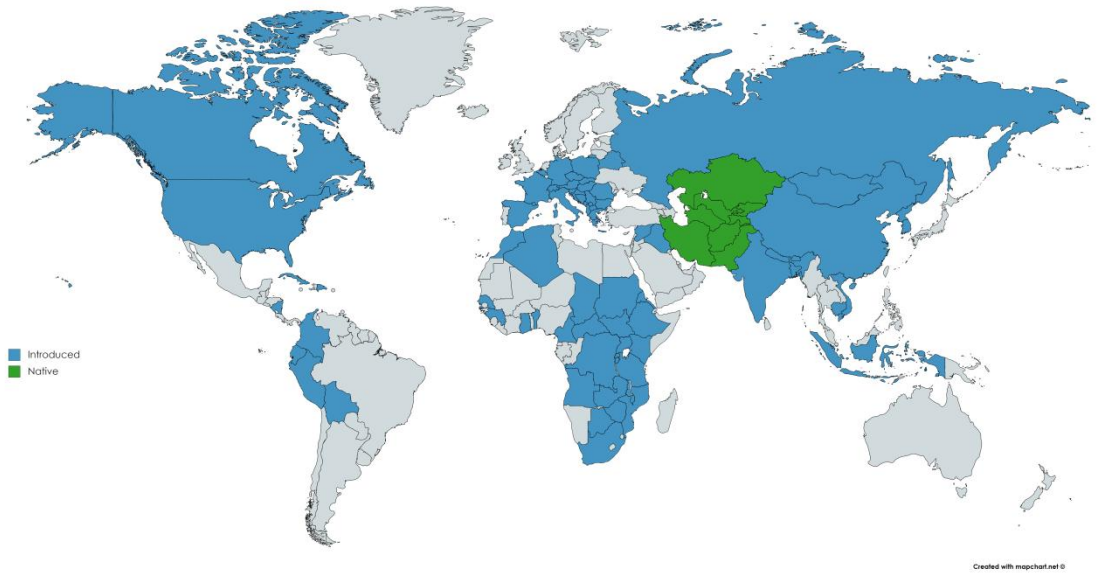


Figure 6. Distribution of *Cannabis sativa* L. Data taken from http://www.plantsoftheworldonline.org. Map created with mapchart.net.

Regarding the use of the plant for human consumption, cannabis derivates can be found in multiple forms. Herb is the name used for the plant leaves and flowering tops. These parts are dried and crumbled and they are usually smoked. Other cannabis products are oils. They are low concentrated preparations, as they are obtained diluting the plant extract or macerating the raw plant in vegetable oils (most commonly olive, sesame or hemp oil), and it is consumed orally. Cannabis resin, also known as hashish or hash, is the concentrated extract of stalked resin glands from the plant. Both the color and the appearance can vary a lot (from dry and hard, to moist and pliable; from almost black, to a dirty yellow) depending on the preparation method or the amount of leftover plant material. It is consumed by smoking a small piece, typically mixed with herbal cannabis or tobacco; or via oral ingestion. Lastly, cannabis or hash oil is the strongest form of marijuana and the least common form of the drug. It is an oleoresin obtained by the extraction of the plant through any of various methods, most involving a solvent, such as butane or ethanol. The appearance depends on both production method and temperature. Color most commonly ranges from transparent golden to tan or black. Hash oil is sold in tiny bottles or cartridges used with pen vaporizers, and it is usually consumed by smoking, vaporizing or eating.

The most common administration routes are the inhalation, through smoking or vaporizing, and ingestion. Other routes include topical, sublingual or rectal and is becoming increasingly common in medical uses. All these routes determine the timing of the onset of the effects, as well as the potency and duration.

2.2. PHARMACOLOGY OF THE CANNABIS PLANT

Whereas cannabis has been consumed for millennia, it was not until 1964 that the Δ^9-tetrahydrocannabinol (THC), the main chemical component responsible for the psychoactive effect, was isolated and its chemical structure elucidated (Gaoni and Mechoulam, 1964, 1971; Poutsma, 1965). Up to now, more than 400 chemical components have been described in cannabis, of which around 80 are unique to this species. Some of these components are characteristic from cannabis plant and are known as cannabinoids. The main and more abundant ones will be further described below. Other cannabinoids include cannabinol (CBN), cannabigerol (CBG) or tetrahydrocannabivarin (THCV), among many others.

Delta-9-tetrahydrocannabinol (THC)

THC is the most known cannabinoid in cannabis plant. It is a derivative from the inactive acid form, which becomes THC when heated. The first isolation of THC in a pure form was reported in 1964, after several efforts of different chemists in elucidating the active principle of the plant (Marshall, 1898; Adams et al., 1940).

THC is the responsible for the major psychoactive and reinforcing effect of cannabis (D'Souza et al., 2004); as well as for some of the therapeutic properties - at least partially - attributed to the plant. THC in rodents produces a widely known range of behavioral symptoms like hypomotility, hypothermia, catalepsy and analgesia (Little et al., 1988). The evaluation of this set of symptoms is commonly known as tetrad test, and is a common feature of cannabinoid agonists (Martin et al., 1991). Other effects are the reduction of ocular pressure, increase in food intake and decrease some tumors growth (Williams et al., 1998; Blasco-Benito et al., 2018; Miller et al., 2018). In humans, THC increases pulse rate, impairs episodic and working memory, and modulates emotion and reward processing. However, a high variability is evident both among studies and across individuals within studies (Freeman et al., 2019). The psychotomimetic effect of THC is usually the most intended effect in recreational cannabis users. Currently, the cannabis available in Europe and USA contains mean concentrations of around 17% THC, about two-fold the concentration that was present 10 years ago (Chandra et al., 2019).

Pharmacological action of THC is primarily due to the partial agonism over cannabinoid type 1 receptors (CB1R) and cannabinoid type 2 receptors (CB2R). The characteristics and distribution of these receptors will be subsequently reviewed (section **2.3.1.**).

Cannabidiol (CBD)

The chemical structure of CBD was elucidated in 1963 (Mechoulam and Shvo, 1963). Similar to THC, CBD is formed when the acid form is heated. By now, several properties have been

attributed to this chemical compound, such as anxiolytic, antidepressant, antipsychotic, anti-inflammatory and anticonvulsive effects (Resstel et al., 2009; Zanelati et al., 2010; Pedrazzi et al., 2015; Devinsky et al., 2016; Petrosino et al., 2018).

Several reports have demonstrated that CBD attenuates many of the effects evoked by THC. CBD has low affinity for CB1R and CB2R orthosteric sites; however, it has been demonstrated that acts as a potent non-competitive CB1R negative allosteric modulator (Laprairie et al., 2015) and it is supposed to be a CB2R modulator (Martínez-Pinilla et al., 2017). It is also known to act as a low-affinity serotonin 1A receptor (5-HT1AR) agonist (Russo et al., 2005), peroxisome proliferator-activated receptor γ (PPARγ) agonist and transient receptor potential voltage channel 1 (TRPV1) agonist (Bisogno et al., 2001). Additionally, it is a high affinity G protein-coupled receptor 55 (GPR55) antagonist (Ryberg et al., 2009).

2.3. THE ENDOCANNABINOID SYSTEM

Despite the advances in the chemistry of synthetic cannabinoids with similar effects as THC during the 70s and the 80s, the molecular basis of cannabinoid activity remained unclear for several decades. During those years it was generally assumed that the high lipophilicity was the basis of their pharmacological action (Paton, 1975). A further conceptual problem, which hindered the work aimed at the discovery of a specific receptor, was the presumed lack of stereospecificity. However, this idea was demonstrated to be false for cannabinoid compounds (Mechoulam et al., 1988) and, indeed, a specific cannabinoid receptor was discovered in 1988 (Devane et al., 1988), known today as CB1R. This discovery gave birth to an exciting endocannabinoid research explosion, which started with the search of the endogenous ligands of this new receptor and continues today. So far, several components have been discovered, such as endogenous ligands, synthesis and degrading enzymes and more cannabinoid receptors. All of them will be described below.

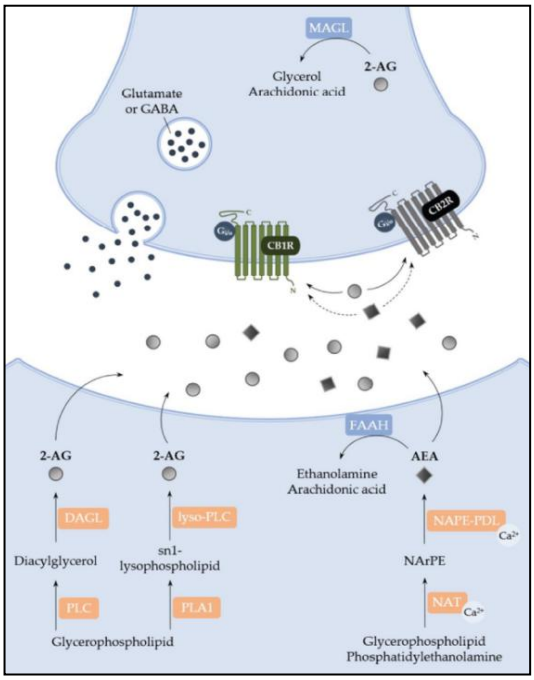


Figure 7. Endocannabinoid retrograde signaling and metabolism. Taken from Rodrigues et al., 2019

It is widely accepted that, in the central nervous system (CNS), endocannabinoid system plays a major role as a tonic regulator of cognitive functioning (Reibaud et al., 1999; Lichtman et al., 2002; Ranganathan and D'Souza, 2006). The most accepted theory of endocannabinoids action is that they are

retrograde neurotransmitters (see **Figure 7**). Cannabinoids are produced in the postsynapses, whereupon they migrate to presynaptic axon terminals, bind to cannabinoid receptors, and inhibit neurotransmitters release (Maejima et al., 2001; Wilson and Nicoll, 2001). The main neuronal types that express cannabinoid receptors in the brain are GABA neurons, followed by glutamatergic neurons (Katona et al., 2000; Robbe et al., 2001; Piomelli, 2003). For this reason, endocannabinoids are thought to be essential to maintain the balance between these two neurotransmitters, that are the most abundant in the brain (Schousboe, 1981).

2.3.1. Components of the endocannabinoid system

Cannabinoid receptor type 1 (CB1R)

CB1R is a member of G protein-coupled receptors (GPCR), formed by seven transmembrane domains connected by three extracellular and three intracellular loops, an extracellular N-terminal tail, and an intracellular C-terminal tail. It was cloned in 1990 (Matsuda et al., 1990) and consists of 472 amino acids in humans (473 amino acids in rat and mouse, with 97–99% amino acid sequence identity among these species) (Howlett et al., 2002). It is widely expressed in the CNS, especially in substantia nigra, globus pallidus and caudate/putamen, as well as in hippocampus, frontal cortex and cerebellum (Herkenham et al., 1990; De Jesús et al., 2006), and it participates in a variety of brain functions related to these areas, including executive, emotional, reward, and memory processing. Apart from CNS, CB1R is expressed in other tissues such as digestive tract (Croci et al., 1998), lungs (Grassin-Delyle et al., 2014), sperm (Rossato et al., 2005) and oocytes (Peralta et al., 2011).

Detailed electron microscope studies in the hippocampus show that CB1R is almost exclusively present on presynaptic terminals of inhibitory GABAergic neurons (Katona et al., 2000). Animal studies are consistent with this observation, and CB1R distribution in frontal cortex and amygdala seem to correlate with hippocampal CB1R. These terminals target dendritic branch of pyramidal output from these areas (Fitzgerald et al., 2019). CB1R are also expressed in a much lesser extent in glutamatergic terminals of areas such as hippocampus, nucleus accumbens and cerebellum (Robbe et al., 2001; Kawamura, 2006).

The distribution of CB1R suggests that this receptor plays a key role in the excitatory/inhibitory modulation that these neurotransmitters wield over dopaminergic and serotonergic systems. Moreover, CB1R are also functionally present in glial cells (Han et al., 2012) and in different cell compartments, such as endosomes and mitochondria (Bénard et al., 2012), thereby increasing the physiological repertoire of CB1R actions.

Cannabinoid receptor type 2 (CB2R)

CB2R, like CB1R, is a GPCR. It was cloned three years after CB1R (Munro et al., 1993) and consists of 360-amino acids in humans. It is quite different from CB1R, especially in its amino terminal domain, where there is no significant conservation. Between transmembrane domains 1 and 7, the CB2R is only 48% identical to that of CB1R, substantially less than between other GPCR types, but enough to be identified as a cannabinoid receptor. It is expressed primarily in spleen and immune cells (specially B lymphocytes) (Galiègue et al., 1995), and it is nearly absent in normal nervous tissue (Howlett et al., 2002). Moreover, it has been demonstrated to be present in human spermatozoa (Agirregoitia et al., 2010) and blood vessel endothelium (Dowie et al., 2010).

Despite CB2R was considered a peripheral cannabinoid receptor, its presence has also been described throughout the CNS of rats (Van Sickle, 2005; Gong et al., 2006) and in microglial cells of human brain (Galve-Roperh et al., 2000; Núñez et al., 2004). Specially, CB2R in the brain is found upregulated under certain pathological conditions, such as gliomas (De Jesús et al., 2010), Alzheimer´s disease (Benito et al., 2003) or neuroinflammatory conditions that course with microglia activation (Dowie et al., 2014; Gómez-Gálvez et al., 2016).

Anandamide (AEA)

After the discovery of CB1R, the search for the endogenous ligand lead to the discovery of arachidonoylethanolamide, an arachidonic acid derivative isolated from porcine brain extract, that was named anandamide (from Sanskrit "ananda", meaning bliss) (Devane et al., 1992). AEA is a high affinity CB1R partial agonist generated from n-acylphosphatidylethanolamines (NAPEs) derived from cell membranes. AEA is expressed in the human brain (Muguruza et al., 2013a), and, to a lesser extent, in the blood, cerebrospinal fluid, oviductal fluid and seminal plasma (Schuel et al., 2002; Monteleone et al., 2005; Romigi et al., 2010). It is known to have a very short half-life due to the rapid re-uptake and subsequent degradation (Di Marzo et al., 1994).

Despite AEA was discovered quite recently, it is known to play a role in key physiological processes such as regulation of feeding behavior (Monteleone et al., 2005), memory processes and sleep patterns (Murillo-Rodríguez et al., 1998), pain relief (Fride and Mechoulam, 1993) and embryo implantation (Maccarrone, 2002). It is also known to have anxiolytic (Kathuria et al., 2003) and vasodilator (Zygmunt et al., 1999) effects.

2-arachidonoylglycerol (2-AG)

2-AG, discovered in 1995 (Mechoulam et al., 1995), is a full agonist of both CB1R and CB2R that binds with low affinity (Gonsiorek et al., 2000). It is synthesized from diagylglycerol

(DAG) and it is present at relatively high levels in the CNS (Stella et al., 1997). Detection of 2-AG in brain tissue is complicated in part due to the relative ease of its isomerization to 1-arachidonoylglycerol (1-AG) during standard lipid extraction conditions. However, last decade a method for the quantification of 2-AG and other endocannabinoids was developed and validated (Lehtonen et al., 2011), and enabled the measure of 2-AG and other endocannabinoids in *postmortem* human brain (Muguruza et al., 2013a).

Studies in animals have demonstrated that 2-AG is essential in memory processes (Stella et al., 1997), stress resilience (Bluett et al., 2017) and regulation of inflammatory and immune responses (Kishimoto et al., 2003; Gokoh et al., 2005).

Both AEA and 2-AG are not stored, as classical neurotransmitters are. Instead, they are rapidly synthesized on demand by neurons in response to depolarization and consequent Ca^{2+} influx (Di Marzo et al., 1998) and released from postsynaptic neurons. Subsequently, they activate presynaptic CB1R by travelling backward across synapses, suppressing neurotransmitter release (Wilson and Nicoll, 2002).

N-acylphosphatidylethanolamine phospholipase D (NAPE-PLD) and diacylglycerol lipase (DAGL)

AEA is produced from the hydrolysis of a pre-formed membrane phospholipid precursor catalyzed by the enzyme NAPE-PLD, which was cloned in 2004 (Okamoto et al., 2004). This enzyme is stimulated with Ca^{2+} and it is equally efficacious with most NAPEs, and hence responsible for the formation of other n-acylethanolamines apart from AEA (Okamoto et al., 2005). Another route of AEA synthesis found in circulating macrophages is cleavage of the NAPE phosphodiester bond by a NAPE-selective phospholipase C (PLC) followed by dephosphorylation of the resulting phospho-AEA to liberate AEA (Liu et al., 2006).

2-AG is synthesized by more than one pathway, while a DAG derivate is considered the biosynthetic precursor for 2-AG (Bisogno et al., 1997). DAGs are then converted into 2-AG by the action of selective DAGL, designated as DAGLα and DAGLβ. While DAGLα is the dominant 2-AG producing lipase in adult brain (Bisogno et al., 2003), DAGLβ plays an important role in the generation of 2-AG in the liver and during immune responses (Gao et al., 2010; Shin et al., 2019). The expression of both enzymes shifts during brain development, as they seem to be co-localized with CB1R in axonal tracts of the embryonic nervous system, and move to a dendritic field location in postsynaptic neurons in the adult brain (Bisogno et al., 2003; Ludányi et al., 2011; Yoshino et al., 2011). This dynamic localization reflects the proposed roles for 2-AG as an autocrine endocannabinoid required for axonal guidance (Brittis et al., 1996; Williams et al., 2003), and as a retrograde messenger in the control of synaptic plasticity in the adult brain (Chevaleyre and Castillo, 2003).

Fatty acid amino hydrolase (FAAH) and monoacylglyrerol lipase (MGL)

AEA degradation in the CNS occurs primarily by the enzyme FAAH (Cravatt et al., 1996), which is an integral membrane protein that hydrolyses AEA, producing arachidonic acid and ethanolamine (McKinney and Cravatt, 2005). However, the hydrolase is not selective and it degrades multiple fatty acid amides (Boger et al., 2000). In the brain, FAAH is mostly situated in the endoplasmic reticulum with a somatodendritic location (Gulyas et al., 2004). More recently, it has been reported that other enzyme, FAAH2, can degrade AEA and related compounds. They have limited sequence homology (~20%), their orientation in the membrane differs, and while FAAH is expressed in the brain, testis, and small intestine, FAAH2 is not present in the brain, it faces the lumen and it is predominant in cardiac tissue and ovary. In addition, FAAH has been reported to have much greater activity with regard to fatty acid ethanolamides such as AEA (Wei et al., 2006)

AEA and related compounds can also be degraded either by the n-acylethanolamine acid amide hydrolase (NAAA), highly expressed in immune cells, specifically in macrophage lysosomes (Muccioli, 2010), or by several of the same enzymes responsible for arachidonic acid oxidation, including cyclooxygenase-2 (COX-2) and the 12- and 15-lipoxygenases (Di Marzo, 2006)

2-AG degradation is primarily due to MGL, which is responsible for inactivation of the major fraction of 2-AG in the brain (Dinh et al., 2004) and it is localized presynaptically, specifically in synaptic terminals (Makara et al., 2005; Ludányi et al., 2011). A minor proportion of 2-AG is thought to be hydrolyzed by two additional enzymes, monoacylglycerol lipase (ABHD6) and lysophosphatidylserine lipase (ABHD12). These enzymes display different localizations, and are believed to control independent pools of 2-AG and signaling events (Blankman et al., 2007).

Additionally, 2-AG can be oxidized by COX-2 more efficiently than AEA, leading to a distinct family of prostaglandins in macrophages, and in a Ca^{2+} dependent manner (Kozak et al., 2000). This mechanism is thought to contribute to the inflammation-induced neurodegeneration (Sang et al., 2007).

The understanding of the endocannabinoid system has been expanded to include related receptors, such as PPAR, TRPV1 or GPR55 (Pertwee et al., 2010; Storozhuk and Zholos, 2018; Karwad et al., 2019). Furthermore, several compounds have been shown to enhance AEA and 2-AG levels by competing with the same degrading enzymes, thus increasing their activity. These compounds include palmitoylethanolamide (PEA), stearoyl ethanolamide (SEA), and oleoylethanolamine (OEA), and are thought to play the so-called “entourage effect” (Ben-Shabat et al., 1998).

2.3.2. Cannabinoid intracellular signaling in the brain

Nowadays, it is known that CB1R and CB2R properties encompass a great molecular, cellular and functional complexity. Despite the constant addition of new players involved in the central endocannabinoid signaling, most of what is known refers to CB1R, which is far more abundant than CB2R in the healthy human brain (Benito et al., 2008).

Stimulation of CB1R and CB2R activates a number of signal transduction pathways associated with heterotrimeric G proteins complex. Cannabinoid receptors are primarily coupled to inhibitory Gαi/o protein subunits, that inhibit adenylyl cyclases, decreasing intracellular cyclic adenosine monophosphate (cAMP) concentration (Howlett et al., 1986; Matsuda et al., 1990; Felder et al., 1993) and subsequently damping protein kinase A (PKA) activation. A-type rectifying potassium (K^+) currents are normally inactivated upon phosphorylation by PKA, so reduced PKA activation produces a net reduction in the phosphorylation of K^+ channels, thus modulating the kinetics of the currents (Deadwyler et al., 1995; Hampson et al., 1995).

Apart from this intracellular response, recent advances suggest that CB1R can couple to different Gα proteins in brain cells, depending on the 'context' - cell type, cellular functional state, pathological condition - where they are activated. In addition, CB1R activation inhibits N-type voltage-gated Ca^{2+} channel, and activates G-protein gated inwardly rectifying K^+ channels (GIRK) (Guo and Ikeda, 2004) independently of cAMP and depending on the ligand used. The relative contribution of each of these inhibitory mechanisms seems to depend on the variance of ion channel expression by cell type and cAMP-dependent interaction with other molecules. Gβ/γ subunits are also thought to play a role in these effects, while there is still some uncertainty about how this process occurs. Under certain conditions, some endocannabinoids and exogenous cannabinoids, such as WIN55,212-2, AEA and THC, can also lead to mobilization of intracellular Ca^{2+} (Lauckner et al., 2005, 2008; Waldeck-Weiermair et al., 2008). This modulation seems to occur indirectly or even independently of CB1R activation (Netzeband et al., 1999; Rao and Kaminski, 2006), and it is thought to vary upon cell type.

The above mentioned effects regarding Ca^{2+} mobilization, together with Ras homolog family member A (RhoA) activation and extracellular signal–regulated kinase (ERK) phosphorylation seem to be mediated by the proper CB1R coupling to Gαq/11 (Lauckner et al., 2005). However, both exo- and endocannabinoids have been also reported to activate GPR55, which is an orphan GPCR that is known to couple to Gα12/13 and, to a lesser extent, to Gαq/11 subunits (Ross, 2009; Ryberg et al., 2009). Other signal transduction observed upon CB1R activation is the activation of mitogen-activated protein kinase (MAPK) or Akt along with

phosphoinositide 3-kinase (PI3K) activation, and nitric oxide (NO) production, depending on the particular type of cell and the agonist used (Zou and Kumar, 2018).

This plethora of intracellular responses extends far beyond the classic view of the cannabinoid receptors coupling exclusively to Gαi/o protein subtypes and decreasing cAMP. The prevailing idea that is gaining ground in GPCR study is that receptors can exist in different activated conformational states capable of coupling to different G proteins. Although the GPCR's preferred coupling partner is usually defined according to the G-protein subtype activated by the endogenous ligand under physiological or experimental conditions, the binding of any single particular agonist may also initiate the activation of multiple different G proteins, as it may be capable of stabilizing more than one conformation of the GPCR (Kenakin, 2011). In addition, a conformation that preferably activates one isoform of Gα may activate another if the preferred is less available, and feedback pathways may result in receptor modifications (e.g., phosphorylation) that alter the G protein preference of the GPCR.

Apart from the agonist, CB1R expression level seems to be important for the specific signaling outcome. In addition, Gαi protein expression, and the isoform of adenylyl cyclase expressed in the cell are also important since it has been described that CB1R may couple to stimulatory Gαs under certain conditions, activating adenylyl cyclase (Rhee et al., 2002; Finlay et al., 2017).

Whereas CB2R have been less studied, upon activation, inhibition of adenylyl cyclase via Gαi/o proteins and activation of MAPK and Akt have been reported, whereas it does not seem to signal via ion channels (Pertwee, 1997; Martínez-Martínez et al., 2016)

In recent decades, the endocannabinoid system has attracted considerable attention as a potential therapeutic target in numerous physiological and pathological conditions. In this context, the vast majority of intracellular responses triggered by endocannabinoids are likely due to the activation of specific G proteins. In human genome, 16 genes encode α subunits of G proteins (Sánchez-Fernández et al., 2014), and it is common for a given cell type to express a dozen or more of these subunits (Straiker et al., 2002). G proteins mediate the earliest step in cell responses to external events, albeit studies focusing on this first event of cannabinoid signaling are scarce. There is an increasing interest in dissecting the intracellular pathways responsible for the multiple effects that both endo- and exocannabinoids exert in the brain, so studies addressing the specific mechanisms underlying each of these effects are still needed.

3. CANNABIS AND SCHIZOPHRENIA

3.1. HISTORICAL OVERVIEW

Back in the early 19th century, the French psychiatrist Jacques-Joseph Moreau (1804-1884) provided the first systematic work on the effects of cannabis intoxication, on his monography "Du hachisch et de l'aliénation mentale: études psychologiques, 1845". On that novel work, Moreau tried to model and understand mental illness using cannabis (J. Moreau, 1845). Since then, many scientists are still making efforts for elucidating the relationship between cannabis and psychosis.

Back in the 70s and 80s, several case reports started to suggest that cannabis could be a complicating factor in schizophrenia (Breakey et al., 1974; Treffert, 1978; Knudsen and Vilmar, 1984). In addition, several studies reporting psychotic episodes triggered by cannabis consumption in non-psychiatric subjects started to appear on scientific literature (PPålsson et al., 1982; Rottanburg, 1982).

In 1987, it was described for the first time that cannabis could play a role in the onset of schizophrenia in a larger longitudinal study (Andréasson et al., 1987). This 15-year follow up cohort-study of Swedish conscripts found that heavy use of marijuana increased the risk of schizophrenia later in life by up to six times in a dose-dependent manner. However, the majority of patients with schizophrenia never consumed cannabis, and only 3% of heavy users developed schizophrenia. These observations evidenced the already known fact that cannabis use is neither necessary nor sufficient to cause schizophrenia, and suggested that cannabis might play a role only in a subgroup of individuals with a preexisting vulnerability to schizophrenia. The Swedish conscript survey is, to date, the longest follow-up of psychotic patients with data on cannabis use prior to incidence of psychosis.

To date, there is rational evidence that in those patients with psychosis, continued use of cannabis is associated with more positive symptoms, more frequent and earlier relapses, and poorer outcome (Caspari, 1999; Grech et al., 2005; Núñez et al., 2016; Setién-Suero et al., 2019).

3.2. CANNABIS CONSUME AS A RISK FACTOR FOR SCHIZOPHRENIA

Cannabis may represent one of the most potentially modifiable risk factors for the development of schizophrenia, so establishing the nature of this association and accurately estimating its magnitude still suppose the main aim of several human studies (Hickman et al., 2009; Gage et al., 2013). Despite the numerous studies in the literature, causality requires certain scientific evidence criteria, some of which remain undemonstrated. Non causal explanations for associations may include reverse causation - where associations actually

reflect psychosis increasing risk of cannabis use -, confounding factors- where other variables that increase risk of both cannabis use and psychosis lead to false associations - and bias - where problems with measurement or sample selection lead to incorrect estimates.

It is important to note that causality cannot be proven by observational studies. The unique experimental design to demonstrate causality in humans is the randomized controlled trial. In this sense, experimental support for causality, in the sense of randomized allocation to exposure, has been demonstrated only for acute outcomes such as induction of transient delusions or hallucinations or cognitive impairment following experimental cannabis use. These studies will be addressed in section **3.2.2.**

3.2.1. Observational studies

Since the first study from Andreasson and colleagues, numerous others have investigated the association between cannabis and schizophrenia. A subsequent follow-up study of the same cohort (Zammit, 2002) still found a dose related increase in risk of psychotic symptoms and schizophrenia with previous cannabis use, even when statistically controlling other potential confounding variables, such as psychiatric symptoms at baseline. Moreover, evidence from following cohort studies from New Zealand (Arseneault et al., 2002; Fergusson et al., 2003, 2005; Caspi et al., 2005), United States (US) (Tien and Anthony, 1990), Netherlands (van Os, 2002), Germany (Henquet et al., 2005) and United Kingdom (Wiles et al., 2006), consistently reported an increased risk of psychosis in people using cannabis. The last study from the Swedish cohort, published in 2014 (Manrique-Garcia et al., 2014), evidenced a more severe course and poorer prognosis in cannabis users already diagnosed with schizophrenia.

Regarding the strength of the association, these cohort studies were reviewed in a careful meta-analysis that addressed studies quality (Moore et al., 2007), which concluded that frequent cannabis consumption was associated with a two-fold risk of a psychotic outcome. A more recent meta-analysis adding two cross-sectional studies (Miettunen et al., 2008; McGrath et al., 2010), and two case-control studies (Di Forti et al., 2009, 2014) confirmed a dose-response relationship, where the frequency of cannabis use correlated positively with the increase of the risk for psychosis (Marconi et al., 2016).

Some reports have pointed that among patients with a FEP, current and daily cannabis use is more prevalent, as well as lifetime use of high potent cannabis (Larsen et al., 2006; Di Forti et al., 2009; Ferraro et al., 2019), thus providing a preliminary evidence that regular cannabis use precedes psychotic outcomes. The most robust study carried out to date (Di Forti et al., 2019), is a large multicentric case-control study that confirmed the contribution of frequent use of cannabis of high potency (more than 10% of THC) to the variation of the incidence of

psychotic disorders. Moreover, data were fully adjusted for age, gender and ethnicity, level of education, employment status, tobacco use, stimulants, legal highs and hallucinogenics.

Despite all this scientific evidence regarding unspecific psychotic outcomes, whether cannabis use 'causes' schizophrenia has been a matter for debate for decades. The fact that is consistent is that cannabis use may precipitate schizophrenia in people who are genetically vulnerable (Vaucher et al., 2018), by means of a family history of schizophrenia (McGuire et al., 1995), or because expressing subtle previous psychotic symptoms (Verdoux et al., 2003). Importantly, a recent study have demonstrated that adolescents with a high genetic liability to schizophrenia that used cannabis most frequently had lower cortical thickness than those who never used cannabis (French et al., 2015). In this context, it has been suggested an additive interaction between genetic risk state for schizophrenia and lifetime regular cannabis use, that indicates that the etiopathogenesis of schizophrenia involves genetic underpinnings that makes individuals more sensitive to the effects of some environmental exposures such as cannabis use (Guloksuz et al., 2019). There is also evidence that the genetic vulnerability for schizophrenia and for cannabis use partly overlap (Verweij et al., 2017; Aas et al., 2018; Gurriarán et al., 2019).

3.2.2. Controlled studies in humans

The first pharmacological observations with individual compounds present in cannabis date from the 40s (Loewe, 1946), albeit research into the pharmacology of cannabinoids increased markedly in the mid-1960s and early 1970s. Since the synthesis of THC, a huge number of studies have put efforts in elucidating the mechanisms underlying its psychoactive effect. Randomized controlled trials provided evidence that this compound induced a range of transient behavioral and cognitive effects in mentally healthy individuals similar to those seen in schizophrenia and other endogenous psychoses (D'Souza et al., 2004). They have shown that both subjects with schizophrenia and first-degree relatives are more susceptible to its psychotropic effects than healthy controls (D'Souza et al., 2005; Veling et al., 2008; Kahn et al., 2011). Moreover, frequent users of cannabis show blunted responses to the psychotomimetic and cognitive impairing effects of THC but not to its euphoric effects (D'Souza et al., 2008b). Importantly, this group addressed important issues regarding THC psychotomimetic properties, such as the D2R implication in the effects (D'Souza et al., 2008a; Gupta et al., 2019), and its impact on cortical processes such as γ-band neural oscillations, sensory gating and working memory (D'Souza et al., 2012; Cortes-Briones et al., 2015; Skosnik et al., 2018).

Studies evaluating binocular depth inversion illusion (BDII) test, which is a measure of impaired visual processing that occurs in some psychotic states, found that cannabis resin, nabilone and dronabinol (a synthetic form of THC) induce BDII similar to that observed in

acute paranoid schizophrenia or schizophreniform psychotic patients (Emrich et al., 1991; Leweke et al., 2000; Koethe et al., 2006). Additionally, THC has also shown to disturb P300 waves amplitude, which has been related to cognitive impairment (Radhakrishnan et al., 2014).

This experimental evidence suggests that the THC present in cannabis plant may cause biological effects that resemble certain symptoms seen in subjects with schizophrenia. However, further work is still necessary to identify the factors that place individuals at higher risk for chronic cannabis consumption, as well as the biological mechanisms underlying the association between this consumption and schizophrenia.

3.3. MECHANISMS UNDERLYING CANNABIS AND PSYCHOSIS RELATIONSHIP

Apart from epidemiological studies, growing body of literature from pharmacological, genetic and *postmortem* approaches suggests that the consumption of exogenous cannabinoids may be involved in the pathophysiology of psychosis and/or schizophrenia.

Before the discovery of cannabinoid receptors, early studies carried out in humans with different THC analogs (Hollister, 1974) provided the basis for correlating their psychotomimetic potency to a valid proxy in animal models, i.e. drug discrimination (Balster and Prescott, 1992). Subsequent studies led to hypothesize that the reinforcing and psychotropic effects of THC in humans are mediated through its agonist effect on CB1R, although it has modest affinity and low intrinsic activity over the receptor (Wiley et al., 1995; Howlett et al., 2002; Spiller et al., 2019). The question that remains unanswered is: how does their activation disrupt network dynamics and information processing to make psychosis more likely?

The CB1R is one of the most abundant GPCR in the CNS, and it is distributed presynaptically, with high density across many brain regions that are relevant to neural circuitry of psychosis and schizophrenia, such as frontal cortex, basal ganglia, anterior cingulate cortex and hippocampus (Herkenham et al., 1990, 1991; Glass et al., 1997; Egertová and Elphick, 2000).

Across all the major brain structures where endocannabinoids signaling has been explored, both glutamatergic and GABAergic terminals are direct targets for exo- and endocannabinoids, and these compounds are known to modulate these excitatory and inhibitory inputs to dopaminergic neurones. Interestingly, striatal GABAergic projection neurons, cortical interneurons and glutamatergic pyramidal cells are the main cell types that have been recently associated with schizophrenia (Skene et al., 2018).

There is suggestive evidence that the sensitization of the mesolimbic dopaminergic system may be one pathway by which the repeated use of cannabis may be related to the onset of psychotic symptoms. Thus, acute CB1R activation stimulates mesolimbic and mesoprefrontal dopaminergic transmission (French et al., 1997; Pistis et al., 2001; Voruganti et al., 2001). Dopaminergic activity is driven by excitatory and inhibitory inputs arising from numerous afferent structures and interneurons that are filtered by local endocannabinoid signaling, and CB1R on GABAergic terminals can facilitate this activity through suppression of inhibitory input on dopaminergic neurons (Covey et al., 2017).

Negative effects of cannabinoids in working memory and other cognitive processes have been related to a disruption of neural synchronization in the PFC (Gonzalez-Burgos and Lewis, 2008). Activation of CB1Rs by cannabinoids in cortical interneurons inhibits GABA release, and may suppress the control that they exert over pyramidal neurons, thereby interfering with associative functions, disrupting normal gating mechanisms and resulting in poor integration of cortical inputs (Pistis et al., 2001). Cannabinoids, indeed, have also been shown to influence glutamatergic synaptic transmission and plasticity in the PFC (Auclair et al., 2000).

As said before, all of these effects are mainly driven by cannabinoid action upon CB1R. This receptor is known to downregulate after chronic activation (Sim-Selley et al., 2006), and cannabis dependent subjects have shown decreased brain CB1R availability (D'Souza et al., 2016). However, this downregulation is thought to be reversible and return to control values after around three weeks of cannabis abstinence in humans and rodents, although behavioral and molecular sensitization have also been reported (Rubino et al., 2001, 2003; Tournier et al., 2016). In this regard, physiological neurodevelopmental processes especially of the frontal cortex and limbic system, that occur during adolescence (Giedd et al., 1999), are thought to be more sensitive to exogenous disturbances such as cannabis use (Fuhrmann et al., 2015). Indeed, the endocannabinoid system seems to have a crucial role in these neurodevelopmental processes (Meyer et al., 2018), as cannabis has particularly deleterious effects during this period (Ashtari et al., 2011; Rubino and Parolaro, 2016).

In summary, it has been shown that exposure to cannabinoids induces several alterations in the functioning of the brain in CB1R enriched regions and in neuromodulator systems relevant to cognition. Alterations in the functionality of the endocannabinoid system, such as receptor downregulation, de-/sensitization and downstream effector changes accompanying the resultant regional neuroadaptations are supposed to underlie cognitive effects in relation with chronic cannabis use. Considering that cannabis regular use is highly prevalent in the population with psychosis, investigations of the factors influencing worse outcomes and early onset are needed in order to advance the search of new treatment options.

These studies suggest that a deregulation of the endocannabinoid system can indeed interact with neurotransmitter systems that are already known to underlie schizophrenia (Fernandez-Espejo et al., 2009; Volk and Lewis, 2016; Fakhoury, 2017).

Studies regarding endocannabinoid system in schizophrenia

Genetic and *postmortem* studies in the brain of subjects with schizophrenia have provided additional evidence for the role of the endocannabinoid system in psychotic and schizophrenia-like symptoms. All the evidences have been extensively reviewed in the Manuscript presented in the **Annex** section of this Doctoral Thesis, and the main findings are the following:

Studies addressing CB1R availability or density on the brain of subjects with schizophrenia are inconclusive, showing a decrease (Eggan et al., 2008, 2010; Ranganathan et al., 2016), an increase (Jenko et al., 2012; Ceccarini et al., 2013; Volk et al., 2014) or no changes (Deng et al., 2007) of this receptor in different regions of the brain. Several confounding factors, including antipsychotic treatment (Urigüen et al., 2009), radiotracer used in the experiments, or cannabis and tobacco use (Ranganathan et al., 2016) are likely underlying the contradictory outcomes. A recent study addressing the functionality of CB1R has not found differences in this regard (Muguruza et al., 2019). However, further research is needed to understand the potential pathophysiological consequences of alterations in CB1R.

Endocannabinoid levels and the enzymes that regulate them have also been studied in the brain of subjects with schizophrenia. While most of the studies of the enzymes are restricted only to mRNA measures, an interesting report has found an increased activity of the enzyme FAAH (Muguruza et al., 2019) in the PFC of these subjects. Additionally, alterations in the levels of the main endocannabinoids, AEA and 2-AG, are evident in this area and others, such as hippocampus and cerebellum of schizophrenia subjects (Muguruza et al., 2013a). These reports point to an imbalance of the endocannabinoid system in schizophrenia, although more studies are needed in order to understand the specific role that this system may have on schizophrenia pathogenesis.

Moreover, certain alleles of the *CNR1* gene have shown to increase susceptibility to schizophrenia, as well as cognitive impairment related to cannabis misuse among subjects with schizophrenia (Ujike et al., 2002; Ho et al., 2011).

Despite all these evidences, still more scientific evidence is needed to fully elucidate the mechanisms by which the endocannabinoid system imbalances are associated with psychotic and schizophrenia-like symptoms.

4. 5-HT2A RECEPTORS

4.1. GENERAL ASPECTS

The serotonin 5-HT2A receptor subtype is a member of the rhodopsin-like or class A superfamily of GPCR. This receptor is a member of the 14 5-HT receptors, classified in seven families based on their structural, operational and transductional features, namely: 5-HT1 (5-HT1A, 5-HT1B, 5-HT1D, 5-HT1E, and 5-HT1F), 5-HT2 (5-HT2A, 5-HT2B, and 5-HT2C), 5-HT3, 5-HT4, 5-HT5 (5-HT5A, 5-HT5B), 5-HT6, and 5-HT7 (see **Figure 8**). All these receptors are differentially expressed throughout the organism, partly explaining the pharmacological complexity of 5-HT action (Hoyer et al., 2002). So far, it is well established the fundamental role of serotonin and its receptors in mood control (Jenkins et al., 2016), food intake (Halford et al., 2005) or blood pressure regulation (Watts et al., 2012). Moreover, serotonin receptors have been related to many diseases, such as depression, anxiety, schizophrenia, obsessive–compulsive and panic disorders, migraine or hypertension (Pithadia, 2009).

Receptor family	Subtypes	Type	Functions
5-HT1	5-HT1A 5-HT1B 5-HT1D 5-HT1E 5-HT1F	G protein-coupled receptor	Inhibitory auto- and heteroreceptor
5-HT2	5-HT2A 5-HT2B 5-HT2C	G protein-coupled receptor	Excitatory heteroreceptor
5-HT3		Ligand-gated ion channel	Excitatory heteroreceptor
5-HT4		G protein-coupled receptor	Excitatory heteroreceptor
5-HT5	5-HT5A 5-HT5B	G protein-coupled receptor	Inhibitory
5-HT6		G protein-coupled receptor	Excitatory
5-HT7		G protein-coupled receptor	Excitatory

Coupled to Gαi/o
Coupled to Gαq/11
Coupled to Gαs
Ion channel

Figure 8. 5-HT receptor subtypes and their mostly reported functions.

The 5-HT2AR is encoded by the *HTR2A* gene, which has been located on human chromosome 13q14 –q21 and comprises of 471 amino acids in rats, mice and humans (Stam et al., 1992).

In the human CNS, 5-HT2AR is abundantly expressed in several brain cortical areas, especially among layers III and V, and the hypothalamus. To a lesser extent, it is also expressed in the hippocampus and striatal structures (Pazos et al., 1987). A revealing study addressing specific location of these receptors in the PFC points predominantly to a postsynaptic location in the proximal apical dendrites of pyramidal neurons (Jakab and Goldman-Rakic, 1998), where they are suggested to play a crucial role on gating mechanisms implicated in latent inhibition processes and working memory. Although in a lower proportion, 5-HT2AR have also been identified on parvalbumin expressing GABAergic interneurons of the middle layers (Williams et al., 2002; Puig and Gulledge, 2011).

In the periphery, 5-HT2AR is present in gastrointestinal tract, vascular and bronchial smooth muscle and blood platelets, where it mediates several functions such as contraction and platelet aggregation (De Clerck et al., 1984; Fiorica-Howells et al., 2002; Cogolludo et al., 2006).

4.2. INTRACELLULAR SIGNALING

The effector that has been best characterized in 5-HT2AR signaling involves the stimulation of Gαq/11 heterotrimeric G-proteins. Upon activation, it promotes PLC-mediated catalysis of the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) to IP3 and DAG, thus activating protein kinase C (PKC) and elevating cytosolic Ca^{2+} (Conn and Sanders-Bush, 1987; Carr et al., 2002). Although this signaling was the first to be established, 5-HT2AR is now known to activate several other signaling pathways that may involve other G proteins and alternative direct coupling to the receptor (Abbas and Roth, 2008). For example, it mediates the release of arachidonic acid (AA) independently of PLC, and presumably through the activation of phospholipase A2 (PLA2) (Felder et al., 1990). This AA release is mediated by a complex mechanism that involves signaling through RhoA and p38 and p42/44 MAPK, as well as ERK signaling, all of them depending on Gα proteins different from Gαq/11, such as Gα12/13 and Gαi/o protein subtypes (Kurrasch-Orbaugh et al., 2003). Studies in cells also suggest that 5-HT2AR interacts with adenosine diphosphate-ribosylation factor 1 (ARF1), leading to the functional activation of phospholipase D (PLD) (Mitchell et al., 1998; Robertson et al., 2003). Interestingly, another study has demonstrated that 5-HT2AR activation also leads to the formation of 2-AG, an endocannabinoid whose formation is partially dependent on the activation of PLC (Parrish and Nichols, 2006).

In cell cultures, 5-HT2AR also has important functional interactions with some proteins, such as the ribosomal protein S6 kinase 2 (S6K2), which seems to induce a 'tonic-brake' in signaling and alter agonist functional selectivity directly phosphorylating the receptor in the i3 loop (Sheffler et al., 2006; Strachan et al., 2009, 2010). Moreover, 5-HT2AR also interacts with caveolin-1 in smooth muscle tissue (Bhatnagar et al., 2004), arrestins and postsynaptic density protein 95 (PSD-95) in cortical neurons (Schmid et al., 2008; Abbas et al., 2009), which are known to modulate 5-HT2AR functionality *in vivo* and internalization processes.

Importantly, human and animal studies have reported that 5-HT2AR mediates the hallucinogenic response of some psychedelic drugs, such as DOI and psilocybin (Willins and Meltzer, 1997; Vollenweider et al., 1998; Nichols, 2004; González-Maeso et al., 2007; López-Giménez and González-Maeso, 2017). Moreover, cortical expression of 5-HT2AR seems to be sufficient to induce these hallucinogenic effects (González-Maeso et al., 2007). Conversely, other 5-HT2AR agonists, such as lisuride, do not induce similar neuropsychological responses (Egan et al., 1998; Shan et al., 2012). These data raised the idea that the different 5-HT2AR agonists exhibit functional selectivity over this receptor in the brain cortex.

Some studies in animals have suggested that 5-HT elicits its responses on 5-HT2AR by assembling proteins such as β–arrestin2, Src and Akt, while hallucinogenic 5-HT2AR agonists do not require this complex recruitment (Schmid and Bohn, 2010). Interaction with the scaffolding protein PSD-95 has also been suggested to be essential for the hallucinogenic response of 5-HT2AR agonists (Abbas et al., 2009). Interestingly, hallucinogenic agonists such as DOI and LSD seem to show some similarities in the cortical transcriptome fingerprint (i.e. the mRNA translation modifications that they exert) which differs from the one shown by behaviorally inactive drug lisuride (Gonzalez-Maeso et al., 2003). Moreover, the differential gene regulation that these hallucinogens exert is mediated by Gαi/o protein subtypes (González-Maeso et al., 2007).

4.3. 5-HT2AR ALTERATIONS IN SCHIZOPHRENIA

Several reports have focused on the study of 5-HT2AR in schizophrenia. Whereas 5-HT was suggested to play a role in schizophrenia on the 50s (Woolley and Shaw, 1954), the main reason for the focus on this receptor has been that atypical antipsychotics block it with high affinity (Meltzer et al., 1989b). Moreover, as indicated before, 5-HT2AR mediates the psychotic-like states exerted by some drugs, such as psilocybin, LSD or DOI, in both humans and rodents (Vollenweider et al., 1998; González-Maeso et al., 2007).

It has been proposed that if 5-HT2AR is central to the pathology of schizophrenia, some genetic associations regarding *HTR2A* gene and the illness should occur. In this way, a

number of studies have shown that mutations in this gene and/or its promoter are associated with schizophrenia (Inayama et al., 1996; Sáiz et al., 2007; Gu et al., 2013), although none of them seem to replicate consistently over distinct populations (Ohara, 1997; Petronis, 2000). Indeed, GWAS carried out in schizophrenia did not find statistical significance for any variation of this gene (Farrell et al., 2015).

Several reports have shown changes in 5-HT2AR expression and density in *postmortem* tissue from subjects with schizophrenia, showing different results. In this context, a decrease (Burnet et al., 1996; Matsumoto et al., 2005; Dean et al., 2008b; Kang et al., 2009), no alterations (Reynolds et al., 1983; Laruelle, 1993) or even an increase (Marazziti et al., 2003; González-Maeso et al., 2008; Muguruza et al., 2013b) of 5-HT2AR expression or density in different regions of the brain cortex have been described. All these conflicting reports are in line with the observation that complex changes in 5-HT2AR density seem to arise because of both the pathology itself and the chronic antipsychotic drug treatment (Selvaraj et al., 2014). Apart from these studies carried out in the brain cortex, there are also evidences of abnormalities of 5-HT2AR in the hippocampus (Burnet et al., 1996; Scarr et al., 2004), striatum (Joyce et al., 1993) or cerebellum (Eastwood et al., 2001) of subjects with schizophrenia.

Several studies have assessed cortical 5-HT2AR density by using selective radiotracers, such as [^{3}H]ketanserin and [^{18}F]altanserin. Studies using [^{3}H]ketanserin have shown a decrease or no changes in cortical 5-HT2AR density (Dean et al., 1998, 2008a; Pralong et al., 2000). It is important to note that this receptor is known to downregulate with chronic treatments with atypical antipsychotics, such as clozapine and risperidone (Gray and Roth, 2001; Van Oekelen et al., 2003; Roth, 2011; García-Bea et al., 2019). Most revealing studies using [^{3}H]ketanserin and accounting for antipsychotics presence in the subjects brain have shown that 5-HT2AR density is indeed increased only in those subjects with schizophrenia which tested negative for antipsychotics in the brain (González-Maeso et al., 2008; Muguruza et al., 2013b).

Conversely, *in vivo* PET studies using [^{18}F]altanserin lead to the opposite conclusion, suggesting a decreased 5-HT2AR density in the brain cortex of drug-naïve FEP subjects (Erritzoe et al., 2008; Rasmussen et al., 2010). Furthermore, in an interesting study carried out with monozygotic twins discordant for schizophrenia, a decreased cortical 5-HT2AR density was described as a state marker (Rasmussen et al., 2016).

From a pharmacological point of view, these apparently discrepant conclusions can be unified. Selective antagonists are probably the most suitable compounds in order to measure the total density of a receptor. Since they do not bind preferentially to either inactive or active

conformational state, these compounds will bind to both receptor subpopulations in a certain tissue to a similar extent. However, both agonists and inverse agonists display a preference for specific subpopulations of a receptor (active and unactive conformational state, respectively) (Kenakin, 2011).

Biochemical findings in *postmortem* frontal cortex suggest that [^{18}F]altanserin behaves as a 5-HT2AR inverse agonist in schizophrenic subjects (Muguruza et al., 2013b). Together, these data suggest that downregulation of [^{18}F]altanserin binding sites may be showing less 5-HT2AR in an inactive conformational state, whereas upregulation of [^{3}H]ketanserin binding sites would correlate better with an increased total density of 5-HT2AR. In line with these observations, studies using the agonist DOI have shown an upregulation of the precoupled 5-HT2AR population (Muguruza et al., 2013b), as well as a hyperfunctionality of these receptors in the cortex of subjects with schizophrenia (García-Bea et al., 2019).

Regarding this recent study, cortical 5-HT2AR of subjects with schizophrenia shows an interesting overactive signal trafficking specifically towards Gαi1, whereas the canonical Gαq signaling pathway of this receptor remains unaltered (García-Bea et al., 2019).

Platelets have been also used as a peripheral marker to investigate alterations in neurotransmitter receptors and regulatory mechanisms in psychiatric disorders (García-Sevilla et al., 2004). Pharmacological properties of 5-HT2AR from platelets seem to be similar to those from brain (Leysen et al., 1983). Some studies evaluating the density of this receptor have consistently pointed out an increased density of 5-HT2AR in platelets of subjects with schizophrenia. However, results concerning the effects of antipsychotic treatment are more controversial, and an increase, a decrease or no changes have been reported after these treatments (Arora and Meltzer, 1993; Pandey et al., 1993; Govitrapong et al., 2000; Arranz et al., 2003).

As previously mentioned, chronic antipsychotic treatment is the major confounding factor on the studies assessing 5-HT2AR in schizophrenia. In this regard, the atypical phenomenon of paradoxical downregulation has been described for this receptor. In contrast to most other GPCRs, chronic blockade of 5-HT2AR has consistently reported to lead to a downregulation of the receptor (Gray and Roth, 2001; Van Oekelen et al., 2003; García-Bea et al., 2019), although the exact nature of the mechanism underlying this atypical downregulation remains poorly understood. In this sense, some, but not all, antipsychotic and antidepressant drugs are able to induce a decrease in 5-HT2AR density after a chronic administration. Thus, it is unclear whether this effect is due to a direct action at 5-HT2AR or via some other molecular interactors (Roth, 2011).

Overall, the reported data about 5-HT2AR in schizophrenia suggest that the modulation of this receptor is complex and may be related to both the pathology itself and the effects of antipsychotic drugs (Dean, 2003).

5. THE Akt/mTOR PATHWAY

5.1. GENERAL ASPECTS

Akt proteins, also known as protein kinase B, are one of the most versatile kinases in the human kinome. They are serine/threonine kinases that play a key role in several crucial aspects of cell physiology, such as cell cycle and survival, cell growth and proliferation, and glucose metabolism. Thus, its signaling has been classically related to prevention of apoptosis and cancer progression (Kennedy et al., 1997; Chang et al., 2003; Manning and Cantley, 2007). The Akt kinase family comprises three isoforms - Akt1 (PKBα), Akt2 (PKBβ), and Akt3 (PKBγ) - encoded by three different genes, *AKT1*, *AKT2* and *AKT3*, which are located in 14q32, 19q13.1-q13.2 and 1q43-q44 respectively (Coffer et al., 1998; Masure et al., 1999), and whose expression vary among different tissues. Akt1 is ubiquitously expressed, and its deficiency has been mechanistically related to alterations in PFC and working memory deficits (Lai et al., 2006). Akt2 is predominantly expressed in brown fat, heart and skeletal muscle (Matheny et al., 2018), correlating with its role in diabetes and glucose metabolism. Akt3 is most abundantly expressed in the brain, and has an important role in attaining normal brain size (Yang et al., 2005).

Signaling of Akt is one of the main outcomes of the activation of PI3K, and mediates a plethora of intracellular pathways that can be activated in response to different extracellular stimuli including growth factors, nutrients and insulin (Burgering and Coffer, 1995). Akt can bind to several targets by virtue of an amino-terminal pleckstrin homology (PH) domain, such as phosphatidylinositol-3,4,5-trisphosphate (PIP3) produced by PI3K or phosphoinositide-dependent protein kinase 1 (PDK1) recruited to the plasma membrane by PIP3. Several stimuli have been demonstrated to activate PI3K, including α/γ subunits of heterotrimeric G proteins coupled to GPCR. This activation leads to Akt recruitment and activation (Murga et al., 1998). Once recruited to the plasma membrane, Akt is phosphorylated at two sites (Thr308 residue by the PDK1 and Ser473 by the mechanistic target of rapamycin (mTOR) complex 2). Once activated, Akt phosphorylates substrates distributed throughout the cell to regulate multiple cellular functions (Manning and Cantley, 2007b) (see **Figure 9**).

In neurons, the activation of Akt is known to be a key outcome which regulates a wide variety of processes such as neural survival and architecture, axonal growth or synaptic strength control (Dudek et al., 1997; Wang et al., 2003; Huang et al., 2019).

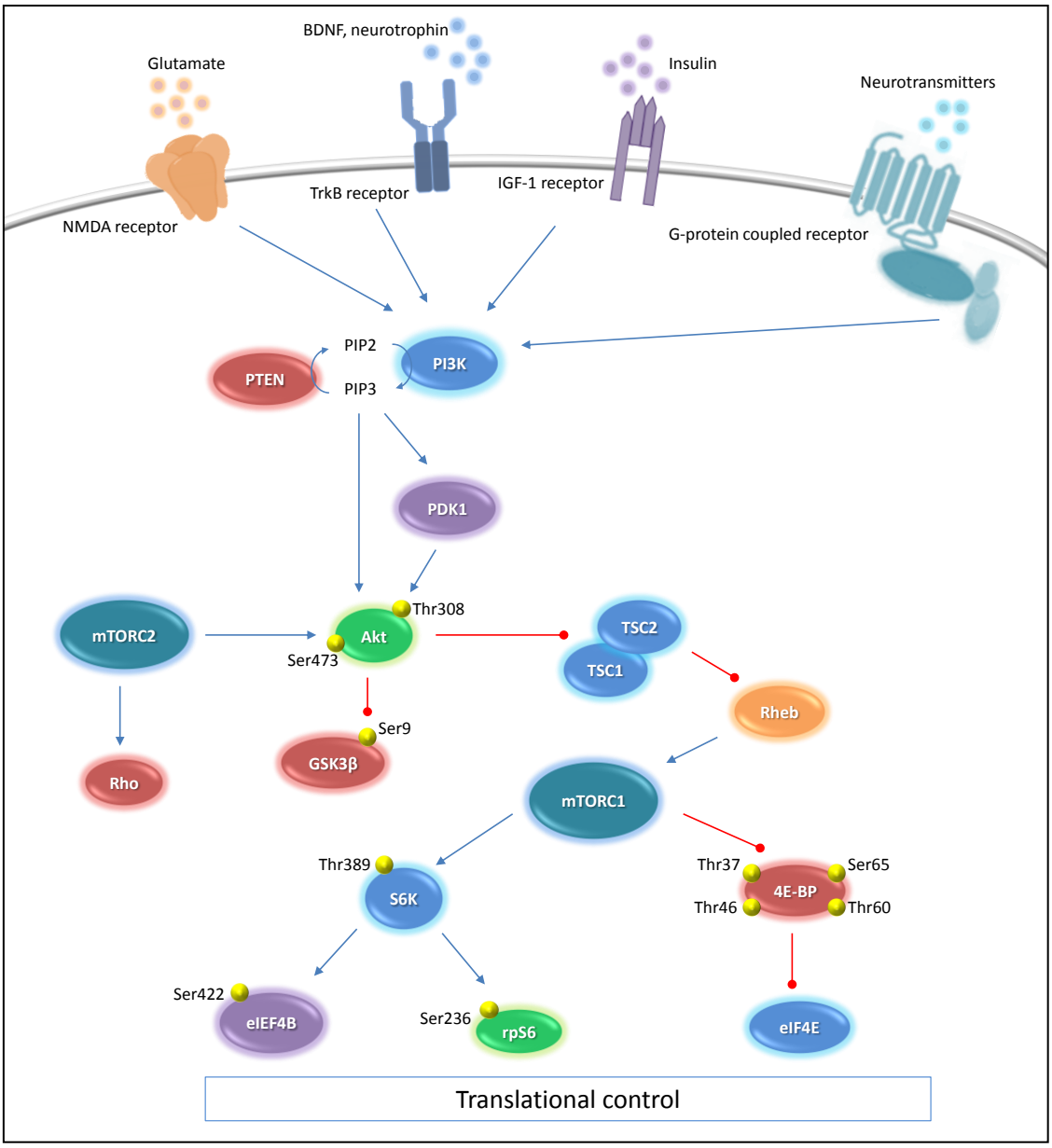


Figure 9. Regulation of the mTOR signaling pathway in the brain. mTORC1 is activated by receptor signaling through the PI3K–Akt pathway. Through its downstream effectors the 4E-BPs and S6K1 and 2, mTORC1 controls neuronal protein synthesis. Text and illustration inspired by Costa-Mattioli et al., 2013.

Once activated, Akt further stimulates mTOR complex 1 through inhibition of either tuberous sclerosis complex (TSC1/2), or proline-rich Akt substrate of 40 kDa (PRAS40), abolishing the tonic inhibition that they exert upon the complex. mTOR is a large (2549 amino acids, ~250 kDa), ubiquitously expressed multi-effector serine/threonine kinase that belongs to the PI3K-related kinase family and interacts with several proteins to form two distinct heteromeric complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The mTOR pathway integrates various external signals and controls diverse cellular processes including translation, apoptosis, autophagy, energy metabolism and cell growth via the assembly of these multi-protein signaling complexes. Both mTOR complexes consist of numerous proteins that control mTOR signaling, dictate subcellular localization, and regulate substrate

specificity; with mTORC1 having six and mTORC2 seven known protein components (Laplante and Sabatini, 2012). The mTOR-containing complexes have different sensitivities to rapamycin as well as upstream inputs and downstream outputs. While mTORC1 is acutely sensitive to rapamycin, chronic exposure to this compound also leads to mTORC2 disruption (Sarbassov et al., 2006).

The mTORC1 is the better characterized of the two mTOR complexes and its most remarkable feature is the number and diversity of upstream signals it senses. As previously mentioned, activation of mTORC1 is mediated mainly by Akt. By integrating inputs from major intracellular and extracellular cues, the best-characterized function of mTORC1 is the regulation of translation and protein synthesis, where it regulates two critical core components of the translation initiation machinery: p70 ribosomal protein S6 kinases (S6K) and the eukaryotic translation initiation factor 4E-binding proteins (4E-BPs). Once active, mTORC1 stimulates these two main downstream substrates, leading to the activation of ribosomal protein S6 (rpS6) and eukaryotic translation initiation factor 4E (eIF-4E) respectively, both involved in the translational machinery for protein synthesis (Costa-Mattioli and Monteggia, 2013; Meyuhas, 2015).

The mTOR signaling has emerged as a critical integrator of synaptic inputs that in turn affects many cellular processes, including autophagy, protein synthesis, transcription, actin dynamics and neuronal morphology (Laplante and Sabatini, 2012). The increasing availability of mTOR inhibitors, such as rapamycin, has enabled to decipher most of what we know about mTOR function in brain disorders. So far, the mTOR pathway has been involved in several aspects of neural development and function including neuronal growth, maintenance and proliferation, dendrite and synapse formation, axonal elongation and plasticity (Hoeffer and Klann, 2010; Kitagishi et al., 2012; Takei and Nawa, 2014). RpS6 phosphorylation is considered as a readout of mTORC1 activity, and it has been recently proposed that rpS6 could participate in the regulation of global translation in the CNS (Puighermanal et al., 2017).

During the last years, many studies have reported abnormalities in the expression and/or activity of its upstream and downstream components in several neurodevelopmental and neuropsychiatric disorders, such as autism spectrum disorders, drug addiction, intellectual disability, major depressive disorder, and schizophrenia (Costa-Mattioli and Monteggia, 2013). In this context, mTORC1 pathway has been shown to be compromised in the PFC of patients with major depressive disorder (Jernigan et al., 2011). In addition, it seems to mediate the therapeutic efficacy of the fast-acting antidepressant ketamine, enhancing the synthesis of excitatory synaptic proteins and the number of dendritic spines in the PFC (Li et al., 2010; Abdallah et al., 2015).

5.2. Akt/mTOR PATHWAY ALTERATIONS IN SCHIZOPHRENIA

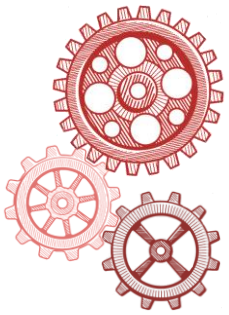
The aetiology of schizophrenia involves a significant neurodevelopmental component which causes the CNS to become particularly susceptible to the effects of various extrinsic risk factors (van Os et al., 2010). It has been suggested that neurodevelopmental causes comprise complex neuromolecular mechanisms that affect normal growth and functioning of the brain. However, the mechanisms underlying the abnormal brain development that increases the risk for developing schizophrenia are still unknown. Diverse molecules that have been previously implicated in schizophrenia, such as glutamate, BDNF or 5-HT, can lead to either over-activation or inhibition of Akt/mTOR signaling pathway. All of the knowledge that has been collected during the last years regarding the role of Akt/mTOR pathway in the CNS leds to hypothesize that alterations in mTOR pathway could have an etiopathological role in schizophrenia (Gururajan and van den Buuse, 2014; English et al., 2015; Pham et al., 2016). However, literature addressing the status of this pathway in the brain of these subjects is scarce.

The first kinase of this pathway that started drawing the attention in the context of schizophrenia since the first discover in 2004 (Emamian et al., 2004a) was Akt. Nowadays, there are several genetic case-control and cohort studies demonstrating an association between certain SNPs - rs1130214, rs10149779, rs3730358 among others-- in *AKT1* gene and schizophrenia in several populations (Schwab et al., 2005; Thiselton et al., 2008; Mathur et al., 2010), although there are also some negative studies mainly in asian populations (Ohtsuki et al., 2004; Loh et al., 2013; Liu et al., 2016). Other human studies have proven that rs1130233 and rs1130214 *AKT1* allelic variants are associated with cognitive impairments, as well as neuroanatomical and functional abnormalities in the PFC (Tan et al., 2008; Pietiläinen et al., 2009). Despite this genetic evidence, literature addressing Akt protein expression or phosphorylation in human brain is more scarce and show discrepant results, showing both a decrease (Emamian et al., 2004a; Thiselton et al., 2008; McGuire et al., 2017; Chadha and Meador-Woodruff, 2020) and an increase (Hino et al., 2016) in this kinase levels or/and functionality. In the same way, other studies carried out in PBMC have shown similar discrepancies (Blasi et al., 2011; Kumarasinghe et al., 2013).

Studies in animals have demonstrated that the signaling towards this pathway can be modulated in the brain cortex by certain antipsychotics (Kozlovsky et al., 2006; Bowling et al., 2014; Liu et al., 2017), although little is known about their chronic effects in subjects with schizophrenia, or whether it is involved in the therapeutic effects of these drugs.

It has been also proposed that the dysfunction of mTORC1 pathway may contribute to an aberrant dendritic organization and a loss of dendritic spines that could lead to a connectivity dysfunction (Dwyer et al., 2013). Interestingly, the only report studying mTOR

pathway in the PFC of subjects with schizophrenia points to a downregulation of both mTORC1 and mTORC2 (Chadha and Meador-Woodruff, 2020). Nonetheless, whether this downregulation translates into less phosphorylated substrates has not been addressed yet.



HYPOTHESIS AND OBJECTIVES

Hypothesis

Chronic THC exposure, especially in specific brain developmental periods such as adolescence, may precipitate the onset of schizophrenia in vulnerable subjects by altering 5-HT2A receptors expression and/or signaling. Likewise, these effects could be mainly mediated by an aberrant Akt/mTOR pathway status.

To prove this hypothesis, the following objectives were set up:

General objective

To study the molecular mechanisms involved in the cannabis-induced increased risk of developing schizophrenia.

Objective 1.

To characterize G-protein subtype stimulation pattern triggered by three different cannabinoid ligands, Δ^9-THC, WIN55,212-2 and ACEA in mouse brain cortex.

Objective 2.

To evaluate the effects of chronic THC administration on psychosis-like symptoms as well as on cortical 5-HT2AR status and functionality in young mice.

Objective 3.

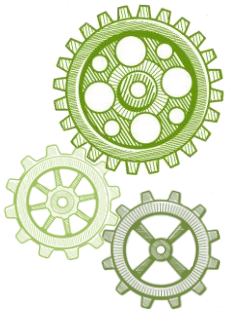
To study the involvement of the Akt/mTOR signaling pathway in the behavioral and molecular effects induced by chronic THC administration in young mice.

Objective 4.

To evaluate the Akt/mTOR signaling pathway status in the *postmortem* prefrontal cortex of subjects with schizophrenia and, subsequently, the effects of antipsychotic treatment on this pathway.

Objective 5.

To evaluate 5-HT2AR protein expression and Akt/mTOR signaling pathway status in blood platelets of subjects with schizophrenia, subjects with cannabis use disorder, and subjects with a dual diagnosis of cannabis use disorder and schizophrenia, and their matched controls.



MATERIALS AND METHODS

1. HUMAN SAMPLES

Two types of human samples have been used along this Doctoral Thesis, *postmortem* brain and blood platelets. This section will focus mainly on information shortly provided in the articles included in the Thesis. The reader is referred to them for obtaining further information.

1.1. HUMAN *POSTMORTEM* BRAIN SAMPLES

Human brain samples were obtained at autopsies performed in the Basque Institute of Legal Medicine (Bilbao, Spain), in compliance with policies of research and ethical boards for *postmortem* brain studies.

A toxicological screening for antidepressants, antipsychotics, other psychotropic drugs, opiates and ethanol present in blood was carried out at the time of death either at the Basque Institute of Legal Medicine or at the National Institute of Toxicology (Madrid, Spain). Screening assays consisted on standard procedures including radioimmunoassay, enzymatic immunoassay, high-performance liquid chromatography and gas chromatography-mass spectrometry. Samples corresponded to the left DLPFC (Brodmann´s area 9) of each subject were dissected at the time of autopsy. After dissection, the tissue was immediately stored at -70ºC until assay.

A total of 56 subjects were selected for this study, based on retrospective searching for previous medical diagnosis and treatment using examiner's information and records from hospitals and mental health centers. Brain samples of 28 subjects with an *antemortem* diagnosis of schizophrenia according to the Diagnostic and Statistical Manual of Mental Disorders DSM-IV or DSM-IV-TR (American Psychiatric Association, 1994, 2000) or ICD-10 criteria (World Health Organization, 1993) were matched to samples of 28 control subjects. Control subjects were chosen based on the absence of diagnosis of neuropsychiatric disorders or drug abuse, and an appropriate gender, age, and *postmortem* interval (PMI) (time between death and tissue freezing) to match each subject in the schizophrenia group.

Schizophrenia subjects (SZ) were subdivided into two groups according to the absence or presence of antipsychotic drugs in blood at the time of death, and classified as antipsychotic-free (AP-F) (n=17) and antipsychotic-treated (AP-T) (n=11).

Samples collection and medical diagnosis searching were confidential at any time. In all the experiments, each subject ascribed to the schizophrenia group was processed in parallel to a matched control subject. A table of group averages of ages, *postmortem* interval and storage time of all the subjects included in this Thesis is available in the **Article 3**, **Table 1**. A demographic full description of all the subjects with schizophrenia and their individually

matched controls is presented in **Supplementary Table 1** and **Supplementary Table 2** of the **Article 3**.

1.2. HUMAN PLATELETS

Platelets were obtained from total blood. Extraction by venipuncture was performed by nurses at the Uribe Mental Health Centre (Getxo, Spain) of the Basque Health Service (Osakidetza) or at the Faculty of Medicine and Nursery of the University of the Basque Country (UPV/EHU) (Leioa, Spain).

A total sample size of 76 subjects (38 cases and 38 controls) was included in this study. All the participants gave written, witnessed, informed consent for the participation in the study. Psychiatrists previously diagnosed all the case subjects included in the study. Subjects who met inclusion criteria for schizophrenia and/or a cannabis use disorder based on Structured Clinical Interview for DSM-IV, DSM-IV-TR or ICD-10 criteria were included in the study. Subjects were subsequently classified as subjects with schizophrenia (SZ) (n=14), subjects with cannabis use disorder (CUD) (n=16), or subjects with a dual diagnosis (DUAL) (n=8) if meeting both diagnostic criteria. Control subjects (n=38) were recruited based on appropriate gender and age in order to match case samples. Exclusion criteria for controls included a history of any neuropsychiatric disorder or any use of cannabis in the last two years prior to the blood sampling.

General information regarding the demographic characteristics of the three experimental groups can be found in the **Article 4**, **Table 1**. A more complete description of the characteristics of all the cases and their matched controls included in the study is available on **Supplementary Tables 1** to **3** of the **Article 4**.

2. ANIMALS

Rats and mice were used along the development of this Doctoral Thesis.

Male Sprague-Dawley rats (220-320 g) were obtained and maintained at the Animal Facilities of the University of the Basque Country (Leioa, Spain). Animals were housed on a 12 h light/dark cycle at 22ºC and 60% humidity with free access to food and water. Animal care and all experimental protocols were performed in agreement with European Union regulations (Directive 2010/63/EU) and approved by the Ethic Committee for Animal Welfare of the University of the Basque Country, UPV/EHU (CEBA 188/2011).

Male CD-1 mice (Charles Rivers, Wilmington, MA, USA) were maintained at the Animal Facilities of the University of the Basque Country and housed (6-8 animals per cage) at 23°C, on a 12 h light/dark cycle, with free access to food and water. All experimental procedures

were performed in accordance with the European Union Directive 2010/63/EU and approved by the Ethic Committee for Animal Welfare of the University of the Basque Country (UPV/EHU) (CEBA 270M/2012, 189/2011).

Male wild-type C57BL/6xCBA mice, CB1-/-, CB2-/- and CB1-/-/CB2-/- (aged 2 months-old) were housed (6–8 animals per cage) at 23°C, on a 12 h light/dark cycle, with free access to food and water at the Animal Facilities of the University of the Basque Country. Experimental procedures were carried out in accordance with the European Union Directive #86/606/EEC and approved by the Ethics Committees for Animal Welfare of the University of the Basque Country (UPV/EHU) (CEBA1882011) and by the Institutional Review Board (INIA) (CEEA2012/021). CB1-/- (Marsicano et al., 2002) and CB2-/- (Buckley et al., 2000) mice were kindly donated by Dr. Ismael Galve-Roperh and Dr. Guillermo Velasco (Universidad Complutense de Madrid, Madrid, Spain), and were crossed to establish the CB1-/-/CB2-/- mouse model.

3. DRUGS AND TREATMENTS

3.1. DRUGS

- **Clozapine:** 8-Chloro-11-(4-methylpiperazin-1-yl)-5H-dibenzo[b,e][1,4]diazepine (Tocris Biosc., Bristol, UK).
- **Risperidone:** 3-[2-[4-(6-fluoro-1,2-benzoxazol-3-yl)piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydropyrido[1,2-a]pyrimidin-4-one (Sigma Aldrich®, Saint Louis, MO, USA).
- **Haloperidol:** 4-[4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl) butan-1-one (Sigma Aldrich®, St. Louis, MO, USA).
- **(±)-DOI:** (±)-1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (Sigma Aldrich®, St. Louis, MO, USA).
- **M100907:** (R)-(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-pipidinemethanol (Sigma Aldrich®, St. Louis, MO, USA).
- **WIN55,212-2:** [(3R)-5-Methyl-3-(4-morpholinylmethyl)-2,3-dihydro[1,4] oxazino[2,3,4-hi]indol-6-yl](1-naphthyl)methanone methanesulfonate (1:1) (Sigma Aldrich®, St. Louis, MO, USA).
- **ACEA:** (5Z,8Z,11Z,14Z)-N-(2-Chloroethyl)-5,8,11,14-icosatetraenamide (Tocris Biosc., Bristol, UK).
- **Δ^9-THC:** (6aR,10aR)-6,6,9-Trimethyl-3-pentyl-6a,7,8,10a-tetrahydro-6H-benzo[c] chromen-1-ol (THCPharm, Frankfurt, Germany).
- **O-2050:** N-{6-[(6aR,10aR)-1-Hydroxy-6,6,9-trimethyl-6a,7,10,10a-tetrahydro-6H-benzo [c]chromen-3-yl]-4-hexyn-1-yl}methanesulfonamide (Tocris Biosc., Bristol, UK).

- **Rapamycin:** (1R,9S,12S,15R,16E,18R,19R,21R,23S,24E,26E,28E,30S,35R)-1,18-Dihydroxy-12-{(2S)-1-[(1S,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-2-propanyl}-19,30-dimethoxy-15,17,21,23,29,35-hexamethyl-11,36-dioxa-4-azat;ricyclo[30.3.1.04,9] hexatriaconta-16,24,26,28-tetraene-2,3,10,14,20-pentone (Selleck Chemicals, Houston, TX, USA).

3.2. CHRONIC ANTIPSYCHOTIC TREATMENTS IN RATS

Rats (n=10 per group) were treated twice-a-day intraperitoneally (i.p.), with saline (1 ml/kg), clozapine (5 mg/kg), risperidone (0.5 mg/kg) or haloperidol (0.5 mg/kg) during 21 days. After 48 hours (clozapine and risperidone) or 72 hours (haloperidol and saline) washout-period, rats were sacrificed, brains removed and cortex dissected and stored at -70ºC until assays.

3.3. ACUTE AND CHRONIC TREATMENTS IN MICE

For acute treatments, a single dose of (±)DOI (0.5 mg/kg) or saline (5 ml/kg) was administered i.p. immediately before behavioral assay.

For chronic treatments, mice were treated i.p. with vehicle (5 ml/kg), THC (10 mg/kg) or rapamycin (5 mg/kg) during 30 days from postnatal day 21. Drugs were dissolved in ethanol:cremophor:saline (0.9% NaCl) at 1:1:18 ratio. THC and vehicle were injected daily, whereas rapamycin was administered 4 days/week in an on/off schedule (Carracedo et al., 2008). A 5-days washout period was established for subsequent behavioral assays and sacrifice of the animals. After sacrifice, brains were removed and cortex dissected and stored at -70ºC until assays.

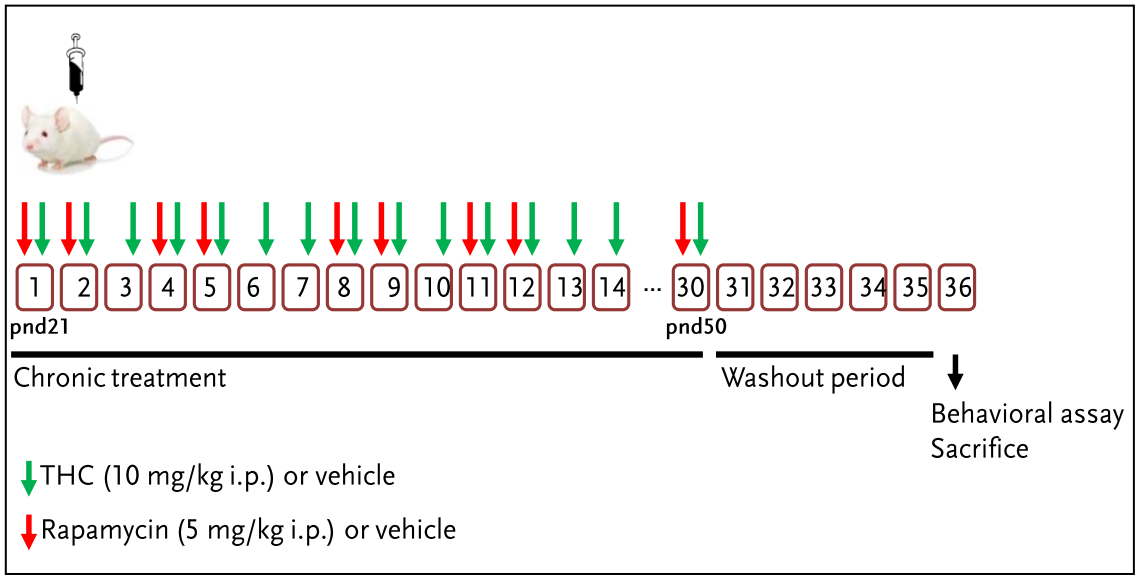


Figure 10. Treatments schedule.

4. MATERIALS

Antibodies

Primary and secondary antibodies used for Western blot experiments in this work are detailed in **Supplementary Table 1** of **Article 2** and **Supplementary Table 3** of **Article 3**. Primary antibodies used for antibody-capture [^{35}S]GTPγS scintillation proximity assay (SPA) experiments are detailed in **Table 1** of **Article 1**.

Radioactive compounds

[^{35}S]-Guanosine 5`-(γthio)triphosphate ([^{35}S]GTPγS) (specific activity: 1250 Ci/mmol) and [^{3}H]ketanserin (specific activity: 67 Ci/mmol) were from PerkinElmer (Waltham, MA, USA).

Other drugs and chemicals

Ammonium persulfate (APS), Bradford Protein Assay, DC Protein Assay Kit, 2x concentrated Laemmli buffer, N-N-N-N´-tetramethylethylenediamine (TEMED) and prestained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) standards were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Methanol was purchased from Carlo Erba Reagents (Barcelona, Spain).

Nitrocellulose membranes (pore size: 0.45 µm) and Whatman™ cellulose papers 3MM were purchased from GE Healthcare (Buckinghamshire, UK).

DL-Dithiothreitol (DTT) and ethylenediamine tetraacetic acid (EDTA) were purchased from Invitrogen (Barcelona, Spain).

Acrilamide 30%-bisacrilamide 0.8% was purchased from National Diagnostics (Atlanta, GA, USA).

Glacial acetic acid, sucrose and HCl (37%) were purchased from Panreac Química S.A.U. (Barcelona, Spain).

Antipain, bovine serum albumin (BSA), 2-butanol, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), chymostatin, (±)DOI, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid - free acid (HEPES), NaOH, cremophor® (polyoxyl 35 hydrogenated castor oil), optiprep™ (60% w/v iodixanol), glycine, guanosine diphosphate (GDP), guanosine 5'-O-(γ-thio)triphosphate (GTPγS), igepal, β-mercaptoethanol, $MgCl_2$, NaCl, NaF, Na_3VO_4, protease Inhibitor cocktail, polyoxyethylene (20) sorbitan monolaurate (Tween 20), sodium deoxycholate, sodium dodecyl sulfate (SDS),

Tris(hydroxymethyl)aminomethane hydrochloride (Tris HCl) and bromophenol blue were purchased from Sigma Aldrich® (Saint Louis, MO, USA).

96-wells isoplates and polyvinyltoluene (PVT) SPA beads coated with protein A were purchased from PerkinElmer (Waltham, MA, USA).

5. METHODS

5.1. PREPULSE INHIBITON OF STARTLE REFLEX TEST

PPI test was performed in a startle chamber (PanLab, Barcelona, Spain) coupled to a StarFear combined system. Animals were previously acclimatized for 1 h to the behavior room, and acoustic stimulus intensity as well as startle response capture equipment were calibrated each day of experiment.

Each session consisted on a previous 10 min habituation period (60 dB white noise background), which was present throughout the entire session. Five pulse trials were presented subsequently in order to achieve a more stable response of the animals. They were then subjected to a pseudo-randomized combination of 10 sets of 3 different trials: (1) 120 dB noise burst alone, 40 ms (pulse); (2) 77, 82, or 87 dB noise bursts, 10 ms (prepulses); and (3) 120 dB burst preceeded 60 ms earlier by a 77, 82, or 87 dB prepulse (prepulse-pulse). Each session lasted 30–33 min.

The maximum response was recorded for every trial, and the average startle response for each 10 sets was used for the analysis. PPI was calculated as follow:

%PPI=100x[1-(startle response to prepulse-pulse trial/startle response to pulse trial)]

5.2. PLATELETS ISOLATION

Blood samples (~15 ml) were extracted by venipuncture in ACD solution A vacutainer® citrate blood collection tubes (Becton Dickinson & Company, Franklin Lakes, NJ, USA). After extraction, blood was carefully layered over a density barrier solution (Optiprep™ (60% w/v iodixanol) (5 vol.) + (0.85% (w/v) NaCl, 20 mM HEPES, 1 mM EDTA; pH 7.4) (22 vol.)) and centrifuged at 350xg for 15 min at 20ºC. The turbid yellowish platelet-containing band was harvested, centrifuged at 600xg for 5 min at 20ºC, and the pellet was stored at -70ºC until assays.

5.3. SAMPLE PREPARATION

5.3.1. Total homogenates

Human platelets

Platelet pellets were washed twice with 0.85% (w/v) NaCl, 20 mM HEPES, 1 mM EDTA, pH 7.4, and homogenized with 80 µl of homogenation buffer (50 mM Tris-HCl, 150 mM NaCl, 5 µl/ml Protease Inhibitor Cocktail, 10 µg/ml antipain, 10 µg/ml chymostatin, 1 mM Na_3VO_4, 1 mM NaF, pH 7.4) using a minipotter (20 strokes). Immediately after, BDC buffer (50 mM Tris-HCl, 150 mM NaCl, 10% Igepal, 5% sodium deoxycholate, 1% SDS, 250 mM CHAPS, pH 7.4) was added (1:30 ratio). Samples were rested on ice during 30 min and centrifuged at 21,000xg and 4ºC for 10 min, after which supernatants were kept. Protein content was determined using DC Protein Assay Kit method, using BSA as a standard. Linear regression analysis and extrapolation of the data were carried out with GraphPad Prism® 5 software (GraphPad Software Inc, San Diego, CA, USA). 6x concentrated Laemmli buffer (9% SDS; 50% glycerol, 0.03% bromophenol blue, 375 mM Tris-HCl, pH 6.8) (20% v/v) were added to 65µg protein aliquots, followed by β-mercaptoethanol (2.5% v/v); and samples were stored at -70ºC until western blot experiment.

Human, rat and mouse cortex

Human PFC (~200 mg), rat cortex (~200 mg) or mouse cortex (~150 mg) tissue samples were thawed at 4ºC and homogenized in homogenation buffer (10 volumes) using a glass/teflon grinder (15 strokes). Immediately after, BCD buffer (0.1 volumes) was added to each sample. Samples were kept on ice for 30 minutes, centrifuged, supernatants kept and protein content determined following the same procedure as previously described. Samples were adjusted to 4 mg protein/ml and aliquoted. 2x concentrated commercial Laemmli buffer (95% v/v) and β-mercaptoethanol (5% v/v) were added to each sample, vortexed and kept at -70ºC until western blot experiments.

5.3.2. Membrane enriched fraction

Mouse brain cortex samples (~200 mg) from seven mice each time were thawed at 4ºC and homogenized with a glass/teflon grinder (10 strokes) in 30 volumes of 50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$ and 1 mM DTT, 250mM sucrose, pH 7.4. The homogenates were centrifuged at 1,100xg and 4ºC for 10 min and the supernatants were then recentrifuged at 40,000xg for 10 min (4ºC). Pellets were resuspended in centrifugation buffer (50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$, 1mM DTT, pH 7.4) (20 volumes) with a glass stick and recentrifuged at 40,000xg for 10 min (4ºC). Pellets were resuspended in five volumes of centrifugation buffer. Protein content was determined by Bradford method with BSA as

standard and 0.5, 1, and 2 mg aliquots were then centrifuged at 21,000xg and 4ºC during 15 min. The supernatant layer was discarded and the pellets stored at -80ºC until assay.

5.4. WESTERN BLOT

Gel electrophoresis

SDS-PAGE was carried out in all the experiments. Gels for electrophoresis consisted of a stacking gel of 5% polyacrylamide buffered solution; and a resolving gel of 12% polyacrylamide (cortex experiments) or 10% polyacrylamide (blood platelets). Samples were heated at 95ºC for 5 min and, after loading, electrical current of 60 V was set up in the first stage of electrophoresis (~30 min in cortex experiments, ~1h in human blood platelets experiments). Afterwards, 120 V current was applied for over 120-140 min. Electrophoresis buffer consisted on 25 mM Tris-HCl, 192 mM glycine, 0.1% SDS, pH 8.3. The amount of protein loaded varied depending upon the type of sample and the antibody used. Apart from the samples of interest, a molecular weight marker and an interexperimental control sample was loaded in each gel.

Transference

Proteins were transferred to nitrocellulose membranes by applying an electric field. Gel and the nitrocellulose membrane were kept tightly packed with cellulose papers into a cassette and the transference was carried out on ice and under constant agitation of the buffer. Amperage of 0.3 A per tray was applied for 60 min. Transference buffer consisted on 25 mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8.3. Tris buffered saline (TBS) (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) was used for washing membranes after transference.

Blocking and immunodetection

Membranes were incubated for 1 hour at room temperature (RT) and agitation in a blocking solution that varied depending upon the type of sample and the antibody used. Membranes were then incubated overnight at 4ºC and agitation in incubation buffer containing the primary antibodies. Next day, membranes were washed with TBST (TBS + 0.1% tween 20) and incubated with fluorescent anti-IgG conjugated antibodies for 60-90 min at RT and agitation. After washing with TBST, the immunoreactive signal was detected and quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences, Nebraska, USA) and integrated intensity values were obtained.

Further details regarding the conditions for western blot experiments are found in **Tables 1, 2** and **3**.

Table 1. Summary table of the specific conditions employed for western blot experiments of **ARTICLE 1.**

TARGET	PROTEIN LOADED	BLOCKING SOLUTION	INCUBATION SOLUTION	1º AB. DILUTION	2º AB. DILUTION
Gαi1	12 µg	3% smp TBS	3% smp TBST	1:100	1:2500
Gαi3	12 µg	3% smp TBS	3% smp TBST	1:750	1:4000
Gαo	12 µg	3% smp TBS	3% smp TBST	1:500	1:4000
Gαz	12 µg	3% smp TBS	3% smp TBST	1:600	1:4000
Gαq/11	12 µg	3% smp TBS	3% smp TBST	1:400	1:4000
Gα12/13	12 µg	3% smp TBS	3% smp TBST	1:500	1:4000
β-actin (mouse)	12 µg	3% smp TBS	3% smp TBST	1:40000	1:6000
β-actin (rabbit)	12 µg	3% smp TBS	3% smp TBST	1:20000	1:6000

Ab. (antibody), smp. (skimmed milk powder), TBS (Tris buffered saline), TBST (Tris buffered saline + 0.1% Tween 20).

Table 2. Summary table of the specific conditions employed for western blot experiments of **ARTICLE 2.**

TARGET	PROTEIN LOADED	BLOCKING SOLUTION	INCUBATION SOLUTION	1º AB. DILUTION	2º AB. DILUTION
5-HT2AR	20 µg	5% smp TBS	5% smp TBST	1:500	1:2500
Akt	20 µg	5% smp TBS	5% smp TBST	1:1000	1:2500
Phospho(Ser^{473})-Akt	20 µg	5% BSA TBS	5% BSA TBST	1:1000	1:4000
S6	20 µg	5% smp TBS	5% smp TBST	1:500	1:2500
Phospho(Ser$^{235/236}$)-	20 µg	5% smp TBS	5% smp TBST	1:500	1:2500
β-actin (mouse)	20 µg	-	-	1:200000	1:10000

BSA (bovine seroalbumin).

Table 3. Summary table of the specific conditions employed for western blot experiments of **ARTICLES 3 and 4.**

	TARGET	PROTEIN LOADED	BLOCKING SOLUTION	INCUBATION SOLUTION	1º AB. DILUTION	2º AB. DILUTION
Human cortex	Akt	30 µg	5% smp TBS	5% smp TBST	1:500	1:4000
	Phospho(Ser^{473})-Akt	35 µg	5% BSA TBS	5% BSA TBST	1:500	1:4000
	S6	30 µg	5% smp TBS	5% smp TBST	1:250	1:4000
	Phospho(Ser$^{235/236}$)-S6	40 µg	5% smp TBS	5% smp TBST	1:250	1:4000
	GSK3α/β	20 µg	5% smp TBS	5% smp TBST	1:500	1:4000
	Phospho(Ser$^{21/9}$)-GSK3α/β	40 µg	5% smp TBS	5% smp TBST	1:250	1:4000
	β-actin (mouse)	-	-	-	1:200000	1:5000-1:10000
	β-actin (rabbit)	-	-	-	1:20000	1:5000
Rat cortex	Akt	20 µg	5% smp TBS	5% smp TBST	1:500	1:5000
	Phospho(Ser^{473})-Akt	20 µg	5% smp TBS	5% smp TBST	1:500	1:5000
	S6	20 µg	5% smp TBS	5% smp TBST	1:500	1:4000
	Phospho(Ser$^{235/236}$)-S6	25 µg	5% BSA TBS	5% BSA TBST	1:500	1:4000
	GSK3α/β	20 µg	5% smp TBS	5% smp TBST	1:500	1:5000
	Phospho(Ser$^{21/9}$)-GSK3α/β	25 µg	5% BSA TBS	5% BSA TBST	1:500	1:5000
	β-actin (mouse)	-	-	-	1:100000-1:200000	1:2500-1:5000
	β-actin (rabbit)	-	-	-	1:20000	1:5000
Human platelets	5-HT2AR	30 µg	5% smp + 0.5% BSA TBS	5% smp + 0.5% BSA TBST	1:10000	1:15000
	Akt	30 µg	5% smp + 0.5% BSA TBS	5% smp + 0.5% BSA TBST	1:500	1:10000
	Phospho(Ser^{473})-Akt	35 µg	5% BSA TBS	5% BSA TBS	1:500	1:10000
	β-actin (mouse)	35 µg	5% BSA TBS	5% BSA TBS	1:100000	1:10000
	β-actin (rabbit)	30 µg	5% smp + 0.5% BSA TBS	5% smp + 0.5% BSA TBST	1:20000	1:10000

5.5. RADIOLIGAND BINDING ASSAY

Membrane pellets (prepared according to section **5.3.2.**) were thawed at 4ºC and resuspended in the corresponding incubation buffer to reach a final protein concentration of 0.15-0.30 mg/ml approximately, depending on the type of experiment. The real final protein content was measured after the experiment according to Bradford's method.

[^{3}H]ketanserin saturation curves (0.05-10 nM, eight concentrations) were performed in a pool of cortex membrane preparations from 8 mice in order to delineate the saturation binding parameters (Kd and Bmax) of the radioligand in the tissue. This maximum [^{3}H]ketanserin concentration was chosen in agreement to previous findings in mouse cortex membrane preparations (González-Maeso et al., 2008) and, according to non-linear regression analysis, this concentration of [^{3}H]ketanserin was suitable to calculate the maximum density of the receptors (Bmax). Incubation was carried out in 96-well plates. After the addition of the membranes (30-60 µg protein per well) in a final volume of 250 µl (incubation buffer: 50 mM Tris-HCl, pH 7.5), reactions were incubated for 60 minutes at 37°C with shaking. The non-specific binding of [^{3}H]ketanserin was determined in the presence of the 5-HT2AR specific antagonist M100907 (1 µM). After incubation, free radioligand was discarded by rapid filtration under vacuum through GF/C glass fiber filters pre-soaked with 0.5% polyethylenimine. Filters were rinsed with cold incubation buffer, air dried 20°C, 1.5 hours in both competition and saturation assays, bagged with BetaPlate Scint scintillation cocktail and radioactivity counted by liquid scintillation spectrometry on a MicroBeta TriLux Scintillation counter. The c.p.m. values were transformed to d.p.m. values by an internal calibration program.

Following the previously described method, competition binding assays were carried out in order to delineate and compare inhibition curves of [^{3}H]ketanserin binding (2 nM) by increasing concentrations of the 5-HT2AR agonist (±)DOI (10^{-12}-10^{-3} M; eighteen concentrations). The concentration of [^{3}H]ketanserin chosen for these competition assays is similar to its affinity (Kd) value for 5-HT2AR (González-Maeso et al., 2008).

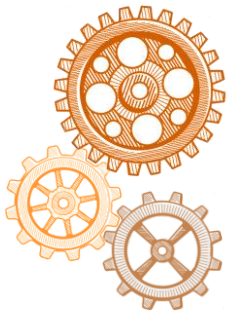
5.6. ANTIBODY CAPTURE [^{35}S]GTPγS SCINTILLATION PROXIMITY ASSAY

Specific activation of different subtypes of Gα proteins was determined using a homogeneous protocol of [^{35}S]GTPγS binding coupled to immunoprecipitation with specific antibodies (known as antibody capture [^{35}S]GTPγS scintillation proximity assay (SPA)). [^{35}S]GTPγS binding was performed in 96-wells plates and in a final incubation volume of 200 µl of buffer containing 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, 0.2 mM DTT, 50 mM Tris-HCl, pH 7.4, 0.4 nM [^{35}S]GTPγS, 15 µg of protein/well, and different concentrations of GDP depending on the Gα subunit subtype tested.

After incubation (2 h, 30ºC), 20 µl of igepal 1% + SDS 0.1% were added to each well, and plates were reincubated (30 min, 22ºC) with gentle agitation. Specific antibody for the Gα subunit of interest was then added to each well an additional incubation was carried out (90 min, RT). Polyvinyltoluene SPA beads coated with protein-A were then added (0.75 mg of beads per well), and plates were incubated for 3h at RT with gentle agitation. Finally, plates were centrifuged for 5 min at 1,000xg, and bound radioactivity was detected on a MicroBeta TriLux Scintillation counter.

In order to test the effects of different cannabinoid ligands on the [^{35}S]GTPγS binding to the different Gα subunit subtypes, a single submaximal concentration (10 µM) of THC, WIN55212-2, ACEA and/or O-2050 was used, based on previous experimental assays in which the standard conditions for these experiments were established (Erdozain et al., 2012). This concentration was the one that provided binding values around the Emax for all the drugs and subunit subtype combination studied. Non-specific binding was defined as the remaining [^{35}S]GTPγS binding in the presence of 100 µM unlabelled GTPγS. Further details regarding antibodies and GDP concentrations used are found in **Table 1** of **Article 1**.

In the same way, for the evaluation of the specific activation of the different Gα protein subtypes by the 5-HT2AR agonist (±)DOI, a concentration of 10 µM was also chosen for obtaining stimulation values around the Emax for any Gα subtype under our experimental conditions (García-Bea et al., 2019). Non-specific binding was defined as the remaining [^{35}S]GTPγS binding in the presence of 100 µM unlabeled GTPγS.



RESULTS

ARTICLE 1

Biased Agonism of Three Different Cannabinoid Receptor Agonists in Mouse Brain Cortex

Rebeca Díez-Alarcia, Inés Ibarra-Lecue, Ángela P López-Cardona,

J Javier Meana, Alfonso Gutiérrez-Adán, Luis F Callado,

Ekaitz Agirregoitia, Leyre Urigüen

Frontiers in Pharmacology (2016), **7**: 415

DOI: https://doi.org/10.3389/fphar.2016.00415

Impact factor 2016: 4.4

Relative position: 33/257, Q1 Pharmacology and Pharmacy

ARTICLE 1

Evidence shows that, for most of GPCRs, including cannabinoid CB1R and CB2R, distinct ligands can regulate several signaling pathways by a selective activation of different intracellular effectors, thus leading to different responses. Several reports aimed to evaluate the neurochemical effects of several cannabinoid drugs different from THC. However, literature addressing the activation pattern differences exerted among them is scarce, and non-existent in native tissue. In this study, we aimed to characterize the pattern of Gα protein subunit stimulation induced by three different cannabinoid ligands (THC, WIN55212-2, and ACEA) in membrane homogenates of mouse brain cortex. For this purpose, stimulation of [^{35}S]GTPγS binding coupled to specific immunoprecipitation with antibodies against different subtypes of Gα proteins (Gαi1, Gαi2, Gαi3, Gαo, Gαz, Gαs, Gαq/11, and Gα12/13) by those three CB1/CB2 cannabinoid agonists (10^{-5} M) was determined. Moreover, mutant mice lacking CB1R, CB2R, and both receptors were used to elucidate the specific cannabinoid receptor mediating each effect. Results of this study showed that, in mouse brain cortex, THC, WIN55,212-2 and ACEA are able to stimulate not only the classical Gαi/o subunits but also other Gα subunits (Gαz, Gαq/11, and Gα12/13) through CB1R and/or CB2R activation. Moreover, the specific pattern of Gα protein subunit activation was different depending on the ligand. While THC activated moderately Gαi1, Gαo, Gαz and Gαq/11 subunits, WIN55,212-2 did it more markedly and also activated Gαi3 and Gα12/13. ACEA activated Gαi1, Gαi3, Gαo and Gαq/11. Results in mutant mice cortex tissue showed that all of the Gα subunit activations induced by THC and WIN55,212-2 were mediated by CB1R or CB2R, as well as ACEA induced Gαi3, Gαo and Gαq/11 activation. However, ACEA induced Gαi1 activation was non-dependent of cannabinoid receptors. These results demonstrate that different exogenous cannabinoid ligands selectively activate different Gα protein subtypes, and may help to understand the specific molecular pathways involved in the pharmacological effects of cannabinoid drugs in the central nervous system.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 04 November 2016
doi: 10.3389/fphar.2016.00415

Biased Agonism of Three Different Cannabinoid Receptor Agonists in Mouse Brain Cortex

*Rebeca Diez-Alarcia[1,2], Inés Ibarra-Lecue[1], Ángela P. Lopez-Cardona[3,4], Javier Meana[1,2], Alfonso Gutierrez-Adán[3], Luis F. Callado[1,2], Ekaitz Agirregoitia[5] and Leyre Urigüen[1,2]**

[1] Department of Pharmacology, University of the Basque Country UPV/EHU, Leioa, Spain, [2] Centro de Investigación Biomédica en Red de Salud Mental, Madrid, Spain, [3] Department of Animal Reproduction, Instituto Nacional de Tecnología Agraria y Alimentaria, Madrid, Spain, [4] G.I. Biogénesis, Universidad de Antioquia, Antioquia, Colombia, [5] Department of Physiology, University of the Basque Country, Leioa, Spain*

Cannabinoid receptors are able to couple to different families of G proteins when activated by an agonist drug. It has been suggested that different intracellular responses may be activated depending on the ligand. The goal of the present study was to characterize the pattern of G protein subunit stimulation triggered by three different cannabinoid ligands, Δ^9-THC, WIN55212-2, and ACEA in mouse brain cortex. Stimulation of the [^{35}S]GTPγS binding coupled to specific immunoprecipitation with antibodies against different subtypes of G proteins ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, $G\alpha_z$, $G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$), in the presence of Δ^9-THC, WIN55212-2 and ACEA (submaximal concentration 10 μM) was determined by scintillation proximity assay (SPA) technique in mouse cortex of wild type, CB_1 knock-out, CB_2 knock-out and CB_1/CB_2 double knock-out mice. Results show that, in mouse brain cortex, cannabinoid agonists are able to significantly stimulate not only the classical inhibitory $G\alpha_{i/o}$ subunits but also other G subunits like $G\alpha_z$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$. Moreover, the specific pattern of G protein subunit activation is different depending on the ligand. In conclusion, our results demonstrate that, in mice brain native tissue, different exogenous cannabinoid ligands are able to selectively activate different inhibitory and non-inhibitory Gα protein subtypes, through the activation of CB_1 and/or CB_2 receptors. Results of the present study may help to understand the specific molecular pathways involved in the pharmacological effects of cannabinoid-derived drugs.

Keywords: CB_1 receptor, CB_2 receptor, functional selectivity, scintillation proximity assay (SPA), G proteins, brain

OPEN ACCESS

Edited by:
Francisco Ciruela, University of Barcelona, Spain

Reviewed by:
Luca Ferraro, University of Ferrara, Italy
Fernando Rodriguez de Fonseca, Instituto de Investigación Biomédica de Málaga, Spain

****Correspondence:***
Leyre Urigüen
leyre.uriguen@ehu.eus

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *11 August 2016*
Accepted: *19 October 2016*
Published: *04 November 2016*

Citation:
Diez-Alarcia R, Ibarra-Lecue I, Lopez-Cardona ÁP, Meana J, Gutierrez-Adán A, Callado LF, Agirregoitia E and Urigüen L (2016) Biased Agonism of Three Different Cannabinoid Receptor Agonists in Mouse Brain Cortex. Front. Pharmacol. 7:415. doi: 10.3389/fphar.2016.00415

INTRODUCTION

During the last decade a wide number of studies have focused on the potential involvement of the endocannabinoid system in a variety of psychiatric and neurological disorders. The putative psychoactive ingredient of *Cannabis sativa* (marijuana plant), Δ^9-tetrahydrocannabinol (Δ^9-THC), as well as the endogenous cannabinoids anandamide (arachidonoyl ethanolamide) and

Abbreviations: 2-AG, 2- arachidonoylglycerol; ACEA, arachidonyl-2-chloroethylamide; $CB_1^{-/-}$, CB_1 knock-out mice; $CB_2^{-/-}$, CB_2 knock-out mice; $CB_1^{-/-}$/$CB_2^{-/-}$, CB_1 and CB_2 double knock-out mice; P1, pellet fraction; P2, membrane enriched fraction; PVT, polyvinyltoluene; RT, room temperature; SDS, sodium dodecyl sulfate; SPA, scintillation proximity assay; TBS, tris-buffered saline; WT, wild type; Δ^9-THC, Δ^9-tetrahydrocannabinol.

2-arachidonoylglycerol (2-AG) act primarily through cannabinoid CB_1 and CB_2 receptors. These cannabinoid receptors are GPCRs mostly coupled to Gi/o proteins (Howlett et al., 2002). The CB_1 receptor is mainly distributed in the CNS, particularly in cortex, basal ganglia, hippocampus, and cerebellum (Mackie, 2005; De Jesus et al., 2006) and generally acts presinaptically inhibiting the release of neurotransmitters. CB_2 receptors are expressed at much lower levels in the CNS compared with CB_1 receptors (reviewed in Atwood and Mackie, 2010). As $G_{i/o}$ coupled GPCRs, CB_1 and CB_2 receptors inhibit adenylyl cyclase, but moreover, both receptors are able to activate MAPK, inhibit voltage gated Ca^{2+} channels and activate inwardly rectifying K^+ channels (Childers et al., 1993).

The activation of CB_1 receptor in the brain leads to the modulation of neuronal excitability, which may be in part responsible of the psychoactive effects of exogenous cannabinoids. In this context, a considerable amount of studies have been performed in order to elucidate the effects of cannabinoids (natural or synthetics) in the development of mental alterations, such as addiction, cognitive deficits, anxiety or psychosis. Importantly, different or opposite behavioral effects have been observed after the administration of Δ^9-THC or synthetic cannabinoid ligands (Fattore et al., 2003; Panagis et al., 2014; Rubino and Parolaro, 2016). It has been demonstrated that for most G protein-coupled receptors, distinct agonists can differentially regulate several signaling pathways through the same receptor by a selective activation of different intracellular effectors. This is a mechanism known as functional selectivity or biased agonism. In this way, cannabinoid receptors have been demonstrated to be capable of coupling to different families of G proteins and/or to beta-arrestin when activated by an agonist drug suggesting that different intracellular responses may be activated depending on the ligand (Glass and Northup, 1999; Bosier et al., 2010). For instance, for the CB_1 receptor has been reported that, whereas 2-AG and WIN55,212 have little preference for inhibition of cAMP and phosphorylation of ERK1/2, anandamide and CP55940 were biased toward cAMP inhibition (Khajehali et al., 2015). Moreover, in a recent study Dhopeshwarkar and Mackie (2016) demonstrated that CB_2 receptor ligands display strong and varied functional selectivity at canonical (inhibition of adenylyl cyclase) and non-canonical (arrestin recruitment) pathways. Moreover, the intracellular signaling activated by a receptor depends on the cellular system where it is expressed, which may vary across different neuronal environments. In this context, it has been demonstrated that opioid and cannabinoid receptors function through the same pool of G proteins when they are co-transfected, whereas in cells endogenously expressing these receptors signaling occurs through distinct pools of G proteins (Shapira et al., 2000). Thus, this fact should be taken into consideration when interpreting results acquired in artificially transfected cells vs. native biological systems.

To our knowledge, no study has compared G protein signaling by different cannabinoid drugs in native brain tissue. Thus, in the current study, we performed [^{35}S]GTPγS scintillation proximity assay (SPAs) coupled with the use of specific antibodies against different Gα protein subunits to evaluate the functional selectivity of different cannabinoid ligands by activating CB_1 and/or CB_2 cannabinoid receptors in mouse brain cortex.

MATERIALS AND METHODS

Animal Procedures

Adult C57BL/6J (WT), CB_1 knock-out ($CB_1^{-/-}$) (Marsicano et al., 2002), CB_2 knock-out ($CB_2^{-/-}$) (Buckley et al., 2000), and CB_1/CB_2 double knock-out ($CB_1^{-/-}/CB_2^{-/-}$) mice were used in this study. Animals (males, aged 7–8 weeks-old) were housed (6–8 animals per cage) in standard cages under controlled conditions of temperature (23 ± 1°C) and photoperiod (light/dark cycle 14 h: 10 h) and free access to standard rodent chow and water.

Animal Welfare and Ethical Statements

All experimental procedures using mice were performed in accordance with the European Directive for the Protection of Vertebrate Animals used for experimental and Other Scientific Purposes (European Union Directive #86/606/EEC) and approved by the Ethics Committees for Animal Welfare of the University of the Basque Country (UPV/EHU) permit number CEBA1882011 and by the Institutional Review Board (INIA), permit number CEEA2012/021.

Rationale for Choice of Cannabinoid Ligands

In the present study, we decided to investigate the effects of three different cannabinoid ligands. Δ^9-THC was chosen for being the main psychoactive component of marijuana plant and the putative responsible of the development of mental disorders in humans. WIN55212-2, a synthetic cannabinoid structurally different from Δ^9-THC, is a potent, non-selective CB_1/CB_2 receptor agonist that is frequently used in the studies that try to elucidate the effects of *Cannabis* in the brain. Finally, we wanted to study a ligand structurally similar to endogenous cannabinoids, such as the synthetic anandamide analog arachidonyl-2-chloroethylamide (ACEA). O-2050 was chosen as a neutral cannabinoid antagonist. O-2050 has been proved to be a neutral CB_1 receptor antagonist in several studies (Canals and Milligan, 2008; Hudson et al., 2010; Brents et al., 2011; Wiley et al., 2011), with quite similar affinity for CB_1 and CB_2 receptors. Although, there is some data in the literature suggesting its activity as inverse or even partial agonist at CB_1 receptors in various tissues (Makwana et al., 2010; Wiley et al., 2011) in a previous work of our group, we showed that O-2050 has no effect over [^{35}S]GTPγS binding and behaves as an antagonist blocking WIN55212-2-mediated activation (Erdozain et al., 2012) (**Figure 1**).

Rationale for Choice of G Protein α Subunit Subtypes

In the present study, we decided to investigate the ability of cannabinoid receptors to activate different G proteins subtype. We chose at least one G protein subtype representative of each

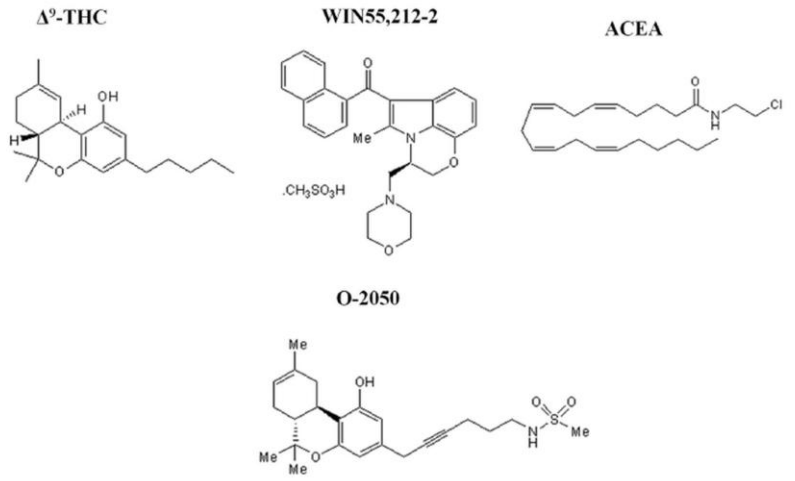


FIGURE 1 | Chemical structure of the different cannabinoid ligands used. Representative scheme of the chemical structure of the different cannabinoid ligands used in this study (Δ^9-tetrahydrocannabinol (Δ^9-THC), WIN55212-2, arachidonyl-2-chloroethylamide (ACEA) and O-2050).

main G protein family and mainly focusing in the inhibitory G proteins for being the cannabinoid canonical pathway (**Figure 2**).

Mouse Brain Cortex Membrane Homogenates Preparation

After sacrifice by cervical dislocation, the brains were rapidly removed, cortices dissected and fresh frozen, and stored immediately at −80°C until use. Preparation of membrane enriched fraction (P2 fraction) was performed as previously described (Gonzalez-Maeso et al., 2000) with minor modifications. Mouse brain cortex samples (approximately 200 mg) from seven mice each time were thawed at 4°C and homogenized with a glass/teflon grinder (IKA labortechnik, Satufen, Germany) (10 strokes at maximum speed) in 30 volumes of homogenization buffer (50 mM Tris-HCl (Invitrogen, Barcelona, Spain), 1 mM EGTA (Sigma-Aldrich, St. Louis, MO, USA), 3 mM $MgCl_2$ (Sigma-Aldrich, St. Louis, MO, USA) and 1 mM DTT (Invitrogen, Barcelona, Spain); pH 7.4; supplemented with 250 mM sucrose (Panreac Química S.A.U, Barcelona, Spain). The homogenates were centrifuged at 1,100 × *g* for 10 min at 4°C (Sorvall RC-5C centrifuge, SM-24 rotor; FisherScientific, Madrid, Spain). The pellets (P1 fraction) were discarded and the supernatants were then recentrifuged at 40,000 × *g* for 10 min (4°C). The resultant pellets were resuspended in 20 volumes of fresh cold centrifugation buffer (50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$ and 1 mM DTT; pH 7.4) with a glass stick and recentrifuged at 40,000 × *g* for 10 min (4°C). The obtained pellets were then resuspended in five volumes of centrifugation buffer. Protein content was determined by the method of Bradford with BSA (Sigma-Aldrich®, St. Louis, MO, USA) as standard. Linear regression analysis and extrapolation of the data were carried out with GraphPad Prism 5® software (GraphPad Software, Inc., San Diego, CA, USA). Finally, aliquots of 0.5, 1, and 2 mg were then centrifuged at 21,000 × *g* (Eppendorf 5810R centrifuge; Eppendorf, Madrid, Spain) during 15 min at 4°C. The supernatant layer was carefully discarded and the pellets stored at −80°C until assay.

Antibody-Capture [^{35}S]GTPγS Scintillation Proximity Assay (SPA)

Specific activation of different subtypes of Gα proteins was determined using a homogeneous protocol of [^{35}S]GTPγS SPA coupled with the use of specific antibodies essentially as previously described (Erdozain et al., 2012). [^{35}S]GTPγS binding was performed in 96-well Isoplates (PerkinElmer Life Sciences, Maanstraat, Germany) and in a final volume of 200 ml containing 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, 0.2 mM DTT, 50 mM Tris-HCl at pH 7.4, 0.4 nM [^{35}S]GTPγS, 15 μg of protein per well, and different concentrations of GDP depending on the Gα subunit subtype tested. At the end of the 2 h incubation period (30°C), 20 μl of Igepal 1% + SDS 0.1% were added to each well, and plates were incubated at 22°C for 30 min with gentle agitation. Specific antibody for the Gα subunit of interest was then added to each well before an additional 90 min RT incubation period (the antibodies and dilutions employed are described in **Table 1**). Polyvinyltoluene (PVT) SPA beads coated with protein A (PerkinElmer, S.L., Tres Cantos, Madrid, Spain) were then added (0.75 mg of beads per well), and plates were incubated for 3 h at RT with gentle agitation. Finally, plates were centrifuged (5 min at 1000 × *g*), and bound radioactivity was detected on a MicroBeta TriLux scintillation counter (PerkinElmer S.L., Tres Cantos, Madrid, Spain). In order to test their effect on the [^{35}S]GTPγS binding to the different Gα subunit subtypes, a single submaximal concentration of the drugs (10 μM) Δ^9-THC, WIN55212-2, ACEA and/or O-2050, was used. This submaximal concentration was chosen as previously reported (Erdozain et al., 2012) in our previous experimental assays in which we established the

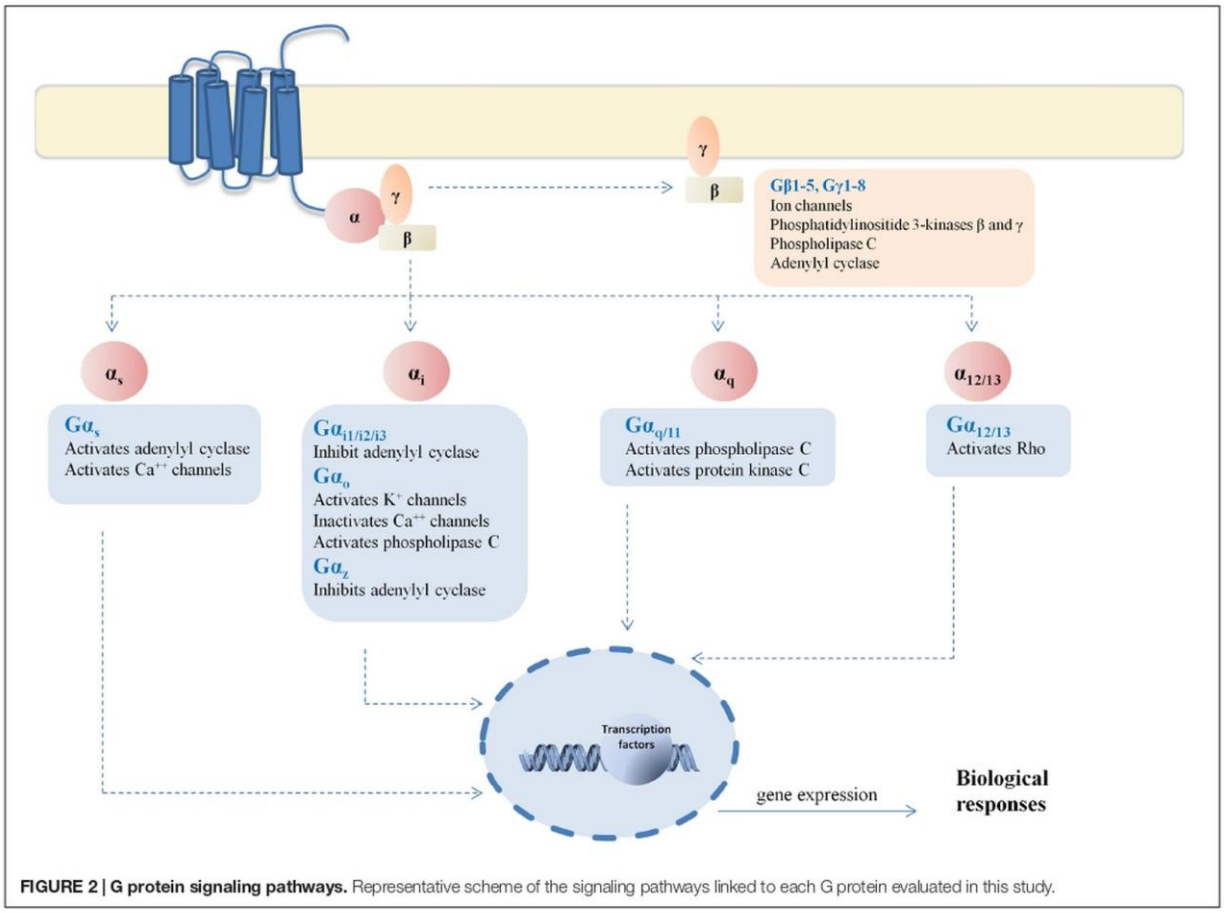


FIGURE 2 | G protein signaling pathways. Representative scheme of the signaling pathways linked to each G protein evaluated in this study.

TABLE 1 | Antibodies, dilutions, and GDP concentrations employed in the [^{35}S]GTPγS scintillation proximity assays.

Target	Description	Commercial firm	Catalog #	[GDP] (μM)	Ab dilution
$G\alpha_{i1}$	Mouse monoclonal anti-$G\alpha_{i1}$	Santa Cruz	sc-56536	100	1:20
$G\alpha_{i2}$	Rabbit polyclonal anti-$G\alpha_{i2}$	Santa Cruz	sc-7276	50	1:20
$G\alpha_{i3}$	Rabbit polyclonal anti-$G\alpha_{i3}$	Santa Cruz	sc-262	200	1:30
$G\alpha_o$	Mouse monoclonal anti-$G\alpha_o$	BIOMOL	SA-280	50	1:75
$G\alpha_z$	Rabbit polyclonal anti-$G\alpha_z$	Santa Cruz	sc-388	100	1:20
$G\alpha_s$	Rabbit polyclonal anti-$G\alpha_s$	Santa Cruz	sc-383	100	1:20
$G\alpha_{q/11}$	Rabbit polyclonal anti-$G\alpha_{q/11}$	Santa Cruz	sc-392	50	1:20
$G\alpha_{12/13}$	Rabbit polyclonal anti-$G\alpha_{12/13}$	Santa Cruz	sc-28588	100	1:20

standard conditions for this assays. This concentration is the one which give us binding values around the Emax for any drug and subunit subtype combination studied (Supplementary Figure S1). Non-specific binding was defined as the remaining [^{35}S]GTPγS binding in the presence of 10 μM unlabelled GTPγS.

Western Blot

For Western blot experiments, membrane enriched fraction (P2 fraction) pellets from mouse brain tissue (cortex) were resuspended in TBS, reaching a concentration of 4 mg protein/ml. Commercial Laemmli 2x (95%) and β-mercaptoethanol (5%) (Sigma-Aldrich®, St. Louis, MO, USA) were added to each sample, reaching a final protein concentration of 2 mg/ml. Finally, all the samples were heated at 95°C for 5 min in a Thermoblock (Biometra, Goettingen, Germany) and kept at −20°C until assay. Electrophoresis was carried out in SDS polyacrylamide gels, composed of 5% stacking (0.5 M Tris-HCl, pH 6.8, 10% SDS) and 12% resolving (1.5 M Tris-HCl, pH 8.8, 10% SDS) gels,

using a miniprotean system (Bio-Rad Laboratories). Equal protein loading in the gel was verified by simultaneous immunodetection of β-actin (mouse monoclonal antibody anti-β-actin, Sigma Biosciences, St. Louis, MO, USA) with the different Gα subunit subtypes. Proteins were then transferred to nitrocellulose membranes (1 h, 0.3 A) using an electrophoretic transfer system (Bio-Rad Laboratories). The non-specific binding sites in the membranes were blocked for 1 h at RT in blocking solution (3% non-fat dry milk, pH = 7.4 in PBS). Membranes were incubated overnight at 4°C in incubation buffer (3% non-fat dry milk + 0.1% Tween-20 in PBS) containing the appropriate dilution of the specific primary anti-Gα subunit antibody. Antibody specificity, as previously described in the literature (Gettys et al., 1994; Valdizan et al., 2010), was confirmed in our experimental conditions by Western blot (data not shown). Membranes were washed with PBS and incubated for 1 h at RT and constant agitation with the fluorescent conjugated secondary antibodies (Alexa Fluor® 680 and/or IRDye 800 conjugated antibodies) suitable diluted in incubation buffer. Finally, membranes were rewashed with PBS and immunoreactivity was detected and quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) and Odyssey Software. Broad-Range pre-stained SDS-PAGE molecular weight standard (Bio-Rad Laboratories, Hercules, CA, USA) was used.

Data Analysis and Statistical Procedures

Data were analyzed with GraphPad Prism™ 5.01 software. In order to allow better interpretation of the data, specific binding obtained from [^{35}S]GTPγS SPAs were transformed to percentage of basal binding (binding values observed in the absence of any exogenous drug) obtained for each Gα protein subunit studied. The statistical comparison of the SPA results was carried out by a two-tailed one sample Student's *t*-test with a significance level of $p < 0.05$. Immunodensity data obtained from Western blotting assays were transformed to percentage of the control, being the control the mean of immunodensities obtained for WT mice. The statistical comparison of the Western blot results was carried out by a one-way ANOVA test, followed by Dunnet's *post hoc* test for multiple comparisons, with a significance level of $p < 0.05$. All data are expressed as mean ± SEM values.

Materials

[^{35}S]GTPγS (4625 × 10^{10} Bq/mmol) was purchased from PerkinElmer Life Sciences (Maanstraat, Germany). Tetrahydrocannabinol (Δ^9-THC) was purchased from THCPharm GmbH (Frankfurt, Germany); WIN55212-2 and GTPγS were purchased from Sigma-Aldrich (St. Louis, MO, USA); Arachidonyl-2-chloroethylamide ACEA and O-2050 were from Tocris Bioscience (Bristol, UK). All other chemical reagents were of analytical quality and were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, MO, USA).

RESULTS

Effects of Δ^9-THC, WIN55212-2, and ACEA on G Protein Activation in Mouse Brain Membranes

Cannabinoid receptor ligands were used for the characterization of the functional coupling of cannabinoid receptors to the different G protein α subunit subtypes ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, $G\alpha_z$, $G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$) in mouse brain tissue. First, we investigated which Gα subunit subtypes were activated by the natural cannabinoid Δ^9-THC (10 μM) in mouse brain cortex membrane homogenates (**Figure 3A**). As expected, we found that Δ^9-THC was able to significantly activate several classical AC inhibitory subunits, as $G\alpha_{i1}$ (113 ± 3%), $G\alpha_o$ (110 ± 2%), and $G\alpha_z$ (113 ± 5%), while exerted no effect on $G\alpha_{i2}$ or $G\alpha_{i3}$. Δ^9-THC was also able to activate the $G\alpha_{q/11}$ subunit (118 ± 5%). However, no changes were observed when we studied AC stimulatory subunit $G\alpha_s$ and the RhoA activator $G\alpha_{12/13}$ subunit. To further test if these effects of Δ^9-THC were cannabinoid-receptor mediated, the same assays were carried out in the presence of a putative neutral antagonist of the CB_1 receptor, O-2050. In all cases, the activation of these G protein subunits was blocked when membranes were co-incubated with the cannabinoid antagonist O-2050. Next, we investigated the effects on G protein subunit activation induced by the synthetic cannabinoid agonist WIN55212-2 (**Figure 3B**). We found that WIN55212-2 significantly increased the binding of [^{35}S]GTPγS to the all the inhibitory subunits $G\alpha_{i1}$ (129 ± 6%), $G\alpha_{i3}$ (129 ± 5%), $G\alpha_o$ (120 ± 4%) and $G\alpha_z$ (134 ± 6%), except $G\alpha_{i2}$. WIN55212-2 was also able to activate the $G\alpha_{q/11}$ subunit (131 ± 7%), but not the AC stimulatory subunit $G\alpha_s$. Surprisingly, WIN55212-2 was also able to significantly stimulate the RhoA activator $G\alpha_{12/13}$ (130 ± 4%). In the same way as previously described for Δ^9-THC, the activation of these G protein subunits by WIN55212-2 was always blocked by the co-incubation with the cannabinoid antagonist O-2050, except for the case of $G\alpha_z$ (106 ± 1%). Finally, we investigated the effect of the synthetic anandamide analog ACEA on G protein subunit activation in mouse brain tissue (**Figure 3C**). When evaluating the classical AC inhibitory subunits, we found that ACEA stimulated $G\alpha_{i1}$ (121 ± 4%), $G\alpha_{i3}$ (120 ± 5%), and $G\alpha_o$ (116 ± 4%). However, as occurred with Δ^9-THC and WIN55212-2, no stimulation was observed in $G\alpha_{i2}$, suggesting that none of the cannabinoids evaluated exert their effects through $G\alpha_{i2}$ signaling. Moreover, ACEA had no effect on $G\alpha_z$. As previously observed for the other two cannabinoid ligands evaluated, ACEA also activated $G\alpha_{q/11}$ subunit (122 ± 7%) while had no effect on $G\alpha_s$. Thus, it seems that none of these cannabinoid ligands are able to activate this AC stimulatory subunit either. No changes were observed when we studied the effects of ACEA on the RhoA activator $G\alpha_{12/13}$ subunit. Again, the activation of these G protein subunits was blocked when membranes were co-incubated with the cannabinoid antagonist O-2050.

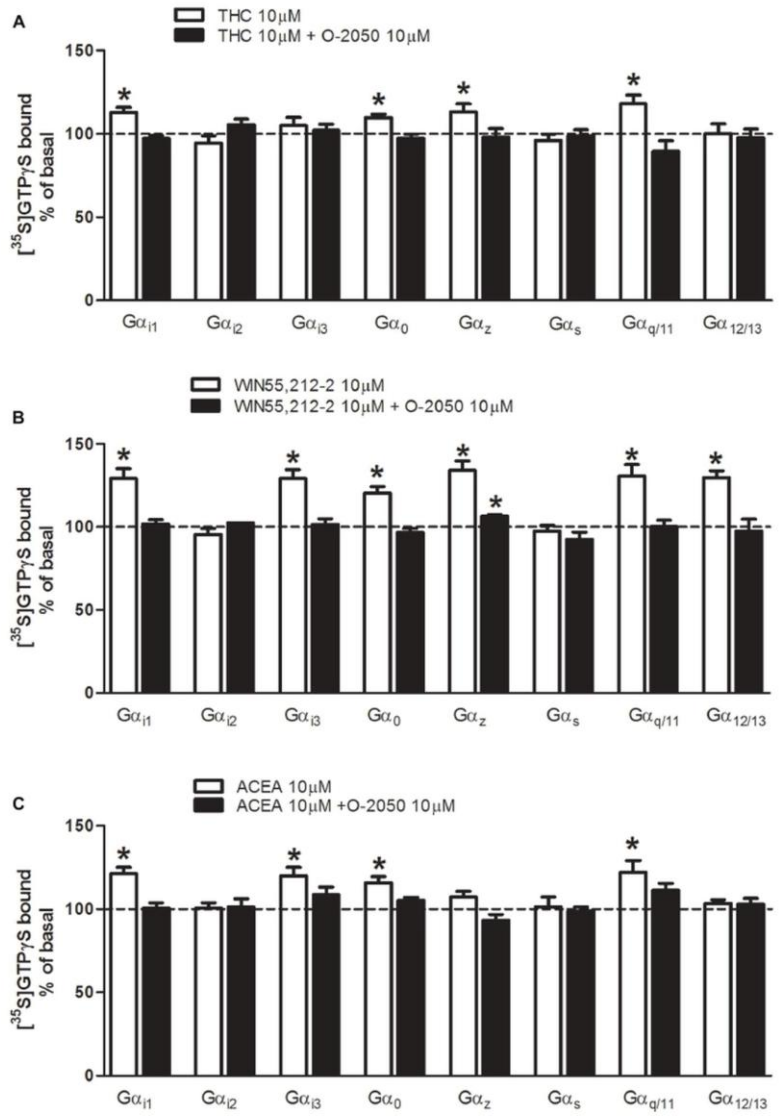


FIGURE 3 | Effects of THC, WIN55212-2, and ACEA on G protein activation in mouse brain membranes. [^{35}S]GTPγS scintillation proximity assays coupled to immunoprecipitation with specific antibodies against different Gα subunits ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, $G\alpha_z$, $G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$) in mouse brain cortical membranes co-incubated with **(A)** THC (10 μM) **(B)** WIN55212-2 (10 μM), or **(C)** ACEA (10 μM) in the presence or absence of the antagonist O-2050 (10 μM). Data are shown as percentage of [^{35}S]GTPγS basal binding values obtained for each specific subunit. Bars represent mean ± SEM of four to six different experiments carried out in triplicate. Asterisks highlight those normalized values of stimulation or inhibition of basal binding which are statistically different from 100% (Student's *t*-test; $^*p < 0.05$).

Effects of the Cannabinoid Antagonist O-2050 on G Protein Activation in Mouse Brain Membrane Homogenates

O-2050 was initially synthesized and described as a neutral CB_1 receptor antagonist, however, there are some evidences suggesting that is able to act as an inverse agonist or even as a partial agonist (Wiley et al., 2011). For this reason, and in order to validate O-2050 as a useful pharmacological tool to antagonize the effect mediated by cannabinoid receptors, [^{35}S]GTPγS SPAs were performed in mouse cortical membranes in the presence of O-2050 (10 μM) alone. Under these experimental conditions, neither stimulation nor inhibition of [^{35}S]GTPγS basal binding values were observed for any of the Gα subunit subtypes studied, with the exception of $G\alpha_z$ (119 ± 1%) (**Figure 4**).

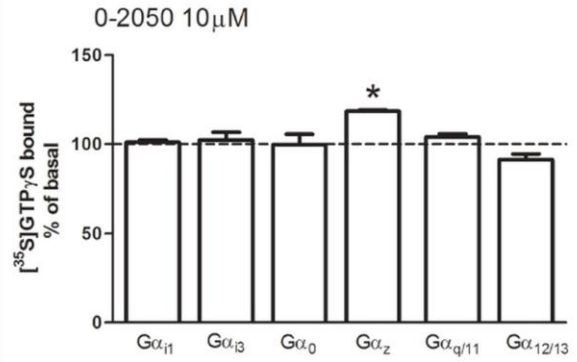


FIGURE 4 | Effects of the cannabinoid antagonist O-2050 on G protein activation in mouse brain membranes. [^{35}S]GTPγS scintillation proximity assays coupled to immunoprecipitation with anti-$G\alpha_{i1}$, $G\alpha_{i3}$, $G\alpha_o$, $G\alpha_z$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ specific antibodies in mouse brain cortical membranes co-incubated with the cannabinoid antagonist O-2050 (10 μM). Data are shown as percentage of [^{35}S]GTPγS basal binding values obtained for each specific subunit. Bars represent mean ± SEM of four to six different experiments carried out in triplicate. Asterisks highlight those normalized values of stimulation or inhibition of basal binding which are statistically different from 100% (Student's *t*-test; $^*p < 0.05$).

Effects of Δ^9-THC, WIN55212-2, and ACEA on G Protein Activation in Cannabinoid Receptors Knockout Mice

To further elucidate the role of each cannabinoid receptor subtype in the agonist-mediated activation of the different Gα subunit subtypes, [^{35}S]GTPγS SPA was performed in brain tissue of $CB_1^{-/-}$, $CB_2^{-/-}$, and $CB_1^{-/-}/CB_2^{-/-}$ mice. For that purpose, brain membranes were incubated with the different cannabinoid ligands (THC, WIN55212-2, or ACEA) and with the specific antibodies against that Gα for which an stimulation with these agonists was observed in WT. **Figure 5A** shows the stimulation of the different Gα subunits when brain membranes of the four genotypes were incubated with Δ^9-THC. The significant stimulation of the inhibitory $G\alpha_{i1}$ subunit observed in the WT mice was completely absent in the $CB_1^{-/-}$ and in the CB_1/CB_2 double ko mice, but was still present in the $CB_2^{-/-}$ (108 ± 2%), which suggests that the Δ^9-THC-mediated stimulation of $G\alpha_{i1}$ is induced by the activation of CB_1 receptor. On the other hand, opposite results were obtained for $G\alpha_{io}$ and $G\alpha_z$, the other two inhibitory subunits that were stimulated by Δ^9-THC. As previously described, there was a significant Δ^9-THC-induced stimulation of $G\alpha_o$ and $G\alpha_z$ subunits in the WT. This stimulation was also observed in the $CB_1^{-/-}$ (115 ± 4% for $G\alpha_o$ and 120 ± 4% for $G\alpha_z$) but not in the $CB_2^{-/-}$ or the $CB_1^{-/-}/CB_2^{-/-}$ mice. These data may indicate that, in mouse brain cortical membranes, Δ^9-THC acts through the CB_2 receptor to stimulate these inhibitory $G\alpha_o$ and $G\alpha_z$ subunits. Finally, the Δ^9-THC-induced activation of the $G\alpha_{q/11}$ subunit observed in the WT mice was not found in the $CB_1^{-/-}$ and $CB_1^{-/-}/CB_2^{-/-}$ mice, while remained unchanged in the $CB_2^{-/-}$ (116 ± 2%). This result indicates that Δ^9-THC stimulates the $G\alpha_{q/11}$ subunit acting mainly through the CB_1 receptor.

Figure 5B shows the stimulation of different Gα subunits when brain membranes of the four genotypes were incubated with the synthetic cannabinoid agonist WIN55212-2. The significant stimulation of the inhibitory $G\alpha_{i1}$ and $G\alpha_{i3}$ subunits induced by WIN55212-2 in the WT mice was not found in the $CB_1^{-/-}$ nor in the $CB_1^{-/-}/CB_2^{-/-}$ mice, but was still present in the $CB_2^{-/-}$ (124 ± 5% for $G\alpha_{i1}$ and 123 ± 5% for $G\alpha_{i3}$). On the contrary, the inhibitory subunits $G\alpha_o$ and $G\alpha_z$, which were significantly stimulated in the WT mice, remained stimulated in the $CB_1^{-/-}$ (111 ± 2% for $G\alpha_o$ and 123 ± 7% for $G\alpha_z$) but not stimulation was found in the $CB_2^{-/-}$ nor in the $CB_1^{-/-}/CB_2^{-/-}$ mice. These results suggest that the inhibitory signaling of WIN55212-2 in the mice brain through $G\alpha_{i1}$ and $G\alpha_{i3}$ activation seems to be mediated by the CB_1 receptor, while the stimulation of $G\alpha_o$ and $G\alpha_z$ would be mediated by the CB_2 receptor activation. The significant activation of the $G\alpha_{q/11}$ subunit induced by WIN55212-2 in the WT mice was completely absent in the $CB_1^{-/-}$ mice, as well as in the CB_1/CB_2 double ko mice. On the contrary, a significant stimulation of $G\alpha_{q/11}$ subunit (120 ± 5%) was observed in the $CB_2^{-/-}$ membranes, suggesting that this stimulation is mediated by the CB_1 receptor. Strikingly, the observed stimulation of the RhoA activator subunit $G\alpha_{12/13}$ by WIN55212-2 in the WT disappeared in the absence of CB_2 receptor (both in $CB_2^{-/-}$ and $CB_1^{-/-}/CB_2^{-/-}$ mice) suggesting an important role of this CB_2 receptor in the intracellular signaling through $G\alpha_{12/13}$ in the brain.

Finally, the same experiments were performed incubating the brain membranes with the synthetic anandamide analog ACEA (**Figure 5C**). Surprisingly, the significant stimulation of $G\alpha_{i1}$ that was observed in the WT mice was still found in all the genotypes evaluated. These results suggest that the inhibitory effect of ACEA mediated by the $G\alpha_{i1}$ subunit activation may be independent of cannabinoid receptors. On the other hand, the significant stimulation of $G\alpha_{i3}$ subunit induced by ACEA was not observed in brain membranes of $CB_1^{-/-}$ mice but was still significant in $CB_2^{-/-}$ membranes (111 ± 3%). No stimulation was observed in the $CB_1^{-/-}/CB_2^{-/-}$ mice. Regarding the $G\alpha_o$ subunit, there was a significant stimulation in the absence of CB_1 receptors (115 ± 1%), while this stimulation was not observed in the brain membranes of $CB_2^{-/-}$ mice, suggesting the necessary role of this receptor in the activation of $G\alpha_o$ induced by the agonist ACEA. The activation of the $G\alpha_{q/11}$ subunit was observed in both $CB_1^{-/-}$ (117 ± 2%) and $CB_2^{-/-}$ (118 ± 4%) but not in the $CB_1^{-/-}/CB_2^{-/-}$ mice. Thus, as for Δ^9-THC and WIN55212-2, the activation of $G\alpha_{i3}$ and $G\alpha_o$ subunits was mediated by their interaction with CB_1 and CB_2 receptors, respectively. However, the stimulation of [^{35}S]GTPγS binding to $G\alpha_{q/11}$ subunit seems to be triggered by the activation of both CB_1 and CB_2 cannabinoid receptors.

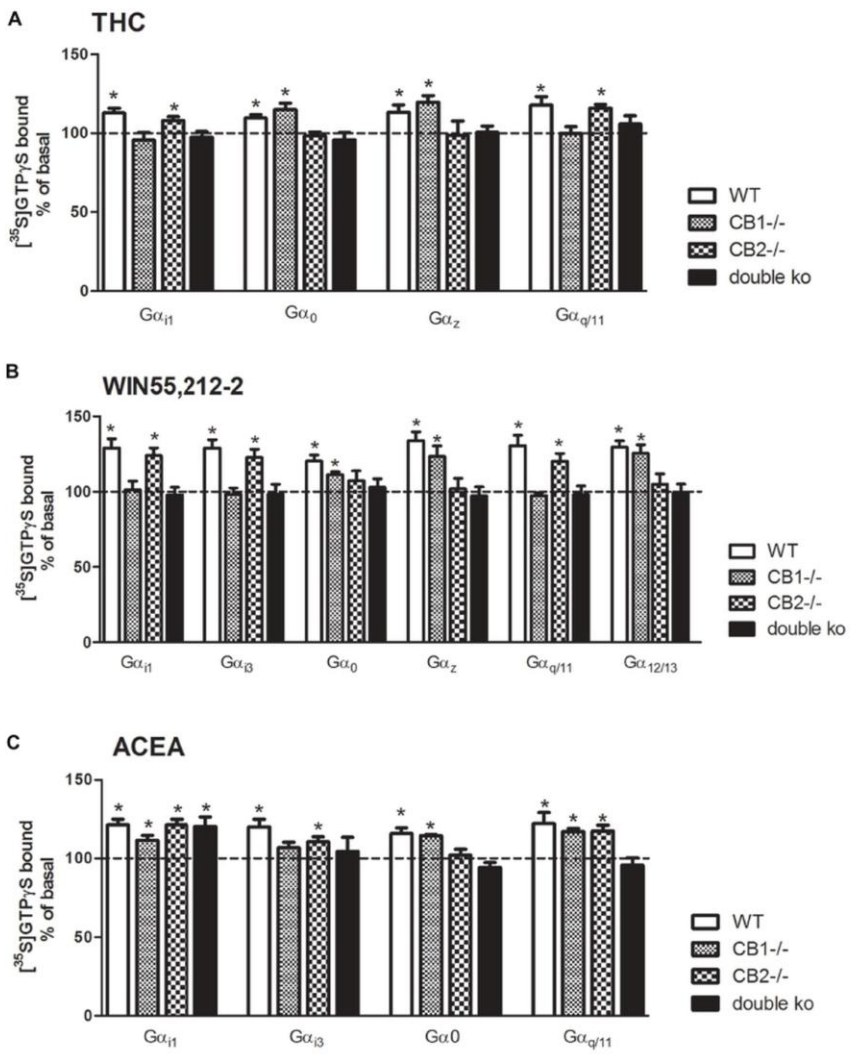


FIGURE 5 | Effects of THC, WIN55, 212-2, and ACEA on G protein activation in cannabinoid receptors knockout mice. [^{35}S]GTPγS scintillation proximity assays coupled to immunoprecipitation with specific antibodies against different Gα subunits in mouse brain cortical membranes of WT, CB_1 knockout ($CB_1^{-/-}$), CB_2 knockout ($CB_2^{-/-}$) and double CB_1 and CB_2 knockout ($CB_1^{-/-}/CB_2^{-/-}$/double KO) co-incubated with **(A)** THC (10 μM) **(B)** WIN55212-2 (10 μM), or **(C)** ACEA (10 μM). Data are shown as percentage of [^{35}S]GTPγS basal binding values obtained for each specific subunit. Bars represent mean ± SEM of four to six different experiments carried out in triplicate. Asterisks highlight those normalized values of stimulation or inhibition of basal binding which are statistically different from 100% (Student's *t*-test; $^*p < 0.05$).

Expression of G Protein Subunits in Knockout Mice for Cannabinoid Receptors

In order to determine if a physiological adaptation of knockout mice to the genetic manipulation to inactivate CB_1 and/or CB_2 receptors may influence our results by the alteration of the expression level of the different Gα protein subunits, Western blotting assays were carried out in brain cortex membranes of WT, $CB_1^{-/-}$, $CB_2^{-/-}$, and $CB_1^{-/-}/CB_2^{-/-}$ mice.

In the case of $G\alpha_{i1}$ (**Figure 6A**), no changes were observed for $CB_1^{-/-}$ and $CB_2^{-/-}$, but a significant reduction (33 ± 10% from WT) of immunodensity was detected in $CB_1^{-/-}/CB_2^{-/-}$ mice brain membrane homogenates. No significant differences were found in the expression of $G\alpha_o$ (**Figure 6C**) or $G\alpha_{12/13}$ (**Figure 6F**) subunits between the WT, $CB_1^{-/-}$, $CB_2^{-/-}$, and $CB_1^{-/-}/CB_2^{-/-}$ mice brain membranes. However, an increase in the expression of $G\alpha_{i3}$ (**Figure 6B**) in both $CB_2^{-/-}$ (139 ± 7%) and $CB_1^{-/-}/CB_2^{-/-}$ mice (140 ± 7%) was found when compared to the WT and $CB_1^{-/-}$ animals. On the contrary,

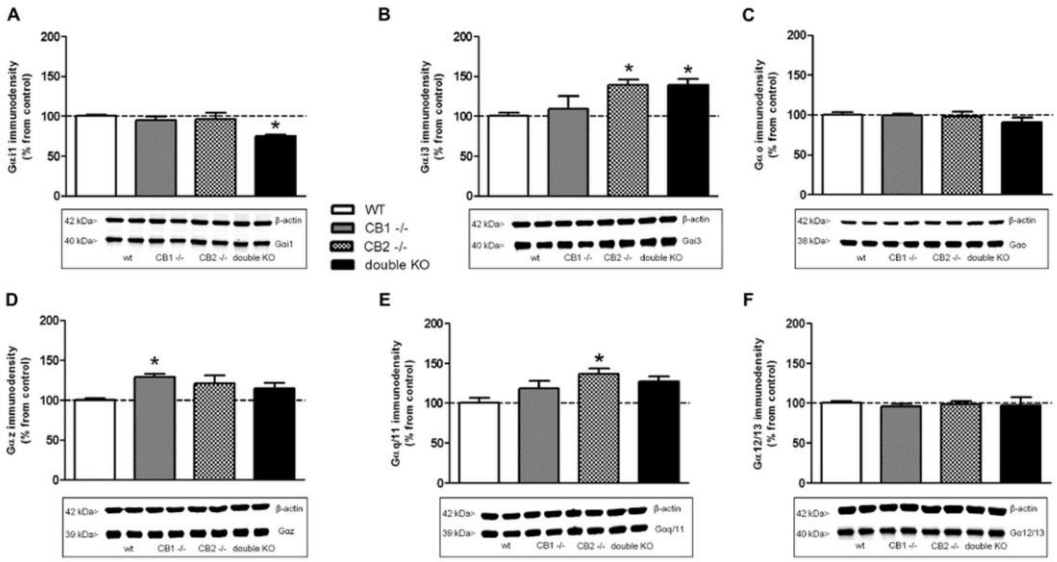


FIGURE 6 | Expression of G protein subunits in cannabinoid receptors knockout mice. Immunoreactive signal and representative images obtained by Western blotting with specific antibodies against different Gα subunits: **(A)** $G\alpha_{i1}$, **(B)** $G\alpha_{i3}$, **(C)** $G\alpha_{o}$, **(D)** $G\alpha_{z}$, **(E)** $G\alpha_{q/11}$, and **(F)** $G\alpha_{12/13}$ in mouse brain cortical membranes of WT, CB_1 knockout ($CB_1^{-/-}$), CB_2 knockout ($CB_2^{-/-}$) and double CB_1 and CB_2 knockout ($CB_1^{-/-}/CB_2^{-/-}$/double KO). Immunoreactivity for β-actin was simultaneously detected on every gel and used as loading control. Normalized values (percentage over controls) of Gα subunits are shown as mean ± SEM of two different experiments carried out in duplicate. Asterisks highlight those values which are statistically different from WT (One-way ANOVA followed by Dunnet's *post hoc* test; $^*p < 0.05$).

$G\alpha_z$ immunodensity was significantly increased in the $CB_1^{-/-}$ mice (129 ± 4%) while no changes were found in the rest of the genotypes when comparing to the WT (**Figure 6D**). Finally, the expression of $G\alpha_{q/11}$ (**Figure 6E**) was significantly increased in brain membranes of $CB_2^{-/-}$ mice (137 ± 7%) but not in WT, $CB_1^{-/-}$ and $CB_1^{-/-}/CB_2^{-/-}$ mice.

DISCUSSION

During the last years, a considerable effort has been made to study the effects of cannabinoids in the brain trying to elucidate the mechanisms by which these compounds may facilitate mental disorders, such as addiction, cognitive deficits, anxiety or psychosis. In this context, these studies have been performed with different cannabinoid ligands (natural or synthetics), in cells or in native tissue and/or in different animal species (mouse, rat, human...).

There is wide evidence supporting the idea that for most of GPCRs, distinct drugs are able to regulate different signaling pathways by the selective activation of different intracellular effectors. The pharmacological relevance of this fact is that the biological responses not only depend on targeting a specific GPCR but also on the particular pathway that this receptor activates. Different studies have focused on the evaluation of the functional selectivity of cannabinoid receptors, but most of them have been performed in transfected cells expressing the CB_1 receptor (Glass and Northup, 1999; Bosier et al., 2010). Moreover, much of these studies explore the signaling pathways activated by different agonists by the evaluation of cAMP production or the phoshorylation of intracellular mediators such as ERK or AKT, with no data about the Gα subtype responsible of these downstream effects. These changes on cAMP concentration or ERK/AKT phosphorylation could be the consequence of the activation of different Gα subtypes, Gβγ dimmers, etc. On the other hand, and interestingly, opposite behavioral effects have been observed after the administration of Δ^9-THC or synthetic cannabinoid ligands (Fattore et al., 2003; Panagis et al., 2014; Rubino and Parolaro, 2016). For example, when evaluating the cannabinoid effects on brain-stimulation reward, Fattore et al. (2003) showed that the potent non-selective CB_1/CB_2 receptor agonists WIN55,212-2 and CP 55,940, but not Δ^9-THC, effectively restored heroin-seeking behavior. In addition, it has been suggested that the signaling of CB_1 receptors may differ between humans and rodents (Straiker et al., 2012).

All these frequently contradictory data highlight the relevance of studying, simultaneously, the effects of different cannabinoid ligands in the same tissue and under the same experimental conditions.

For that reason the goal of the present study was to compare the pattern of G protein subunit stimulation triggered by three structurally different cannabinoids, Δ^9-THC, WIN55212-2 and ACEA in mouse brain cortex. To our knowledge, this is the first study evaluating the cannabinoid-induced stimulation of the different Gα subunits in mouse brain tissue.

WIN55212-2, a synthetic cannabinoid structurally different from Δ^9-THC, is a potent, non-selective CB_1/CB_2 receptor agonist that has been used in many studies of cannabinoid receptor function (Pertwee et al., 2010). The synthetic anandamide analog ACEA is a highly selective agonist for the CB_1 receptor with a low affinity for CB_2 receptors (Hillard et al., 1999).

This study demonstrates that each ligand displays functional selectivity acting as biased agonist for a subset of different G protein subunits. It represents the first characterization of the activation of individual Gα subunits by endogenous cannabinoid receptors in brain cortex. Firstly, we demonstrated that phytocannabinoid Δ^9-THC differs from the synthetic agonists WIN55212-2 and ACEA in its ability to stimulate $G\alpha_{i/o}$ protein subunits in brain cortex.

The $G\alpha_i$ subfamily members $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$ were originally identified by their ability to inhibit AC activity (Plummer et al., 2012; Busnelli et al., 2013; Minetti et al., 2014). Our results show that Δ^9-THC, WIN55212-2, and ACEA significantly stimulate $G\alpha_{i1}$ subunit. Moreover, data from knockout mice suggest that this effect may be CB_1-mediated in the case of Δ^9-THC and WIN55 212-2. However, the $G\alpha_{i1}$ stimulation is still significant in membranes of all genotypes incubated with ACEA, suggesting that this is a non-CB_1 non-CB_2 dependant effect and supporting putative actions of ACEA over other receptors (Pertwee et al., 2010). $G\alpha_{i3}$ subunit was also stimulated in the presence of WIN55212-2 and ACEA, but not of Δ^9-THC. This stimulation seems to be mediated by CB_1 receptors as is blocked in the presence of O-2050 and absent in $CB_1^{-/-}$ or $CB_1^{-/-}/CB_2^{-/-}$ mice. In the case of $G\alpha_{i2}$, it has been previously described that WIN55212-2 is able to activate this subunit in rat (Prather et al., 2000) and in human brain cortical membranes (Erdozain et al., 2012). However, none of the agonists in the present study stimulated the $G\alpha_{i2}$ subunit. This discrepancy may be due to inter-specie and/or regional differences, suggesting that WIN55212-2 may signal through different G protein pools in human and mouse brain cortex.

These three $G\alpha_i$ subunits form the $G\alpha_{i/o}$ subfamily with the neuronal α-subunit $G\alpha_o$, which corresponds to the most abundant Gα protein in brain (Sternweis and Robishaw, 1984). In our experimental approach, and in accordance with other studies (Glass and Northup, 1999; Presley et al., 2016), Δ^9-THC, WIN55212-2 and ACEA significantly stimulated $G\alpha_o$. Results obtained in knockout animals show that the stimulation of $G\alpha_o$ in mouse cortex is mediated, at least in part, by CB_2 receptors, suggesting a necessary role of this receptor in the cannabinoid-induced activation of $G\alpha_o$.

The $G\alpha_z$ subtype is the most divergent member of the inhibitory subfamily and is distributed primarily in neuronal and neuroendocrine cells (Hinton et al., 1990). While Δ^9-THC and WIN55212-2, similarly, stimulated $G\alpha_z$, no stimulation of this subunit was observed when membranes were incubated with ACEA, suggesting that ACEA may not signal through this subunit. Additionally, results obtained with knockout mice suggest that the stimulation of $G\alpha_z$ by Δ^9-THC and WIN55212-2 may be induced by a CB_2-mediated mechanism.

Unlike $G\alpha_z$, the $G\alpha_s$ family is ubiquitously expressed and couples receptors to AC in a stimulatory fashion (Milligan and Kostenis, 2006). Under the present assay conditions, nor Δ^9-THC, WIN55212-2 or ACEA were able to activate this stimulatory subunit. Thus, there is no evidence of $G\alpha_s$ coupling of cannabinoid receptors in the presence of any of these drugs in brain tissue. There are contradictory results about the ability of cannabinoid drugs to activate $G\alpha_s$ proteins. In this way, there are data from both CHO cell lines (Rinaldi-Carmona et al., 1996; Bonhaus et al., 1998) and HEK cells (Presley et al., 2016) expressing CB_1 receptor, showing the absence of effect as well as a modest but significant coupling of CB_1 to $G\alpha_s$ triggered by different cannabinoids. It has been proposed that ACEA may elevate cAMP through a non-CB_1 mechanism, since there is an increase in cAMP in both cells transfected and non-transfected with CB_1 and pretreated with pertussis toxin (Presley et al., 2016). It is important to point out that all these studies have been performed in cell lines. Moreover, they use the accumulation of cAMP in the presence of the $G\alpha_{i/o}$ inhibitor pertussis toxin as an indirect evaluation of potential coupling of CB_1 receptors to $G\alpha_s$. This increase in cAMP production can be mediated by a mechanism different from $G\alpha_s$ activation, as they did not explore directly the activation of this subunit. Therefore, the possible increase in cAMP induced by other actors different from $G\alpha_s$ subunits could not be discarded.

The $G\alpha_{q/11}$ proteins, widely expressed through the CNS, mediate PLC activation, leading to the activation of downstream calcium signaling pathways including PKC and MAPKs activation (Sanchez-Fernandez et al., 2014). In this study, a significant stimulation of $G\alpha_{q/11}$ was observed in the presence of the three cannabinoids evaluated. It has been previously reported that WIN55212-2 induces the coupling of CB_1 to $G\alpha_{q/11}$ in different cellular types (Lauckner et al., 2005; McIntosh et al., 2007). Our results show that not only WIN55212-2 but also Δ^9-THC and ACEA can activate $G\alpha_{q/11}$ subunit in mouse brain. Moreover, the activation of this subunit induced by Δ^9-THC and WIN55212-2 seem to be mediated by the CB_1 receptor, as demonstrate the data obtained with knockout animals. In the case of ACEA, our data suggest that ACEA modulate $G\alpha_{q/11}$ through both CB_1 and CB_2 cannabinoid receptors.

The $G\alpha_{12/13}$ proteins regulate important signaling events by the activation of the small GTPase protein RhoA, involved in the regulation of the actin cytoskeleton and cell motility (Kozasa et al., 2011; Yu and Brown, 2015). Under our experimental conditions, a significant stimulation of $G\alpha_{12/13}$ subunit was observed when membranes were incubated with WIN55212-2 but not with Δ^9-THC or ACEA. To our knowledge, this is the first study reporting that WIN55212-2 signals through $G\alpha_{12/13}$ in brain cortex. These data are concordant with other studies suggesting that cannabinoids induce the stimulation of this RhoA-activator (Dalton et al., 2013; Roland et al., 2014). Moreover, our results from knockout mice show that the WIN55212-2-induced signaling through $G\alpha_{12/13}$ in the brain seems to be mediated, mainly, by the CB_2 receptor.

Although O-2050 had been described as a CB_1 antagonist, it displays a complex pharmacological profile. In this context, its good affinity for CB_2 receptors complicates its use as a tool

to evaluate the unique contribution of CB_1 receptor (Wiley et al., 2011). We observed that, when alone, O-2050 activated the $G\alpha_z$ subunit. Therefore, in co-incubations, O-2050 behaved always as an antagonist of the effects of Δ^9-THC, WIN55212-2 and ACEA over all the studied Gα subunit subtypes, except for the $G\alpha_z$ subunit. When WIN55212-2 and O-2050 were co-incubated, the stimulation of $G\alpha_z$ was lower but still significant. In this way, the blockade exerted by O-2050 pharmacologically confirmed the involvement of cannabinoid receptors in the observed stimulations.

Studies in knockout mice provide very valuable data in basic research but in addition to the absence of the targeted protein, we cannot discard the appearance of putative neurodevelopmental compensatory mechanisms. In this work, we have used CB_1 and/or CB_2 receptor knockout mice to elucidate the role of each receptor in the observed effects of different ligands on the stimulation of Gα subunits. Moreover, Western blotting assays were carried out in order to unmask the role of a possible adaptation of cannabinoid receptors knockout mice affecting the expression level of the different Gα subtypes on the different genotypes. The observed stimulations in knockout mice may not be influenced by putative neurodevelopmental compensatory mechanisms involving G proteins density. In this way, although expression of some Gα subunits in knockout mice is different from the WT, these changes do not explain the absence of stimulation in CB_1 or CB_2 knockout mice. The convergence of our pharmacological and genetic data demonstrate that the results obtained herein with the cannabinoid receptors knockout mice are likely due to the absence of the CB_1 and/or CB_2 receptors and not to non-specific changes due to neurodevelopmental adaptations.

CONCLUSION

Our results demonstrate that, in mouse brain native tissue and under our experimental conditions different exogenous cannabinoids are able to selectively activate different inhibitory and non-inhibitory Gα protein subtypes, through the activation of CB_1 and/or CB_2 receptors. However, it is important to be aware of potential limitations. It has been suggested that the signaling of CB_1 receptors is significantly diminished in humans compared to that of rodents, a finding that may have implications for the use of rodent models for studies of CB_1 receptor function related to human disease and therapy (Straiker et al., 2012).

Results of the present study may help to dissect the specific signaling pathways involved in the different pharmacological actions of cannabinoids. Moreover, the knowledge of the specific molecular target responsible of these different physiological effects will help in the design of new biased cannabinoid drugs with more specific therapeutic effect and a reduced range of adverse effects.

AUTHOR CONTRIBUTIONS

RD-A, II-L, and AL-C performed the experiments, LC, EA, AG-A, JM, and LU designed the study, JM, LC, RD-A, AG-A, and LU analyzed and interpreted the results, RD-A and LU drafted the manuscript. All the contributors revised critically and gave their approval to the final version of the manuscript.

FUNDING

This work was supported by Plan Estatal de I+D+i 2013–2016 and Instituto de Salud Carlos III-Subdirección General de Evaluación y Fomento de la Investigación, Spanish Ministry of Economy, and FEDER (grant number PI13/01529 to LU) and the Basque Government (grant number IT616/13). II-L is a recipient of a Predoctoral Fellowship from the Basque Government. AL-C acknowledges the financial support given by Universidad de Antioquia, COLCIENCIAS through the Francisco José de Caldas fellowship 512/2010.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Guillermo Velasco and Dr. Ismael Galve-Roperh (Universidad Complutense de Madrid) for kindly donating $CB_1^{-/-}$ and $CB_2^{-/-}$ mice. These lines were used to set up a permanent colony and establish the $CB_1^{-/-}/CB_2^{-/-}$ double knockout mouse model in our lab.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphar.2016.00415/full#supplementary-material

REFERENCES

Atwood, B. K., and Mackie, K. (2010). CB2: a cannabinoid receptor with an identity crisis. *Br. J. Pharmacol.* 160, 467–479. doi: 10.1111/j.1476-5381.2010.00729.x

Bonhaus, D. W., Chang, L. K., Kwan, J., and Martin, G. R. (1998). Dual activation and inhibition of adenylyl cyclase by cannabinoid receptor agonists: evidence for agonist-specific trafficking of intracellular responses. *J. Pharmacol. Exp. Ther.* 287, 884–888.

Bosier, B., Muccioli, G. G., Hermans, E., and Lambert, D. M. (2010). Functionally selective cannabinoid receptor signalling: therapeutic implications and opportunities. *Biochem. Pharmacol.* 80, 1–12. doi: 10.1016/j.bcp.2010.02.013

Brents, L. K., Reichard, E. E., Zimmerman, S. M., Moran, J. H., Fantegrossi, W. E., and Prather, P. L. (2011). Phase I hydroxylated metabolites of the K2 synthetic cannabinoid JWH-018 retain in vitro and in vivo cannabinoid 1 receptor affinity and activity. *PLoS ONE* 6:e21917. doi: 10.1371/journal.pone.0021917

Buckley, N. E., McCoy, K. L., Mezey, E., Bonner, T., Zimmer, A., Felder, C. C., et al. (2000). Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB(2) receptor. *Eur. J. Pharmacol.* 396, 141–149. doi: 10.1016/S0014-2999(00)00211-9

Busnelli, M., Peverelli, E., Mantovani, G., Spada, A., and Chini, B. (2013). Deciphering the specific role of G(alphai/o) isoforms: functional selective

oxytocin ligands and somatostatin SST5 receptor mutants. *Biochem. Soc. Trans.* 41, 166–171. doi: 10.1042/BST20120306

Canals, M., and Milligan, G. (2008). Constitutive activity of the cannabinoid CB1 receptor regulates the function of co-expressed Mu opioid receptors. *J. Biol. Chem.* 283, 11424–11434. doi: 10.1074/jbc.M710300200

Childers, S. R., Pacheco, M. A., Bennett, B. A., Edwards, T. A., Hampson, R. E., Mu, J., et al. (1993). Cannabinoid receptors: G-protein-mediated signal transduction mechanisms. *Biochem. Soc. Symp.* 59, 27–50.

Dalton, G. D., Peterson, L. J., and Howlett, A. C. (2013). CB(1) cannabinoid receptors promote maximal FAK catalytic activity by stimulating cooperative signalling between receptor tyrosine kinases and integrins in neuronal cells. *Cell. Signal.* 25, 1665–1677. doi: 10.1016/j.cellsig.2013.03.020

De Jesus, M. L., Salles, J., Meana, J. J., and Callado, L. F. (2006). Characterization of CB1 cannabinoid receptor immunoreactivity in postmortem human brain homogenates. *Neuroscience* 140, 635–643. doi: 10.1016/j.neuroscience.2006.02.024

Dhopeshwarkar, A., and Mackie, K. (2016). Functional selectivity of CB2 cannabinoid receptor ligands at a canonical and noncanonical pathway. *J. Pharmacol. Exp. Ther.* 358, 342–351. doi: 10.1124/jpet.116.232561

Erdozain, A. M., Diez-Alarcia, R., Meana, J. J., and Callado, L. F. (2012). The inverse agonist effect of rimonabant on G protein activation is not mediated by the cannabinoid CB1 receptor: evidence from postmortem human brain. *Biochem. Pharmacol.* 83, 260–268. doi: 10.1016/j.bcp.2011.10.018

Fattore, L., Spano, M. S., Cossu, G., Deiana, S., and Fratta, W. (2003). Cannabinoid mechanism in reinstatement of heroin-seeking after a long period of abstinence in rats. *Eur. J. Neurosci.* 17, 1723–1726. doi: 10.1046/j.1460-9568.2003.02607.x

Gettys, T. W., Sheriff-Carter, K., Moomaw, J., Taylor, I. L., and Raymond, J. R. (1994). Characterization and use of crude alpha-subunit preparations for quantitative immunoblotting of G proteins. *Anal. Biochem.* 220, 82–91. doi: 10.1006/abio.1994.1302

Glass, M., and Northup, J. K. (1999). Agonist selective regulation of G proteins by cannabinoid CB(1) and CB(2) receptors. *Mol. Pharmacol.* 56, 1362–1369.

Gonzalez-Maeso, J., Rodriguez-Puertas, R., Gabilondo, A. M., and Meana, J. J. (2000). Characterization of receptor-mediated [35S]GTPgammaS binding to cortical membranes from postmortem human brain. *Eur. J. Pharmacol.* 390, 25–36. doi: 10.1016/S0014-2999(99)00827-4

Hillard, C. J., Manna, S., Greenberg, M. J., DiCamelli, R., Ross, R. A., Stevenson, L. A., et al. (1999). Synthesis and characterization of potent and selective agonists of the neuronal cannabinoid receptor (CB1). *J. Pharmacol. Exp. Ther.* 289, 1427–1433.

Hinton, D. R., Blanks, J. C., Fong, H. K., Casey, P. J., Hildebrandt, E., and Simons, M. I. (1990). Novel localization of a G protein, Gz-alpha, in neurons of brain and retina. *J. Neurosci.* 10, 2763–2770.

Howlett, A. C., Barth, F., Bonner, T. I., Cabral, G., Casellas, P., Devane, W. A., et al. (2002). International union of pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol. Rev.* 54, 161–202. doi: 10.1124/pr.54.2.161

Hudson, B. D., Hebert, T. E., and Kelly, M. E. (2010). Physical and functional interaction between CB1 cannabinoid receptors and beta2-adrenoceptors. *Br. J. Pharmacol.* 160, 627–642. doi: 10.1111/j.1476-5381.2010.00681.x

Khajehali, E., Malone, D. T., Glass, M., Sexton, P. M., Christopoulos, A., and Leach, K. (2015). Biased agonism and biased allosteric modulation at the CB1 cannabinoid receptor. *Mol. Pharmacol.* 88, 368–379. doi: 10.1124/mol.115.099192

Kozasa, T., Hajicek, N., Chow, C. R., and Suzuki, N. (2011). Signalling mechanisms of RhoGTPase regulation by the heterotrimeric G proteins G12 and G13. *J. Biochem.* 150, 357–369. doi: 10.1093/jb/mvr105

Lauckner, J. E., Hille, B., and Mackie, K. (2005). The cannabinoid agonist WIN55,212-2 increases intracellular calcium via CB1 receptor coupling to Gq/11 G proteins. *Proc. Natl. Acad. Sci. U.S.A.* 102, 19144–19149. doi: 10.1073/pnas.0509588102

Mackie, K. (2005). Distribution of cannabinoid receptors in the central and peripheral nervous system. *Handb. Exp. Pharmacol.* 168, 299–325. doi: 10.1007/3-540-26573-2_10

Makwana, R., Molleman, A., and Parsons, M. E. (2010). Evidence for both inverse agonism at the cannabinoid CB1 receptor and the lack of an endogenous cannabinoid tone in the rat and guinea-pig isolated ileum myenteric plexus-longitudinal muscle preparation. *Br. J. Pharmacol.* 160, 615–626. doi: 10.1111/j.1476-5381.2010.00717.x

Marsicano, G., Wotjak, C. T., Azad, S. C., Bisogno, T., Rammes, G., Cascio, M. G., et al. (2002). The endogenous cannabinoid system controls extinction of aversive memories. *Nature* 418, 530–534. doi: 10.1038/nature00839

McIntosh, B. T., Hudson, B., Yegorova, S., Jollimore, C. A., and Kelly, M. E. (2007). Agonist-dependent cannabinoid receptor signalling in human trabecular meshwork cells. *Br. J. Pharmacol.* 152, 1111–1120. doi: 10.1038/sj.bjp.0707495

Milligan, G., and Kostenis, E. (2006). Heterotrimeric G-proteins: a short history. *Br. J. Pharmacol.* 147(Suppl. 1), S46–S55. doi: 10.1038/sj.bjp.0706405

Minetti, G. C., Feige, J. N., Bombard, F., Heier, A., Morvan, F., Nurnberg, B., et al. (2014). Galphai2 signalling is required for skeletal muscle growth, regeneration, and satellite cell proliferation and differentiation. *Mol. Cell. Biol.* 34, 619–630. doi: 10.1128/MCB.00957-13

Panagis, G., Mackey, B., and Vlachou, S. (2014). Cannabinoid regulation of brain reward processing with an emphasis on the role of CB1 receptors: a step back into the future. *Front. Psychiatry* 5:92. doi: 10.3389/fpsyt.2014.00092

Pertwee, R. G., Howlett, A. C., Abood, M. E., Alexander, S. P., Di Marzo, V., Elphick, M. R., et al. (2010). International union of basic and clinical pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB(1) and CB(2). *Pharmacol. Rev.* 62, 588–631. doi: 10.1124/pr.110.003004

Plummer, N. W., Spicher, K., Malphurs, J., Akiyama, H., Abramowitz, J., Nurnberg, B., et al. (2012). Development of the mammalian axial skeleton requires signalling through the Galpha(i) subfamily of heterotrimeric G proteins. *Proc. Natl. Acad. Sci. U.S.A.* 109, 21366–21371. doi: 10.1073/pnas.1219810110

Prather, P. L., Martin, N. A., Breivogel, C. S., and Childers, S. R. (2000). Activation of cannabinoid receptors in rat brain by WIN 55212-2 produces coupling to multiple G protein alpha-subunits with different potencies. *Mol. Pharmacol.* 57, 1000–1010.

Presley, C. S., Abidi, A. H., and Moore, B. M. II. (2016). Cannabinoid receptor 1 ligands revisited: Pharmacological assessment in the ACTOne system. *Anal. Biochem.* 498, 8–28. doi: 10.1016/j.ab.2015.12.019

Rinaldi-Carmona, M., Calandra, B., Shire, D., Bouaboula, M., Oustric, D., Barth, F., et al. (1996). Characterization of two cloned human CB1 cannabinoid receptor isoforms. *J. Pharmacol. Exp. Ther.* 278, 871–878.

Roland, A. B., Ricobaraza, A., Carrel, D., Jordan, B. M., Rico, F., Simon, A., et al. (2014). Cannabinoid-induced actomyosin contractility shapes neuronal morphology and growth. *Elife* 3: e03159. doi: 10.7554/eLife.03159

Rubino, T., and Parolaro, D. (2016). The impact of exposure to cannabinoids in adolescence: insights from animal models. *Biol. Psychiatry* 79, 578–585. doi: 10.1016/j.biopsych.2015.07.024

Sanchez-Fernandez, G., Cabezudo, S., Garcia-Hoz, C., Beninca, C., Aragay, A. M., Mayor, F., et al. (2014). Galphaq signalling: the new and the old. *Cell. Signal.* 26, 833–848. doi: 10.1016/j.cellsig.2014.01.010

Shapira, M., Vogel, Z., and Sarne, Y. (2000). Opioid and cannabinoid receptors share a common pool of GTP-binding proteins in cotransfected cells, but not in cells which endogenously coexpress the receptors. *Cell. Mol. Neurobiol.* 20, 291–304. doi: 10.1023/A:1007058008477

Sternweis, P. C., and Robishaw, J. D. (1984). Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. *J. Biol. Chem.* 259, 13806–13813.

Straiker, A., Wager-Miller, J., Hutchens, J., and Mackie, K. (2012). Differential signalling in human cannabinoid CB1 receptors and their splice variants in autaptic hippocampal neurones. *Br. J. Pharmacol.* 165, 2660–2671. doi: 10.1111/j.1476-5381.2011.01744.x

Valdizan, E. M., Castro, E., and Pazos, A. (2010). Agonist-dependent modulation of G-protein coupling and transduction of 5-HT1A receptors in rat dorsal raphe nucleus. *Int. J. Neuropsychopharmacol.* 13, 835–843. doi: 10.1017/S1461145709990940

Wiley, J. L., Breivogel, C. S., Mahadevan, A., Pertwee, R. G., Cascio, M. G., Bolognini, D., et al. (2011). Structural and pharmacological analysis of O-2050, a putative neutral cannabinoid CB(1) receptor antagonist. *Eur. J. Pharmacol.* 651, 96–105. doi: 10.1016/j.ejphar.2010.10.085

Yu, O. M., and Brown, J. H. (2015). G protein-coupled receptor and RhoA-stimulated transcriptional responses: links to inflammation, differentiation, and cell proliferation. *Mol. Pharmacol.* 88, 171–180. doi: 10.1124/mol.115.097857

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY MATERIAL

Biased Agonism of Three Different Cannabinoid Receptor Agonists in Mouse Brain Cortex

Rebeca Díez-Alarcia[1,2], Inés Ibarra-Lecue[1], Ángela P Lopez-Cardona[3,4], J Javier Meana[1,2], Alfonso Gutiérrez-Adán[3], Luis F Callado[1,2], Ekaitz Agirregoitia[5], Leyre Urigüen[1,2]

[1]*Department of Pharmacology, University of the Basque Country UPV/EHU, Leioa, Spain*

[2]*Centro de Investigación Biomédica en Red de Salud Mental, Madrid, Spain*

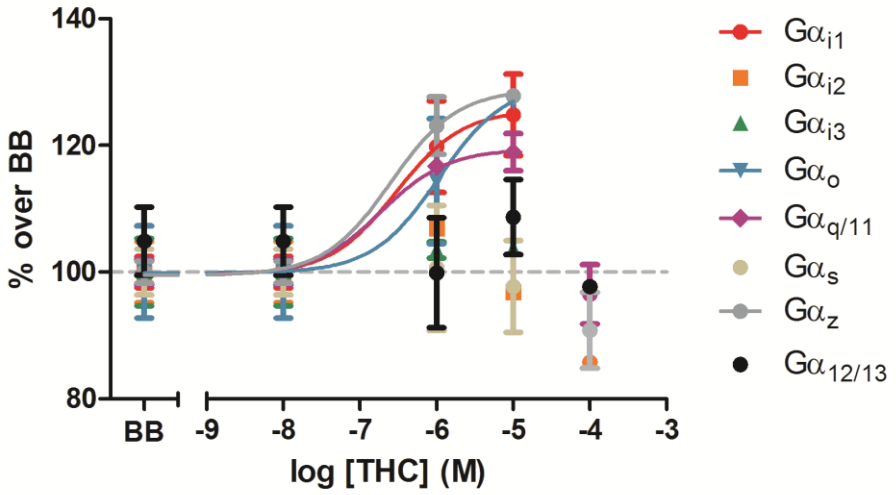
[3]*Department of Animal Reproduction, Instituto Nacional de Tecnología Agraria y Alimentaria, Madrid, Spain*

[4]*G.I. Biogénesis, Universidad de Antioquia, Antioquia, Colombia*

[5]*Department of Physiology, University of the Basque Country, Leioa, Spain*

Content:

Supplementary Figure 1



	Gαi1	Gαo	Gαq/11	Gαz
Bottom	99.59	99.86	99.53	99.47
Top	125.7	129.9	119.5	128.9
LogEC50	-6.526	-5.969	-6.726	-6.597

Concentration-dependent curves of stimulation of the different G α subunits ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{io}$, $G\alpha_{q/11}$, $G\alpha_{s}$, $G\alpha_{z}$, $G\alpha_{12/13}$) obtained for THC by [^{35}S]GTPγS SPA coupled to immunoprecipitation with specific antibodies in mouse brain tissue. Data are shown as percentage of [^{35}S]GTPγS binding stimulationn over basal binding values (100%). Data correspond to the mean ± of three experimental triplicates.

ARTICLE 2

Chronic cannabis promotes pro-hallucinogenic signaling of 5-HT2A receptors through Akt/mTOR pathway

Inés Ibarra-Lecue, Irene Mollinedo-Gajate, J Javier Meana, Luis F Callado, Rebeca Díez-Alarcia, Leyre Urigüen

Neuropsychopharmacology (2018), **43**(10): 2028-2035

DOI: https://doi.org/10.1038/s41386-018-0076-y

Impact factor 2017: 6.5

Relative position: 13/261, D1 Pharmacology and Pharmacy

10/142, D1 Psychiatry

21/261, D1 Neurosciences

ARTICLE 2

Cannabis abuse during adolescence, especially the consumption of strains with high concentrations of THC, increases the risk of developing schizophrenia later in life. But despite this evidence is widely accepted, the mechanisms underlying this relationship remain poorly understood. With the aim of deepen the knowledge of these mechanisms, in this study we evaluated in mice the effects of an early chronic THC exposure (10 mg/kg i.p. daily, 30 days, from postnatal day 21 to postnatal day 51), focusing on two molecular targets that have been previously associated with schizophrenia or psychotomimetic effects of cannabis: cortical 5-HT2AR and Akt/mTOR signaling pathway. Chronic exposure to THC was demonstrated to exacerbate schizophrenia-like responses, such as PPI impairment induced by 5-HT2AR stimulation. The study of (±)DOI induced Gα protein subunit stimulation showed that chronic THC promoted a selective hyperfunctionality of cortical 5-HT2AR towards inhibitory Gαi1, Gαi3, Gαo, and Gαz proteins, whereas canonical Gαq/11 protein signaling pathway did not show a significant modulation. In addition, THC evoked a hyperactive state of cortical Akt/mTOR signaling pathway. In order to evaluate whether this signaling pathway was implicated on the effects of chronic THC on 5-HT2AR functionality, a co-treatment with the specific mTOR inhibitor rapamycin (5 mg/kg i.p. alternate days, 30 days) was carried out. Subsequent experiments revealed that the effective blockade of Akt/mTOR signaling prevents the 5-HT2AR signaling pattern modulation as well as the supersensitivity to schizophrenia-like effects induced by the chronic THC administration. These results provide a novel and plausible mechanism through which chronic cannabis exposure in early life could increase the risk of developing schizophrenia.

Neuropsychopharmacology

www.nature.com/npp

ARTICLE OPEN

Chronic cannabis promotes pro-hallucinogenic signaling of 5-HT2A receptors through Akt/mTOR pathway

Inés Ibarra-Lecue[1], Irene Mollinedo-Gajate[1], J Javier Meana[1,2], Luis F Callado[1,2], Rebeca Diez-Alarcia[1,2] and Leyre Urigüen[1,2]

Long-term use of potent cannabis during adolescence increases the risk of developing schizophrenia later in life, but to date, the mechanisms involved remain unknown. Several findings suggest that the functional selectivity of serotonin 2A receptor (5-HT2AR) through inhibitory G-proteins is involved in the molecular mechanisms responsible for psychotic symptoms. Moreover, this receptor is dysregulated in the frontal cortex of schizophrenia patients. In this context, studies involving cannabis exposure and 5-HT2AR are scarce. Here, we tested in mice the effect of an early chronic Δ^9-tetrahydrocannabinol (THC) exposure on cortical 5-HT2AR expression, as well as on its in vivo and in vitro functionality. Long-term exposure to THC induced a pro-hallucinogenic molecular conformation of the 5-HT2AR and exacerbated schizophrenia-like responses, such as prepulse inhibition disruption. Supersensitive coupling of 5-HT2AR toward inhibitory Gαi1-, Gαi3-, Gαo-, and Gαz-proteins after chronic THC exposure was observed, without changes in the canonical Gαq/11-protein pathway. In addition, we found that inhibition of Akt/mTOR pathway by rapamycin blocks the changes in 5-HT2AR signaling pattern and the supersensitivity to schizophrenia-like effects induced by chronic THC. The present study provides the first evidence of a mechanistic explanation for the relationship between chronic cannabis exposure in early life and increased risk of developing psychosis-like behaviors in adulthood.

Neuropsychopharmacology (2018) 0:1–8; https://doi.org/10.1038/s41386-018-0076-y

INTRODUCTION

Cannabis consumption especially in early adolescence [1], a period of increased vulnerability to its effects, increases the risk of developing schizophrenia [2]. Both cannabis extracts and Δ^9-tetrahydrocannabinol (THC) can evoke transient psychotic states in healthy subjects [3] and worsen symptoms in schizophrenia patients [4]. However, the neuronal and molecular mechanisms underlying psychotic symptoms elicited by cannabis abuse during adolescence remain unknown.

Several findings suggest that serotonin 2A receptors (5-HT2AR) are involved in the molecular mechanisms responsible for psychotic symptoms. In addition to the canonical signaling pathway through Gαq/11-proteins, hallucinogenic 5-HT2AR agonists, such as lysergic acid diethylamide (LSD) and 2,5-dimethoxy-4-iodoamphetamine ((±)-DOI), which trigger mental states resembling psychotic symptoms [5] activate inhibitory Gαi/o proteins [6]. Conversely, non-hallucinogenic 5-HT2AR agonists exclusively activate the canonical signaling pathway through Gαq/11-proteins. This differential signaling mechanism responsible for the unique effects of hallucinogens is known as biased agonism.

The disruption of 5-HT2AR functionality in psychosis is supported by genetic studies describing differential epigenetic methylation and gene polymorphisms in subjects with schizophrenia [7]. Animal models of schizophrenia have shown increased 5-HT2AR density and/or functionality [8, 9]. Moreover, it has been demonstrated that the active conformation of the 5-HT2AR is up-regulated in prefrontal cortex of antipsychotic-free schizophrenia subjects [10]. Furthermore, atypical antipsychotics display high affinity as antagonists of 5-HT2AR. All these data demonstrate that upregulation and/or increased functionality of 5-HT2AR could predispose to psychosis or schizophrenia.

Akt/mTOR signaling pathway mediates several functions, including synaptic plasticity and axonal branching [11]. Akt protein dysregulation has been linked to schizophrenia [12, 13], and, in addition, *AKT1* genetic variations have been associated with increased psychotic symptoms after smoking cannabis [14]. Importantly, acute THC activates this signaling pathway in mouse brain [15].

Considering these findings, we investigated whether chronic THC exposure at young ages could lead to an over-functionality of brain cortical 5-HT2AR with biased agonism toward pro-hallucinogenic G-protein pathways. In addition, the possible involvement of Akt/mTOR signaling pathway in the induction of this psychosis-like status was studied.

MATERIALS AND METHODS

Animals

All experimental procedures were performed in accordance with the European Directive for the Protection of Vertebrate Animals used for experimental and Other Scientific Purposes (European Union Directive 2010/63/EU) and approved by the Ethic Committee for Animal Welfare of the University of the Basque Country (UPV/EHU) (CEBA 270M/2012, 189/2011). Male CD-1 mice (Charles Rivers, Wilmington, MA, USA) were housed (6–8 animals per cage) under controlled temperature (23 ± 1 °C), on a normal 12 h light/dark cycle, with free access to food and water. Only male mice

[1]Department of Pharmacology, University of the Basque Country UPV/EHU and Centro de Investigación Biomédica en Red de Salud Mental CIBERSAM, Leioa, Spain and [2]Biocruces Health Research Institute, Bizkaia, Spain
Correspondence: Leyre Urigüen (leyre.uriguen@ehu.eus)

Received: 13 February 2018 Revised: 13 April 2018 Accepted: 15 April 2018
Published online: 27 April 2018

were used in this study to avoid the effects of the female estrous cycles that can interfere with the pharmacological parameters.

Treatments
THC, rapamycin and vehicle were administered for 30 days starting at postnatal day 21. Drugs were dissolved in ethanol:cremophor: saline (0.9% NaCl) at 1:1:18 ratio. THC (THCPharm. GmbH. Frankfurt, Germany) (10 mg/kg ip) [15] was injected daily. Rapamycin (Shelleck Chemicals LLC, Houston, TX, USA) (5 mg/kg ip) was administered in a 1 day on/1 day off schedule [16], 30 min before THC injection. For prepulse inhibition (PPI) experiments, (±)-DOI (Sigma-Aldrich, St. Louis, MO, USA) (0.5 mg/kg ip) [17] was dissolved in saline and administered immediately before the behavioral assay. Experiments were carried out after a 5-day washout period to avoid the interference of acute effects of THC.

Experimental design
Experimental design of each assay was conducted as follows. A first batch of two groups of animals (vehicle and THC, $n = 16$ animals per group) was treated with THC or vehicle for 30 days. After a 5-day washout period, a number of animals ($n = 8$ per group, vehicle and THC) were killed, brains removed and THC concentrations measured. The rest of the animals ($n = 8$ per group, vehicle and THC) were used to perform basal PPI, 5-HT2AR density, radioligand binding and functional coupling experiments ($n = 8$ per group, pooled for in vitro assays). Briefly, after the 5-day washout period, PPI experiments were carried out and later, animals were killed, brains removed, and in vitro assays performed.

Another different batch of four groups of animals (vehicle-vehicle, vehicle-THC, rapamycin-vehicle and rapamycin-THC) ($n = 8$ per group) was stablished to carry out basal PPI, western blot experiments (Akt, p-Akt, rpS6, p-rpS6) and functional coupling experiments ($n = 8$ per group, pooled for in vitro assays) following these different pharmacological treatments.

Finally, another batch of four groups (vehicle-vehicle, vehicle-THC, rapamycin-vehicle and rapamycin-THC) was used to accomplish PPI experiments after acute DOI in a timely matched manner ($n = 8$ per group).

Quantification of THC concentration in mouse brain by liquid chromatography–tandem mass spectrometry
Pooled homogenized THC-free mouse brain samples ($n = 6$–8) were used for the development and validation. Experiments were carried out with a 1290 Infinity II ultrahigh performance liquid chromatography (UHPLC) system coupled to a 6495 iFunnel triple Quad mass spectrometer equipped with a JetStream electrospray ionization source (Agilent Technologies, Santa Clara, CA, USA). A Kinetex EVOC18 100A 3 × 100 mm (2.6 µm) column was selected. Sample analysis in positive ionization mode was performed using as mobile phase water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B) with elution gradient mode. The flow rate was 0.5 ml/min; injection volume was 5 µl, and the column temperature was maintained at 30 °C. The calibration curve was prepared by spiking 20 µl of standard working solution to obtain brain THC final concentrations of 8–2000 ng/g. Quality control samples (QCs) were prepared by spiking brain samples containing 60 and 1000 ng/g as the final THC concentration.

Prepulse inhibition of the startle reflex
Prepulse inhibition (PPI) test was performed in a startle chamber (PanLab, Barcelona, Spain) where a 60-dB background-noise was present for a 10 min acclimatization period, as well as throughout the entire experiment. Each session began with five startle pulse-alone trials to achieve a more stable response of the animals and were not included in the analysis. Mice were then subjected to a pseudo-randomized combination of: ten pulse-alone trials consisted of a white noise burst (120 dB, 40 ms); ten prepulse-alone trials for each prepulse intensity (10 ms at 77, 82, or 87 dB); ten prepulse-pulse trials for each prepulse intensity with an interval of 60 ms between both and ten no-stimulus trials, in which only the background noise was present. The inter-trial intervals were 10, 12, 15, 20, and 25 s. The maximum amplitude of the startle reaction was recorded for every trial, and the average startle for each trial subgroup was used for the analysis. No-stimulus trials and prepulse-alone trials did not exert any startle response and data were not included in the analysis. PPI was calculated as a percentage score: % PPI = 100—[(startle response to prepulse-pulse trial/startle response to pulse-alone trial) × 100]. Inhibition of the PPI response was induced by administration of the 5-HT2AR agonist (±)-DOI (0.5 mg/kg, i.p.) immediately before acclimation period. Each session lasted 30–33 min.

Brain cortex membranes preparation
Animals were killed by cervical dislocation, brains removed, cortex dissected, and samples stored at −80 °C until use. Membrane-enriched fraction (P2) was prepared as described [18].

Radioligand binding
[^{3}H]Ketanserin saturation binding assays were performed as previously reported [19] with minor modifications. [^{3}H]Ketanserin binding (0.05–10 nM; eight concentrations) was used to calculate density (Bmax) and affinity (Kd) of 5-HT2AR. Non-specific binding was determined in the presence of the 5-HT2AR antagonist M100907 (1 µM). Competition curves of [^{3}H]ketanserin binding (2 nM) with increasing concentrations of the agonist (±)-DOI (10^{-12}–10^{-3} M) were also performed in order to delineate both the G-protein coupled and uncoupled 5-HT2AR conformations. Briefly, after incubation (60 min, 37 °C), free radioligand was discarded by rapid filtration under vacuum (1450 FilterMate Harvester, Perkin Elmer, Waltham, MA, USA). Filters were then rinsed, dried and bagged in Sample Bag with BetaPlate Scint scintillation cocktail. Radioactivity was detected by liquid scintillation spectrometry using a MicroBeta TriLux counter (Perkin Elmer, Waltham, MA, USA).

Antibody-capture [^{35}S]GTPγS scintillation proximity assay (SPA)
Specific activation of different subtypes of Gα-proteins by (±)-DOI (10 µM) was determined using a previously described protocol [18]. The concentration of (±)-DOI (10 µM) was chosen for being the one which induces stimulation values around the Emax for any Gα subunit subtype under our experimental conditions [19]. [^{35}S]GTPγS binding was performed in buffer containing 0.4 nM [^{35}S]GTPγS, 15 µg of protein/well and different GDP concentrations depending on the Gα-subtype tested. Specific antibody for each Gα-subunit and polyvinyltoluene SPA beads coated with protein-A were added and incubated (3 h, room temperature). Plates were centrifuged and bound radioactivity detected on a MicroBeta TriLux scintillation counter. Non-specific binding was defined as the remaining [^{35}S]GTPγS binding in the presence of 100 µM unlabeled GTPγS. The antagonist ketanserin (10 µM) was used to confirm the 5-HT2AR involvement in the observed stimulations (data not shown).

Western blot
Western blot experiments were performed with total homogenate fractions (S1) from mouse brain cortex tissue. Samples (30 µg) were heated (95 °C), loaded onto polyacrylamide gel (12%) and submitted to SDS-PAGE. Nitrocellulose membranes were blocked (5% non-fat dry-milk or/and 0.5% BSA) in TBS buffer followed by overnight incubation with primary antibodies (4 °C). Antibodies against 5-HT2AR, Akt, phospho-Akt(Ser473), rpS6, phospho-rpS6 (Ser235/236) and β-actin were used. Incubation with fluorescent anti-IgG secondary antibodies was performed at room temperature (1 h). Immunoreactivity was quantified using an Odyssey

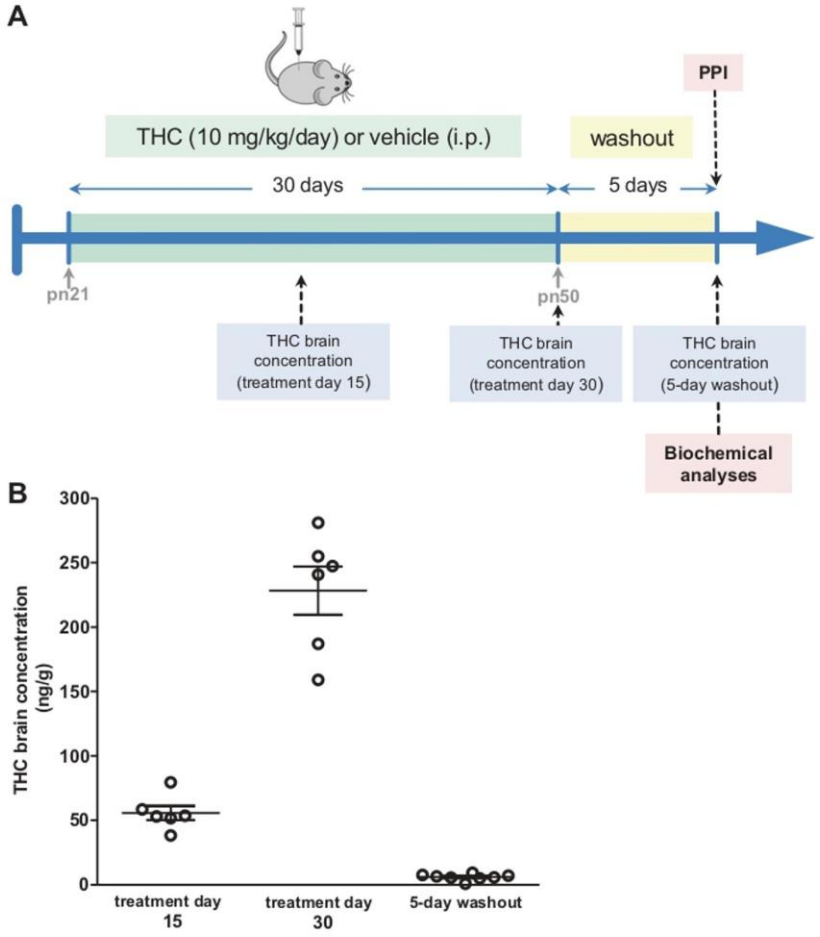


Fig. 1 Chronic THC treatment protocol and concentration. **a** Representation of the treatment design. **b** THC concentrations in mouse brain ($n = 6–8$) during treatment and after the washout period. Points represent individual data for each animal and lines represent means

Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) (further details in Supplementary Table 1).

Data analysis and statistical procedures

Data were analyzed with GraphPad Prism™ 5.01, and InVivoStat software. Before the statistical analyses, the data were inspected for outliers (critical value, $Z > 1.96$) using Grubb's test (GraphPad Software, www.graphpad.com/quickcalcs/grubbs1.cfm). In PPI experiments, data from animals (between 0 and 3 animals per group) showing an outlier value for a particular dB or for the startle amplitude to the pulse were discarded for further analysis. Two animals were also discarded for not showing any response to the startle stimulus. Two-group comparisons were made by unpaired Student's *t*-test. Multiple groups' comparisons were studied by one-way, two-way or three-way analysis of variance (ANOVA), followed by Bonferroni's or Benjamini-Hochberg's post hoc analyses. Statistical significance was set at $p < 0.05$. Displacement curves were analyzed using a nonlinear fit, and the selection between models was made by the extrasum-of-squares (*F*-test). Following the nonlinear curve fitting, Ki values for (±)-DOI were calculated from the corresponding IC50 values. Differences in the binding profiles were assessed by unpaired Student's *t*-test of normalized (log) parameters. Specific binding values obtained from SPA assays were transformed to percentage of basal binding (binding values observed without agonist drug) obtained for each Gα-protein and analyzed by one-sample or two-sample Student's *t*-test. The immunodensitometric values of the different target proteins were normalized to the intra-assay values obtained with anti-β-actin antibody and expressed as mean ± SEM of the percentages over an inter-assay normalization sample included in every experiment. Each sample was analyzed at least in two independent experiments.

RESULTS

Time-dependent concentration of THC in mouse brain cortex

In order to validate the chronic administration protocol, brain THC concentrations were measured at treatment days 15 and 30, and after a 5-day washout period (Fig. 1a). THC reached 56 ng/g after 15 days of daily-chronic treatment. This concentration increased fourfold at day 30 (228 ng/g), while brain THC levels largely disappeared (6.05 ng/g) after the washout period (Fig. 1b).

Chronic THC potentiates (±)-DOI-induced decrease in PPI

Loss of PPI in rodents is considered a valid proxy for the study of the neurobiology of impaired sensorimotor gating in schizophrenia patients [20, 21]. Disruptions of the PPI response by acute administration of the 5-HT2AR agonist (±)-DOI were evaluated in vehicle and THC-treated animals. We used a dose of 0.5 mg/kg (±)-DOI that has demonstrated, under our experimental

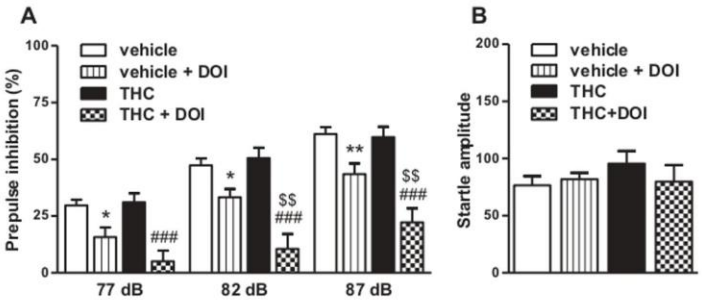


Fig. 2 Chronic THC increases the disruption of PPI induced by the 5-HT2AR agonist (±)-DOI. **a** Chronic THC exacerbated the (±)-DOI-induced PPI disruption (three-way repeated measures ANOVA: THC × (±)-DOI; $F(1,51) = 6.48$, $p < 0.05$; $n = 8–16$). Benjamini-Hochberg's post hoc comparisons: $^{*}p < 0.05$, $^{**}p < 0.01$ vs. vehicle. ###$p < 0.001$ vs. THC, $$$p < 0.01$ vs. vehicle-DOI (see Supplementary Table 3 for further details on ANOVAs). **b** No differences were found in the startle amplitude among groups. Bars represent mean ± SEM

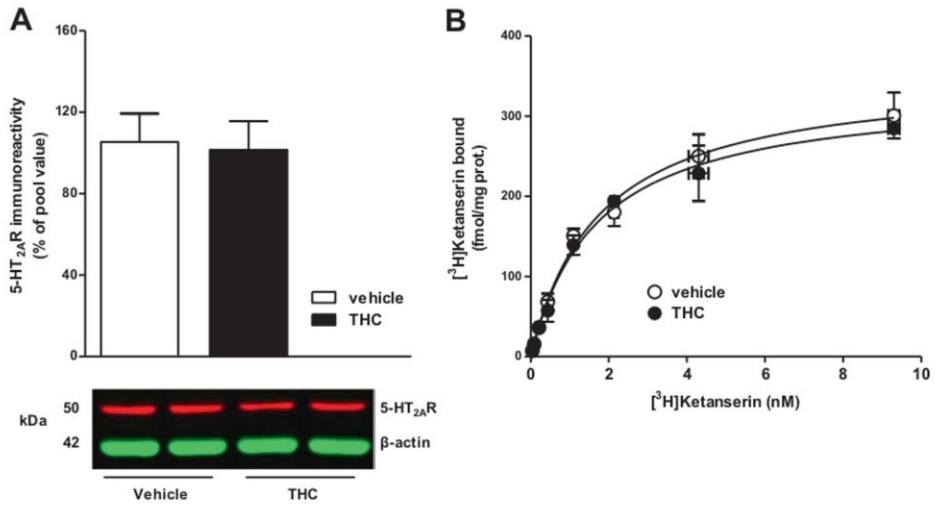


Fig. 3 Chronic THC does not modulate 5-HT2AR protein density. **a** 5-HT2AR immunodensity in brain total homogenates of vehicle- and THC-treated mice ($n = 8$ each group). Representative immunoblot of 5-HT2AR and β-actin in vehicle and THC-treated mice. **b** Specific [^{3}H] ketanserin binding saturation curves in cortical membranes of vehicle- and THC-treated mice (pool of $n = 8$ each group). Bars and points represent mean ± SEM

conditions, to induce a significantly decrease in the PPI (Supplementary Figure 1). Chronic THC significantly exacerbated the (±)-DOI-induced disruption of PPI (Fig. 2a). Chronic THC alone did not alter basal PPI, similar to previous findings [22]. No significant differences among groups were found in the amplitudes of the startle reflex (Fig. 2b).

Chronic THC does not alter total 5-HT2AR density in brain cortex

Next, we investigated the effects of chronic THC on cortical 5-HT2AR, the main molecular target of (±)-DOI. Protein immunodetection of 5-HT2AR showed a single band at ~53 kDa, as previously described [23]. Chronic THC did not alter 5-HT2AR protein expression (Fig. 3a). In order to confirm this result, saturation binding experiments with the 5-HT2AR antagonist [3H] ketanserin were performed in brain cortical membranes. No significant differences in density (~340 fmol/mg protein) were observed between both groups (Fig. 3b).

Chronic THC modulates the functional coupling of cortical 5-HT2AR

In order to deeper evaluate the molecular conformation of the 5-HT2AR, we examined the pharmacological parameters of (±)-DOI displacing [^{3}H]ketanserin binding in brain cortex membranes. The agonist (±)-DOI displaced the antagonist [^{3}H]ketanserin binding in a biphasic manner (Fig. 4a). Comparing both the high- and low-affinity binding populations dissected by the agonist in vehicle- and THC-treated animals, the affinity of (±)-DOI for the high-affinity 5-HT2AR population was significantly increased after chronic THC. No differences were found in any other pharmacological parameter (Supplementary Table 2).

We next evaluated the functionality of 5-HT2AR by studying the agonist-induced functional coupling to different Gα-protein subtypes. The agonist (±)-DOI produced a selective activation of Gαi1-, Gαi3-, Gαz-, and Gαq/11-proteins but not of Gαo- and Gαs-proteins in vehicle-treated mice. The activation of all these

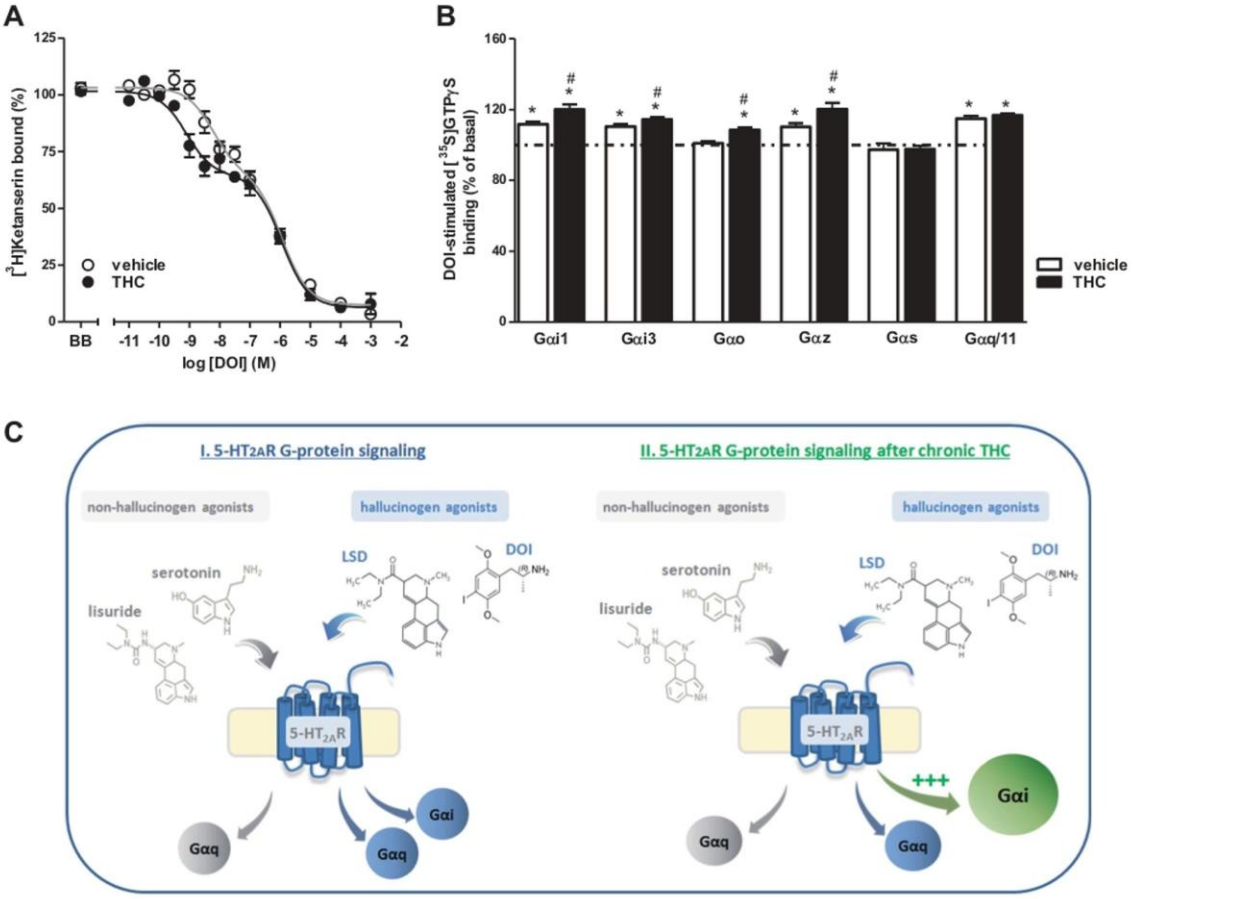


Fig. 4 Chronic THC promotes 5-HT2AR signaling through inhibitory G-proteins. **a** Competition curve of [^{3}H]ketanserin specific binding (2 nM) by (±)-DOI in brain membranes of vehicle- and THC-treated mice (pool of $n = 8$ each group). In both cases, data were best fit to a biphasic compared to monophasic displacement curve (*F*-test, $p < 0.0001$, for both curves). (±)-DOI showed supersensitivity for the high-affinity subpopulation of 5-HT2AR (**$p < 0.01$) (see Supplementary Table 2 for details) after chronic THC. **b** (±)-DOI (10 μM) induced Gαi1-, Gαi3-, Gαz-, and Gαq/11-protein activation (*$p < 0.05$ vs. basal) in brain cortical membranes of vehicle-treated mice (pool of $n = 8$ each group). Chronic THC elicited a supersensitivity for (±)-DOI-induced Gαi1-, Gαi3-, Gαo-, and Gαz protein activation (#$p < 0.05$ vs. vehicle). Points and bars represent mean ± SEM of 4–6 different experiments carried out in triplicate. **c** Suggested model of the 5-HT2AR signaling modulation after chronic THC

Gα-proteins was antagonized in the presence of ketanserin (10 μM) (data not shown). The Gαq/11-protein stimulation showed no significant differences between groups. By contrast, after THC chronic treatment, stimulation of Gαi1-, Gαi3-, Gαo-, and Gαz-proteins by (±)-DOI was significantly increased. Stimulation of Gαo-protein was only observed in THC-treated mice (Fig. 4b). Therefore, chronic THC exposure induced an overstimulation of inhibitory G-protein-mediated signaling (Fig. 4c).

Akt/mTOR signaling pathway is involved in the sensitization of 5-HT2AR induced by chronic THC

In order to tackle the putative role of Akt/mTOR pathway in this functional modulation, mice were treated with both THC and the mTOR inhibitor rapamycin. Chronic THC significantly activated (increase of phosphorylated forms) immunoreactivity of both Akt and ribosomal protein S6 (rpS6), a downstream effector of mTOR, in cortical tissue. Moreover, this activation was abolished in the presence of rapamycin (Fig. 5a, b). Rapamycin also blocked the effect of THC treatment in the PPI response to (±)-DOI and restored it to vehicle-values (Fig. 5c), without changing the startle response (Fig. 5d). Finally, rapamycin treatment fully blocked the THC-induced hyperactivation of Gαi1-, Gαi3-, and Gαz-proteins by (±)-DOI, but not that of Gαo-protein (Fig. 5e). For the effects of chronic rapamycin alone, see Supplementary Figures 2-4.

DISCUSSION

Several pharmacological and behavioral effects of THC have been well documented in animal models, although little is known about the mechanism that promote psychosis-like behaviors [24]. Our study demonstrates under in vitro and in vivo conditions that chronic THC promotes a functional sensitization of 5-HT2AR responses to the hallucinogenic agonist (±)-DOI. To our knowledge, these data provide the first evidence of a psychosis-like alteration of 5-HT2AR signaling after chronic THC administration. Hallucinogenic effects of 5-HT2AR agonists occurs by activation of inhibitory G-proteins [6, 19]. Thus, the specific alteration of agonist-induced signaling bias of 5-HT2AR observed after THC may explain the higher susceptibility to psychosis-like states. In fact, an increased density of the functional conformation of 5-HT2AR has been demonstrated in brain of schizophrenia patients [10]. On the other hand, THC is able to activate Akt/mTOR pathway [15]. Our results also demonstrate that this signaling pathway is implicated in the chronic THC-induced

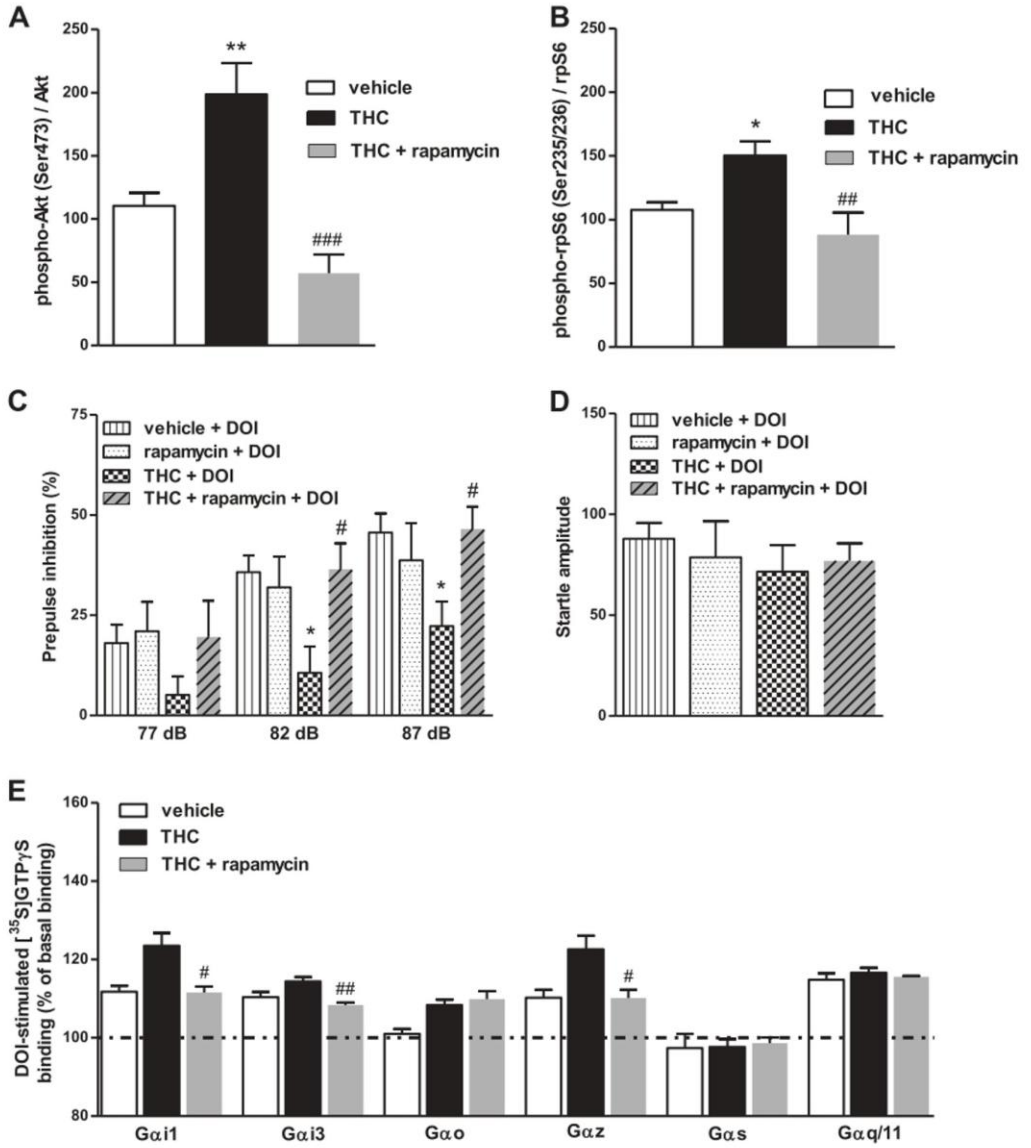


Fig. 5 Akt/mTOR signaling pathway is involved in the THC-induced sensitization of 5-HT2AR signaling. Chronic THC increased phosphorylation of (**a**) Akt and (**b**) rpS6, and concomitant treatment with mTORC1 inhibitor rapamycin blocked these effects (one-way ANOVA: phospho-Akt/Akt ratio $F(2,20) = 15.68$, $p < 0.0001$; phospho-rpS6/rpS6 ratio $F(2,16) = 8.04$, $p < 0.01$; $n = 5–8$). Bonferroni's post hoc comparisons: $^{*}p < 0.05$, $^{**}p < 0.01$ vs. vehicle. $\#\#p < 0.01$, $\#\#\#p < 0.001$ vs. THC. **c** Rapamycin blocked the sensitization to (±)-DOI effect on PPI in THC-treated mice, restoring it to vehicle-values among all the prepulses (three-way repeated measures ANOVA: THC × rapamycin; $F(1,35) = 4.50$, $p < 0.05$; $n = 8–16$). Benjamini-Hochberg's post hoc comparisons: $^{*}p < 0.05$ vs. vehicle + DOI. $\#p < 0.05$ vs. THC + DOI (see Supplementary Table 3 for further details on ANOVAs). Data of vehicle + DOI and THC + DOI groups are also in Fig. 2a. **d** No changes were found in the startle amplitude among groups. Data of vehicle + DOI and THC + DOI groups are also in Fig. 2b. **e** Rapamycin blocked the (±)-DOI-induced hyperactivation of Gαi1-, Gαi3-, and Gαz- ($\#p < 0.05$, $\#\#p < 0.01$ vs. THC), but not that of Gαo proteins (pool of $n = 8$ each group). Bars represent mean ± SEM of 4–6 different experiments carried out in triplicate

modulation of 5-HT2AR functionality, since the mTOR inhibitor rapamycin blocked the sensitization.

Sensorimotor gating alterations are recognized as an endophenotype of schizophrenia [25] and loss-of-normal PPI occurs in schizophrenia patients [26]. PPI evaluation shows high-translational validity, as it can be assessed in both rodents and humans [20, 21, 27]. In line with other studies [22, 28], chronic THC per se does not alter sensorimotor gating process. However, decreases [29, 30] or no effects [31, 32] have been reported after chronic synthetic cannabinoids administration (WIN55,212–2 or CP55,940). This discrepancy may be explained because distinct cannabinoid ligands can activate different G-protein subtypes [18]. At this point, as the main psychoactive component and the key player in the psychotomimetic effects of cannabis, the effects of THC should be considered as more representative of the alterations induced by chronic cannabis abuse in humans.

Interactions between the endocannabinoid and serotonergic systems have been widely reported in rodents and recently, a

CB1R-5-HT2AR heterodimer has been proposed [33]. However, studies assessing cannabinoid effects on 5-HT2AR functionality are limited. Chronic synthetic cannabinoids seem to potentiate (±)-DOI-mediated responses [34]; whereas, the acute administration of some cannabinoids inhibits (±)-DOI-induced head-twitch responses [35]. On the other hand, the antipsychotic risperidone, a 5-HT2AR inverse agonist, reverses acute THC-induced PPI deficits [36]. Moreover, cannabis-induced psychosis in humans is also responsive to treatment with atypical antipsychotics [37]. Therefore, it seems that psychotic effect induced by acute THC should be differentiated from other modulation processes induced after chronic exposure.

Although disruptions of PPI after acute (±)-DOI administration are widely described [38, 39], our study demonstrates a marked supersensitivity to this disruption after chronic THC. The responses to (±)-DOI become increased together with an enhanced high-affinity conformation of the 5-HT2AR. Classically, the high-affinity conformation of G-protein receptors is assumed as the functionally active state and represents the receptor fraction coupled to G-proteins [40]. Here, the enhanced coupling is selectively observed toward inhibitory G-proteins, which is considered the pro-hallucinogenic signaling pathway of this receptor. Accordingly, no changes were observed in total expression and density of 5-HT2AR. In this context, an upregulation of cortical 5-HT2AR mRNA and protein expression has been observed following a 7-day treatment with the synthetic cannabinoid CP55,940 [41]. However, as we observed in the present study, it has been described that chronic THC (10 mg/kg) do not modulate 5-HT2AR density in mice brain [42]. This fact supports again the use of THC, rather than other cannabinoid ligand, to evaluate the effects of cannabis abuse. The washout period becomes crucial in studies assessing chronic effects of cannabinoids, as they are able to accumulate in adipose tissue and may induce effects due to the residual presence of the drug. Our data about THC brain concentrations reached during treatment strongly support that the observed effects were due to enduring brain changes derived from the chronic exposure to THC.

The present is the first demonstration that chronic THC leads to supersensitive coupling of 5-HT2AR to inhibitory G-proteins whereas Gαq/11-protein signaling pathway remains unaltered. 5-HT2AR canonically activates phospholipase C via Gαq/11-proteins but it can also activate phospholipase A2, as well as other G-protein subtypes, depending on the ligand used [43]. Hallucinogenic and non-hallucinogenic serotonergic drugs activate the same population of 5-HT2AR in cortical pyramidal neurons, but differ in the receptor-dependent pattern of G-protein signaling and gene transcription induction that they elicit. In human and murine cortical neurons, non-hallucinogenic 5-HT2AR agonists induce *c-fos* expression mediated by Gαq/11-protein-dependent phospholipase C activation. In contrast, hallucinogen 5-HT2AR agonists such as (±)-DOI or LSD, acting at the 5-HT2AR, not only activate *c-fos* but also induce *egr-2* expression, which is a Gαi/o-protein-dependent response. This signaling pattern has been proposed as a specific fingerprint of hallucinogenic pharmacological properties [44].

Although further testing is needed, the selective overactivation of inhibitory G-proteins by (±)-DOI after THC treatment provides a crucial aspect regarding how chronic THC exposure could increase vulnerability to psychosis. Remarkably, selective hypersensitive 5-HT2AR coupling to Gαi/o proteins has also been demonstrated in post mortem frontal cortex of schizophrenia patients [45].

Our results also demonstrate the involvement of the Akt/mTOR signaling pathway in the molecular mechanisms underlying the THC-induced modulation of 5-HT2AR. Rapamycin inhibits mTOR complex 1 (mTORC1), a protein complex that plays an important role in protein synthesis, cell-cycle progression, cell growth and proliferation [46, 47]. The protein kinase Akt activates mTORC1 that subsequently activates ribosomal protein S6, and regulates axonal branching [11]. This complex has been involved in THC-induced memory deficits [15]. Moreover, polymorphisms in the *AKT1* gene are known to increase cannabis-induced psychotic responses [48, 49]. Interestingly, ablation of both phospho (Ser473)-Akt and S6 kinase (S6K), whose target substrate is the S6 ribosomal protein, is able to alter 5-HT2AR functionality and signaling [50]. Our data demonstrate that rapamycin prevents the THC-induced activation of Akt/mTOR pathway and restores the alterations in the 5-HT2AR functionality. Nonetheless, the involvement of other signaling pathways, as well as the modulation of other cellular mechanisms induced by the chronic administration of rapamycin cannot be discarded.

In summary, the present study demonstrates that chronic THC exposure leads to the overactivation of a pro-hallucinogenic signaling pathway of 5-HT2AR through the regulation of Akt/mTOR pathway. Findings of the present work describe, for the first time, the molecular mechanisms underlying the link between cannabis abuse and susceptibility to schizophrenia-like symptoms.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Arkaitz Carracedo for scientific advice regarding rapamycin experiments and the human and technical support provided by the Central Service of Analysis of Alava -SGIker- of UPV/EHU. LU., R.D.-A. and L.F.C. designed experiments. I.I.-L., LU., and J.J.M. wrote the manuscript. Chronic treatments were performed by I.I.-L. Behavioral experiments were performed by I.I.-L. and I.M.-G. with assistance from L.U. Biochemical assays were performed by I.I.-L. and R.D.-A. All the contributors revised critically and gave their approval to the final version of the manuscript. This study was funded by the Instituto de Salud Carlos III, FEDER (PI13/01529 to L.U.) and the Basque Government (IT616/13 to J.J.M.). I.I.-L. and I.M.-G. are recipients of Predoctoral Fellowships from the Basque Government and the University of the Basque Country, respectively.

ADDITIONAL INFORMATION

Supplementary information accompanies this paper at (https://doi.org/10.1038/s41386-018-0076-y)

Competing interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

REFERENCES

1. Arseneault L, Cannon M, Poulton R, Murray R, Caspi A, Moffitt TE. Cannabis use in adolescence and risk for adult psychosis: longitudinal prospective study. BMJ. 2002;325:1212–3.
2. Di Forti M, Marconi A, Carra E, Fraietta S, Trotta A, Bonomo M, et al. Proportion of patients in south London with first-episode psychosis attributable to use of high potency cannabis: a case-control study. Lancet Psychiatry. 2015;2:233–8.
3. Hall W, Degenhardt L. Adverse health effects of non-medical cannabis use. Lancet. 2009;374:1383–91.
4. Foti DJ, Kotov R, Guey LT, Bromet EJ. Cannabis use and the course of schizophrenia: 10-year follow-up after first hospitalization. Am J Psychiatry. 2010;167:987–93.
5. Nichols DE. Psychedelics. Pharmacol Rev. 2016;68:264–355.
6. Gonzalez-Maeso J, Weisstaub NV, Zhou M, Chan P, Ivic L, Ang R, et al. Hallucinogens recruit specific cortical 5-HT(2A) receptor-mediated signaling pathways to affect behavior. Neuron. 2007;53:439–52.
7. Abdolmaleky HM, Yaqubi S, Papageorgis P, Lambert AW, Ozturk S, Sivaraman V, et al. Epigenetic dysregulation of HTR2A in the brain of patients with schizophrenia and bipolar disorder. Schizophr Res. 2011;129:183–90.
8. Malkova NV, Gallagher JJ, Yu CZ, Jacobs RE, Patterson PH. Manganese-enhanced magnetic resonance imaging reveals increased DOI-induced brain activity in a mouse model of schizophrenia. Proc Natl Acad Sci USA. 2014;111:E2492–500.
9. Santini MA, Ratner C, Aznar S, Klein AB, Knudsen GM, Mikkelsen JD. Enhanced prefrontal serotonin 2A receptor signaling in the subchronic phencyclidine mouse model of schizophrenia. J Neurosci Res. 2013;91:634–41.
10. Muguruza C, Moreno JL, Umali A, Callado LF, Meana JJ, Gonzalez-Maeso J. Dysregulated 5-HT(2A) receptor binding in postmortem frontal cortex of schizophrenic subjects. Eur Neuropsychopharmacol. 2013;23:852–64.

11. Grider MH, Park D, Spencer DM, Shine HD. Lipid raft-targeted Akt promotes axonal branching and growth cone expansion via mTOR and Rac1, respectively. J Neurosci Res. 2009;87:3033–42.
12. Emamian ES, Hall D, Birnbaum MJ, Karayiorgou M, Gogos JA. Convergent evidence for impaired AKT1-GSK3beta signaling in schizophrenia. Nat Genet. 2004;36:131–7.
13. Gururajan A, van den Buuse M. Is the mTOR-signalling cascade disrupted in Schizophrenia? J Neurochem. 2014;129:377–87.
14. Morgan CJ, Freeman TP, Powell J, Curran HV. AKT1 genotype moderates the acute psychotomimetic effects of naturalistically smoked cannabis in young cannabis smokers. Transl Psychiatry. 2016;6:e738.
15. Puighermanal E, Busquets-Garcia A, Gomis-González M, Marsicano G, Maldonado R, Ozaita A. Dissociation of the pharmacological effects of THC by mTOR blockade. Neuropsychopharmacol. 2013;38:1334–43.
16. Carracedo A, Ma L, Teruya-Feldstein J, Rojo F, Salmena L, Alimonti A, et al. Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. J Clin Invest. 2008;118:3065–74.
17. Kurita M, Holloway T, García-Bea A, Kozlenkov A, Friedman AK, Moreno JL, et al. HDAC2 regulates atypical antipsychotic responses through the modulation of mGlu2 promoter activity. Nat Neurosci. 2012;15:1245–54.
18. Diez-Alarcia R, Ibarra-Lecue I, Lopez-Cardona AP, Meana J, Gutierrez-Adan A, Callado LF, et al. Biased agonism of three different cannabinoid receptor agonists in mouse brain cortex. Front Pharmacol. 2016;7:415.
19. Gonzalez-Maeso J, Ang RL, Yuen T, Chan P, Weisstaub NV, Lopez-Gimenez JF, et al. Identification of a serotonin/glutamate receptor complex implicated in psychosis. Nature. 2008;452:93–97.
20. Powell SB, Zhou X, Geyer MA. Prepulse inhibition and genetic mouse models of schizophrenia. Behav Brain Res. 2009;204:282–94.
21. Swerdlow NR, Braff DL, Taaid N, Geyer MA. Assessing the validity of an animal model of deficient sensorimotor gating in schizophrenic patients. Arch Gen Psychiatry. 1994;51:139–54.
22. Long LE, Chesworth R, Huang XF, McGregor IS, Arnold JC, Karl T. Transmembrane domain Nrg1 mutant mice show altered susceptibility to the neurobehavioural actions of repeated THC exposure in adolescence. Int J Neuropsychopharmacol. 2013;16:163–75.
23. Aira Z, Buesa I, Garcia del Cano G, Salgueiro M, Mendiable N, Mingo J, et al. Selective impairment of spinal mu-opioid receptor mechanism by plasticity of serotonergic facilitation mediated by 5-HT2A and 5-HT2B receptors. Pain. 2012;153:1418–25.
24. Rubino T, Parolaro D. The impact of exposure to cannabinoids in adolescence: insights from animal models. Biol Psychiatry. 2016;79:578–85.
25. Braff DL, Geyer MA. Sensorimotor gating and schizophrenia. Human and animal model studies. Arch Gen Psychiatry. 1990;47:181–8.
26. Ludewig K, Geyer MA, Vollenweider FX. Deficits in prepulse inhibition and habituation in never-medicated, first-episode schizophrenia. Biol Psychiatry. 2003;54:121–8.
27. Halberstadt AL, Geyer MA. Effect of Hallucinogens on Unconditioned Behavior. Current Topics in BehavioralNeurosciences.. Berlin, Heidelberg: Springer; 2016. p. 1–41.
28. Silva L, Black R, Michaelides M, Hurd YL, Dow-Edwards D. Sex and age specific effects of delta-9-tetrahydrocannabinol during the periadolescent period in the rat: The unique susceptibility of the prepubescent animal. Neurotoxicol Teratol. 2016;58:88–100.
29. Klein J, Hadar R, Gotz T, Manner A, Eberhardt C, Baldassarri J, et al. Mapping brain regions in which deep brain stimulation affects schizophrenia-like behavior in two rat models of schizophrenia. Brain Stimul. 2013;6:490–9.
30. Schneider M, Koch M. Chronic pubertal, but not adult chronic cannabinoid treatment impairs sensorimotor gating, recognition memory, and the performance in a progressive ratio task in adult rats. Neuropsychopharmacology. 2003;28:1760–9.
31. Llorente-Berzal A, Fuentes S, Gagliano H, Lopez-Gallardo M, Armario A, Viveros MP, et al. Sex-dependent effects of maternal deprivation and adolescent cannabinoid treatment on adult rat behaviour. Addict Biol. 2011;16:624–37.
32. Bortolato M, Aru GN, Frau R, Orru M, Luckey GC, Boi G, et al. The CB receptor agonist WIN 55,212-2 fails to elicit disruption of prepulse inhibition of the startle in Sprague- Dawley rats. Psychopharmacology. 2005;177:264–71.
33. Galindo L, Moreno E, Lopez-Armenta F, Guinart D, Cuenca-Royo A, Izquierdo-Serra M, et al. Cannabis Users Show Enhanced Expression of CB1-5HT2A Receptor Heteromers in Olfactory Neuroepithelium Cells. Mol Neurobiol. 2018; https://doi.org/10.1007/s12035-017-0833-7.
34. Hill MN, Sun JC, Tse MT, Gorzalka BB. Altered responsiveness of serotonin receptor subtypes following long-term cannabinoid treatment. Int J Neuropsychopharmacol. 2006;9:277–86.
35. Darmani NA. Cannabinoids of diverse structure inhibit two DOI-induced 5-HT(2A) receptor-mediated behaviors in mice. Pharmacol Biochem Behav. 2001;68:311–7.
36. Nagai H, Egashira N, Sano K, Ogata A, Mizuki A, Mishima K, et al. Antipsychotics improve Delta9-tetrahydrocannabinol-induced impairment of the prepulse inhibition of the startle reflex in mice. Pharmacol Biochem Behav. 2006;84:330–6.
37. Berk M, Brook S, Trandafir AI. A comparison of olanzapine with haloperidol in cannabis- induced psychotic disorder: a double-blind randomized controlled trial. Int Clin Psychopharmacol. 1999;14:177–80.
38. de Oliveira RP, Nagaishi KY, Barbosa Silva RC. Atypical antipsychotic clozapine reversed deficit on prepulse inhibition of the acoustic startle reflex produced by microinjection ofDOI into the inferior colliculus in rats. Behav Brain Res. 2017;325:72–78.
39. Farid M, Martinez ZA, Geyer MA, Swerdlow NR. Regulation of sensorimotor gating of the startle reflex by serotonin 2A receptors. Ontogeny and strain differences. Neuropsychopharmacology. 2000;23:623–32.
40. De Lean A, Stadel JM, Lefkowitz RJ. A ternary complex model explains the agonist specific binding properties of the adenylate cyclase-coupled beta-adrenergic receptor. J Biol Chem. 1980;255:7108–17.
41. Franklin JM, Carrasco GA. Cannabinoid-induced enhanced interaction and protein levels of serotonin 5-HT(2A) and dopamine D(2) receptors in rat prefrontal cortex. J Psychopharmacol. 2012;26:1333–47.
42. Dowie MJ, Howard ML, Nicholson LF, Faull RL, Hannan AJ, Glass M. Behavioural and molecular consequences of chronic cannabinoid treatment in Huntington's disease transgenic mice. Neuroscience. 2010;170:324–36.
43. Kurrasch-Orbaugh DM, Watts VJ, Barker EL, Nichols DE. Serotonin 5-hydroxytryptamine 2A receptor-coupled phospholipase C and phospholipase A2 signaling pathways have different receptor reserves. J Pharmacol Exp Ther. 2003;304:229–37.
44. Gonzalez-Maeso J, Yuen T, Ebersole BJ, Wurmbach E, Lira A, Zhou M, et al. Transcriptome fingerprints distinguish hallucinogenic and nonhallucinogenic 5-hydroxytryptamine 2A receptor agonist effects in mouse somatosensory cortex. J Neurosci. 2003;23:8836–43.
45. Meana J, Miranda-Azpiazu P, Garcia-Bea A, Muguruza C, Callado L, Gabilondo A, et al. Selective up-regulation of serotonin 5-HT2A receptor coupling to G alpha (i1)-proteins in postmortem brain of subjects with schizophrenia. Eur Neuropsychopharmacol. 2014;24:S483–4.
46. Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell. 2006;124:471–84.
47. Li J, Kim SG, Blenis J. Rapamycin: one drug, many effects. Cell Metab. 2014;19:373–9.
48. Di Forti M, Iyegbe C, Sallis H, Kolliakou A, Falcone MA, Paparelli A, et al. Confirmation that the AKT1 (rs2494732) genotype influences the risk of psychosis in cannabis users. Biol Psychiatry. 2012;72:811–6.
49. van Winkel R, Genetic R, Outcome of Psychosis I. Family-based analysis of genetic variation underlying psychosis-inducing effects of cannabis: sibling analysis and proband follow-up. Arch Gen Psychiatry. 2011;68:148–57.
50. Strachan RT, Sciaky N, Cronan MR, Kroeze WK, Roth BL. Genetic deletion of p90 ribosomal S6 kinase 2 alters patterns of 5-hydroxytryptamine 2A serotonin receptor functional selectivity. Mol Pharmacol. 2010;77:327–38.

SUPPLEMENTARY MATERIAL

Chronic cannabis promotes pro-hallucinogenic signaling of 5-HT_{2A} receptors through Akt/mTOR pathway

Inés Ibarra-Lecue B.Sc.[1], Irene Mollinedo-Gajate Ph.D.[1], J Javier Meana M.D. Ph.D.[1,2], Luis F Callado M.D. Ph.D.[1,2], Rebeca Díez-Alarcia Ph.D.[1,2] and Leyre Urigüen Ph.D.[1,2]

[1]Department of Pharmacology, University of the Basque Country UPV/EHU and Centro de Investigación Biomédica en Red de Salud Mental CIBERSAM, Spain

[2]Biocruces Health Research Institute, Bizkaia, Spain

Content:

Supplementary Table 1. Details of the antibodies used and their dilutions.

Antibody type	Target	Company	Reference	Dilution
Primary	5-HT_{2A}R	Santa Cruz Biotech.	Sc-166775	1:20,000
Primary	Akt	Cell Signaling	9272	1:1,000
Primary	Phospho(Ser^{473})-Akt	Cell Signaling	4060	1:1,000
Primary	rpS6	Cell Signaling	2317	1:500
Primary	Phospho($Ser^{235/236}$)-rpS6	Cell Signaling	4858	1:500
Primary (rabbit)	β-actin	Abcam	Ab8227	1:20,000
Primary (mouse)	β-actin	Sigma Aldrich	A1978	1:200,000
Secondary	Alexa Fluor 680 anti-Rabbit Ig-G	Thermo Fisher Sc.	A21076	1:2,500
Secondary	Alexa Fluor 680 anti-Mouse Ig-G	Thermo Fisher Sc.	A21057	1:4,000
Secondary	DyLight 800 Anti-Rabbit Ig-G	Rockland Immunoc.	610-745-127	1:5,000
Secondary	DyLight 800 Anti-Mouse Ig-G	Rockland Immunoc.	610-745-002	1:10,000

5-HT_{2A}R = Serotonin 2A receptor; Ig-G = Immunoglobulin G; Biotech. = Biotechnology; Sc. = Scientific; Immunoc. = Immunochemicals

Supplementary Table 2.

Pharmacological parameters of [^{3}H]ketanserin binding displacement curves by (±)-DOI in brain cortex membrane preparations of THC treated (10 mg/kg, i.p., 30 days) and control (vehicle) mice.

	Vehicle	THC
log $K_{i\text{-}high}$	-8.52 ± 0.17	-9.41 ± 0.14**
Fraction high (%)	41.83 ± 3.77	39.27 ± 2.94
log $K_{i\text{-}low}$	-6.23 ± 0.13	-6.25 ± 0.12
Top (fmol/mg prot)	254.60 ± 30.68	244.00 ± 22.58
Bottom (fmol/mg prot)	49.07 ± 8.97	56.62 ± 9.51

***p*<0.01; unpaired Student's *t*-test.

Data were best fit to a biphasic compared to monophasic displacement curve by *F*-test (F (2,48)=41.14, *p*<0.0001 for vehicle curves; F (2,42)=73.10, *p*<0.0001 for THC curves).

All values represent means ± SEM of four experiments carried out in duplicates.

Supplementary Table 3.

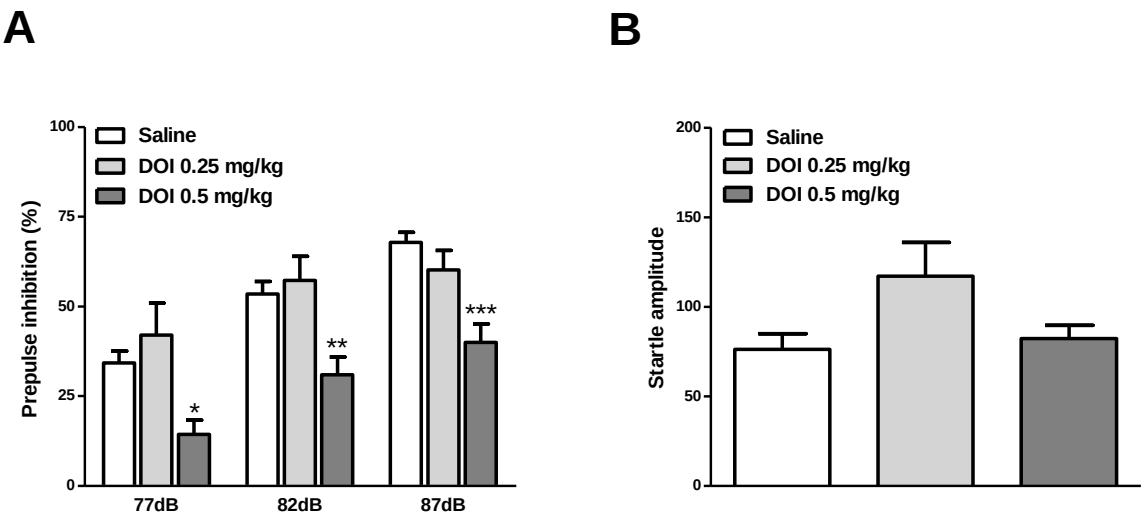
Summary of three-way repeated measures ANOVA analysis performed to test the effects of dB, THC, (±)-DOI and rapamycin as well as the potential interactions in %PPI.

	Source of variation	DFn	DFd	F	*p*
Figure 2A	THC	1	51	0.7	0.4057
	DOI	**1**	**51**	**38.56**	**<0.001**
	dB	**2**	**102**	**118.86**	**<0.001**
	THC x DOI	**1**	**51**	**6.48**	**0.0140**
	THC x dB	2	102	0.89	0.4141
	DOI x dB	2	102	2.00	0.1410
	THC x DOI x dB	2	102	1.68	0.1909
Figure 5C	Rapamycin	1	35	1.25	0.2719
	THC	1	35	3.57	0.0673
	dB	**2**	**70**	**48.03**	**<0.001**
	Rapamycin x THC	**1**	**35**	**4.50**	**0.0410**
	Rapamycin x dB	2	70	0.09	0.9150
	THC x dB	2	70	0.44	0.6444
	Rapamycin x THC x dB	2	70	2.32	0.1056

Significant *p* values are shown in bold.

Supplementary Figure 1.

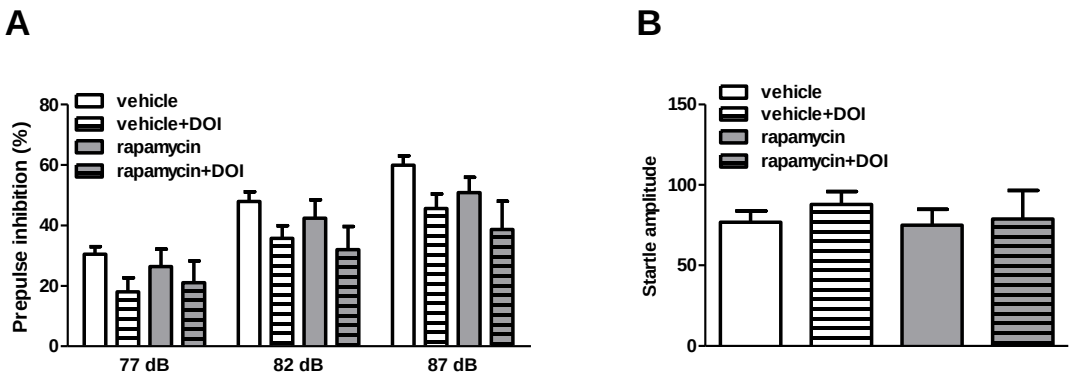
%PPI and startle amplitude of mice treated with two different doses of (±)-DOI (0.25 and 0.5 mg/kg, i.p.).



(**A**) Acute (±)-DOI exerted similar effects in all the prepulse intensities. Significant effect was only found with (±)-DOI 0.5 mg/kg (two-way ANOVA: DOI effect; $F(2,48)=25.70$, $p<0.0001$; n=5 each group). (**B**) Startle amplitude was not significantly modulated. Bonferroni's *post-hoc* comparisons: *$p<0.05$, **$p<0.01$ and ***$p<0.001$ *vs*. saline.

Supplementary Figure 2.

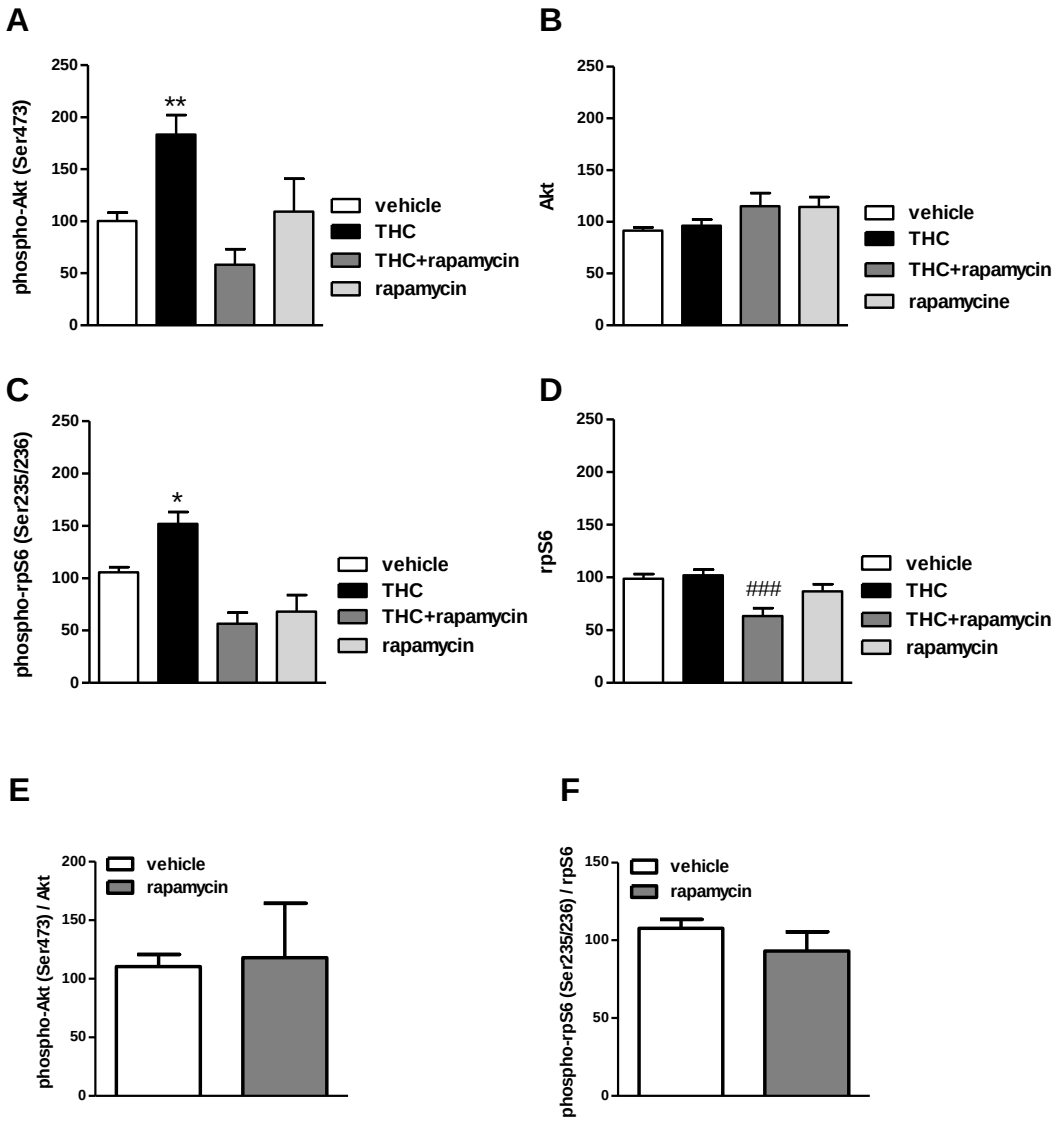
%PPI and startle amplitude of mice treated with rapamycin (5 mg/kg, 4 days/week, 30 days) or vehicle.



(**A**) Acute (±)-DOI exerted similar effects on PPI, independently of the pretreatment with vehicle or rapamycin. (two-way ANOVA: DOI effect; 82 dB $F(1,51)=6.18$; 87 dB $F(1,51)=6.30$, $p<0.05$; n=7-16). (**B**) Startle amplitude was not modulated.

Supplementary Figure 3.

Immunoreactivity of phospho-Akt (Ser473), total Akt, phospho-rpS6 (Ser235/236) and rpS6 in brain cortical tissue homogenates of the different groups and their ratios for rapamycin-treated and control animals.

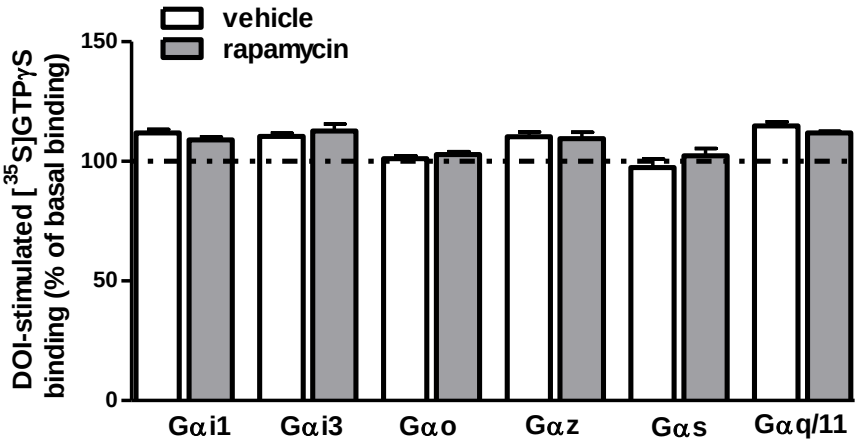


(**A**) Phospho-Akt immunoreactivity was increased in cortical tissue of THC-treated mice, whereas this increase was totally abolished with rapamycin concomitant treatment (two-way ANOVA: rapamycin x THC; $F(1,26)=11.67$, $p<0.01$; n=7-8). (**B**) No changes on total Akt were observed (n=8 each group). (**C**) Phospho-rpS6 was also increased after chronic THC and blocked with rapamycin (two-way ANOVA: rapamycin x THC; $F(1,22)=6.70$, $p<0.05$; n=6-7). (**D**) Rapamycin treatment decreased total rpS6 in THC-treated mice (two-way ANOVA: rapamycin x THC; $F(1,24)=4.61$, $p<0.05$; n=7 each group). (**E**) Ratios of phospho-Akt/Akt and (**F**) phospho-rpS6/rpS6 in cortical tissue of rapamycin-

treated mice showed no changes compared with control animals. Bonferroni's *post-hoc* comparisons: **p*<0.05 and ***p*<0.01 *vs*. vehicle; ###*p*<0.001 *vs*. THC.

Supplementary Figure 4.

(±)-DOI induced [^{35}S]GTPγS binding stimulation coupled to immunoprecipitation with specific antibodies against different Gα-protein subtypes in cortical membranes of mice chronically treated with rapamycin (5 mg/kg, 4 days/week, 30 days) or vehicle.



No differences were found in the stimulation of any Gα-protein subtype between both groups.

ARTICLE 3

Ribosomal protein S6 hypofunction in *postmortem* human brain links mTORC1-dependent signaling and schizophrenia

Inés Ibarra-Lecue, Rebeca Díez-Alarcia, Benito Morentin, J Javier Meana, Luis F Callado, Leyre Urigüen

Frontiers in Pharmacology (2020), **11**: 344

DOI: https://doi.org/10.3389/fphar.2020.00344

Impact factor 2018: 3.8

Relative position: 59/267, Q1 Pharmacology and Pharmacy

ARTICLE 3

The Akt/mTOR signaling pathway has been involved in several cellular functions, such as protein synthesis, cell growth and proliferation, and the development of the CNS in early stages. In this context, some studies have also suggested that Akt/mTOR signaling pathway dysfunction could be involved in the pathophysiology of schizophrenia. However, studies assessing the protein expression of Akt/mTOR pathway related kinases in human brain tissue are extremely scarce. The main goal of the study was to evaluate the Akt/mTOR signaling pathway status in subjects with schizophrenia. For that purpose, we quantified by western blot technique the protein expression and phosphorylation status of the mTOR upstream kinase Akt, the mTOR downstream effector rpS6, as well the status of GSK3β (an Akt-dependent kinase which activation is classically mTOR independent) in *postmortem* PFC samples of subjects with schizophrenia in comparison with matched controls. Akt kinase was found to be slightly hyperactive (significant changes only in phospho(Ser473)Akt / Akt ratio) in PFC of subjects with schizophrenia. Conversely, a strong hypofunctional status was found for rpS6, with a decrease in the expression of both total rpS6 and its active form phospho(Ser235/236)rpS6, thus leading to a decrease in phospho(Ser235/236)rpS6 / rpS6 ratio comparing with matched controls values., GSK3β did not differ between both groups. When subjects with schizophrenia were separated regarding the presence or absence of antipsychotics in blood at the time of death, a decrease in total rpS6 protein expression was found only in schizophrenic subjects with antipsychotics, while no significant differences were found in any other target between both groups. In order to evaluate whether chronic antipsychotic treatment exerts any effect on Akt/mTOR signaling pathway, an additional evaluation of the status of these proteins was performed in brain cortex of rats chronically treated (daily, during 21 days, i.p.) with haloperidol (1 mg/kg), clozapine (10 mg/kg) or risperidone (1 mg/kg). As a result, haloperidol decreased Akt functional status and increased total GSK3β protein expression, while risperidone and clozapine did not exert any significant effect on these two kinases. Additionally, S6 was not significantly modulated with any antipsychotic treatment. Altogether, these results suggest that a hypofunctional rpS6 may have a role in the pathophysiology of schizophrenia.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 24 March 2020
doi: 10.3389/fphar.2020.00344

Ribosomal Protein S6 Hypofunction in Postmortem Human Brain Links mTORC1-Dependent Signaling and Schizophrenia

Inés Ibarra-Lecue[1], *Rebeca Diez-Alarcia*[1,2], *Benito Morentin*[2,3], *J. Javier Meana*[1,2], *Luis F. Callado*[1,2] *and Leyre Urigüen*[1,2]*

[1] Department of Pharmacology, University of the Basque Country UPV/EHU and Centro de Investigación Biomédica en Red de Salud Mental CIBERSAM, Leioa, Spain, [2] Biocruces Bizkaia Health Research Institute, Barakaldo, Spain, [3] Section of Forensic Pathology, Basque Institute of Legal Medicine, Bilbao, Spain

OPEN ACCESS

Edited by:
Richard Lowell Bell,
Indiana University, United States

Reviewed by:
Sridhar Natesan,
King's College London,
United Kingdom
Karen K. Szumlinski,
University of California, Santa Barbara,
United States
Clare Beasley,
University of British Columbia, Canada

***Correspondence:**
Leyre Urigüen
leyre.uriguen@ehu.eus

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: 10 January 2020
Accepted: 09 March 2020
Published: 24 March 2020

Citation:
Ibarra-Lecue I, Diez-Alarcia R, Morentin B, Meana JJ, Callado LF and Urigüen L (2020) Ribosomal Protein S6 Hypofunction in Postmortem Human Brain Links mTORC1-Dependent Signaling and Schizophrenia. Front. Pharmacol. 11:344. doi: 10.3389/fphar.2020.00344

The mechanistic target of rapamycin (also known as mammalian target of rapamycin) (mTOR)-dependent signaling pathway plays an important role in protein synthesis, cell growth, and proliferation, and has been linked to the development of the central nervous system. Recent studies suggest that mTOR signaling pathway dysfunction could be involved in the etiopathogenesis of schizophrenia. The main goal of this study was to evaluate the status of mTOR signaling pathway in postmortem prefrontal cortex (PFC) samples of subjects with schizophrenia. For this purpose, we quantified the protein expression and phosphorylation status of the mTOR downstream effector ribosomal protein S6 as well as other pathway interactors such as Akt and GSK3β. Furthermore, we quantified the status of these proteins in the brain cortex of rats chronically treated with the antipsychotics haloperidol, clozapine, or risperidone. We found a striking decrease in the expression of total S6 and in its active phosphorylated form phospho-S6 (Ser235/236) in the brain of subjects with schizophrenia compared to matched controls. The chronic treatment with the antipsychotics haloperidol and clozapine affected both the expression of GSK3β and the activation of Akt [phospho-Akt (Ser473)] in rat brain cortex, while no changes were observed in S6 and phospho-S6 (Ser235/236) protein expression with any antipsychotic treatment. These findings provide further evidence for the involvement of the mTOR-dependent signaling pathway in schizophrenia and suggest that a hypofunctional S6 may have a role in the etiopathogenesis of this disorder.

Keywords: ribosomal protein S6, schizophrenia, mTORC1, postmortem tissue, antipsychotics

INTRODUCTION

The mechanistic target of rapamycin (mTOR), also known as mammalian target of rapamycin, represents a critical integrator of neuronal activity and synaptic inputs that plays a role in schizophrenia and other neuropsychiatric diseases (Costa-Mattioli and Monteggia, 2013; Gururajan and Van Den Buuse, 2014; Pham et al., 2016; Chadha and Meador-Woodruff, 2020). The canonical activation of mTOR pathway results from an activation cascade of upstream proteins, including receptor tyrosine kinases, phosphatidylinositol-3-kinase (PI3K), and Akt (also known as protein kinase B, PKB). Akt is a serine/threonine kinase classically related to cellular survival, growth and proliferation, prevention of apoptosis, and cancer progression (Kennedy et al., 1997; Manning and Cantley, 2007). The Akt signaling is one of the key outcomes of the activation of PI3K. It supposes an intermediator of cellular pathways activated in response to different extracellular stimuli including growth factors, insulin, or Gβ/γ subunits of G-protein coupled receptors (GPCR) (Burgering and Coffer, 1995; Murga et al., 1998). In neurons, Akt regulates a wide variety of processes such as neural survival and architecture, axonal growth, or synaptic strength control.

A genetic association between variants of the AKT1 gene and schizophrenia has been shown in several populations (Ikeda et al., 2004; Schwab et al., 2005; Thiselton et al., 2008; Mathur et al., 2010). Moreover, AKT allelic variants have been also associated with cognitive impairments and morphological abnormalities in neural networks of the prefrontal cortex (PFC) (Pietilainen et al., 2009). Additionally, a deficiency in Akt protein function seems to promote alterations in PFC and schizophrenia-like behaviors in animal models (Lai et al., 2006). However, literature tackling Akt expression or functional/phosphorylation status in human brain shows discrepant results (Emamian et al., 2004; Zhao et al., 2006; Thiselton et al., 2008; Balu et al., 2012; Hino et al., 2016; McGuire et al., 2017). Akt negatively regulates the activity of GSK3β, which strongly influences the neural function, including gene expression, neuronal architecture, plasticity, and survival. In this context, a lower expression/density and activity of Akt (Zhao et al., 2006) and GSK3β (Emamian et al., 2004) has been described in the PFC of subjects with schizophrenia, although there are contradictory reports (Ide et al., 2006; Amar et al., 2008; Kitagishi et al., 2012; McGuire et al., 2017).

mTOR is a serine/threonine kinase that binds to several interacting proteins, forming two different heteromeric complexes, mTORC1 and mTORC2. The mTORC1 regulates protein synthesis by phosphorylating downstream effectors directly involved in translation control. Once active, mTORC1 phosphorylates ribosomal protein S6 kinase (S6K) and eukaryotic translation initiation factor 4E (eIF-4E)-binding protein 1 (4E-BP1), both involved in the translational machinery for protein synthesis (Meyuhas, 2015; Qin et al., 2016). The first identified substrate of S6K was ribosomal protein S6, a component of the 40S ribosome, which phosphorylation is considered as a readout of mTORC1 activity, and has become a widely used marker of neuronal activity. Phosphorylation of S6 regulates the translation of a subset of mRNA in the central nervous system (CNS) (Puighermanal et al., 2017) and regulates diverse biological processes important for cell growth, ribosome biogenesis, and protein translation (Ruvinsky and Meyuhas, 2006). This downstream effector of mTORC1 is regulated by several environmental factors and extracellular stimuli, and in the CNS it regulates neuronal plasticity associated to cognitive processes, such as learning and memory (Meyuhas, 2015; Pirbhoy et al., 2016; Pirbhoy et al., 2017).

PI3K/Akt/GSK3/mTOR pathway has been linked to the development of the CNS, neuronal growth, maintenance, and proliferation (Kitagishi et al., 2012). Moreover, the dysfunction of mTORC1/S6 pathway may contribute to an aberrant dendritic reorganization and the loss of dendritic spines, what finally would lead to dysfunctions in synaptic connectivity (Antion et al., 2008; Magdalon et al., 2017; Chadha and Meador-Woodruff, 2020). Furthermore, diverse molecules that have been previously implicated in schizophrenia, such as glutamate, reelin, BDNF, serotonin, and/or their respective receptors can lead to either over-activation or inhibition of this signaling pathway (Gururajan and Van Den Buuse, 2014). All these data suggest that this pathway could have a significant role in schizophrenia (Costa-Mattioli and Monteggia, 2013; Gururajan and Van Den Buuse, 2014; Chadha and Meador-Woodruff, 2020).

To go through this hypothesis, we quantified the protein expression and phosphorylation of the mTOR downstream effector ribosomal protein S6 as well as other pathway interactors such as Akt and GSK3β, in postmortem PFC from subjects with schizophrenia and control subjects. To decipher the effect of the long-term treatment with different antipsychotics on this pathway, we also evaluated the status of the target proteins in the brain cortex of rats chronically treated with the antipsychotics haloperidol, clozapine or risperidone.

MATERIALS AND METHODS

Postmortem Human Brain Samples

Human brain samples were obtained at autopsies performed in the Basque Institute of Legal Medicine, Bilbao, in compliance with policies of research and ethical boards for postmortem brain studies. Deaths were subjected to retrospective searching for previous medical diagnosis and treatment using examiner's information and records from hospitals and mental health centers. Brain samples of 28 subjects with an antemortem diagnosis of schizophrenia according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR) (American Psychiatric Association, 2000) were matched to samples of 28 control subjects in a paired design. Control subjects were chosen based on the absence of diagnosis of neuropsychiatric disorders or drug abuse, and an appropriate gender, age, and postmortem interval (time between death and tissue dissection/freezing; PMI) to match each subject in the schizophrenia group. A blood toxicological screening was performed in all the subjects to determine the presence of

antipsychotics, other drugs, and ethanol (National Institute of Toxicology, Madrid, Spain). Schizophrenia subjects (SZ) were divided into two groups according to the absence [antipsychotic-free (AP-F, n = 17)] or presence [antipsychotic-treated (AP-T, n = 11)] of antipsychotic drugs in blood at the time of death. Demographic characteristics and PMI values did not significantly differ between schizophrenia and control groups, nor between AP-F and AP-T subjects (**Table 1**). Samples of dorsolateral PFC (DLPFC) were dissected at autopsy (0.5–1 g tissue) following standard procedures (Rajkowska and Goldman-Rakic, 1995), and immediately stored at −80°C until assay. A demographic full description of subjects with schizophrenia (AP-F and AP-T) and their individually matched controls is summarized in **Supplementary Tables 1** and **2**.

Animals and Treatments

Male Sprague-Dawley rats (222–318 g) (Animal Facility of the University of the Basque Country, Leioa, Spain) were housed on a 12-h light/dark cycle at 22°C and 60% humidity with food and water available *ad libitum*. All experimental procedures were performed in accordance with the European Union Directive 2010/63/EU and approved by the Ethic Committee for Animal Welfare of the University of the Basque Country, UPV/EHU (CEBA 188/2011). Animals were treated twice-a-day (i.p., volume 1 ml/kg), during 21 days, with saline (1 ml/kg), clozapine (5 mg/kg) (Tocris. Bristol, UK), risperidone (0.5 mg/kg), or haloperidol (0.5 mg/kg) (Sigma Aldrich®. Missouri, USA). These doses have been previously used in the literature (Parikh et al., 2004) and yield serum concentrations comparable to those observed in humans receiving these treatments (Kapur et al., 2003). Clozapine was dissolved in a few drops of glacial acetic acid (Panreac Química S.A. Barcelona, Spain) and prepared in distilled water; risperidone and haloperidol were dissolved in saline. After 48 h (clozapine and risperidone) or 72 h (haloperidol and saline) washout-period, rats were sacrificed, brains removed, and cortex dissected and stored at −80°C until assay.

Preparation of Total Homogenates

Brain homogenates were obtained as previously described (Urigüen et al., 2009) with minor modifications. Briefly, human PFC (~200 mg) or rat cortex (~130 mg) tissue samples were thawed at 4°C and homogenized in 8 µl/mg of homogenization buffer using a Potter (15 pulses). Immediately after, 0.08 µl/mg of BCD buffer (homogenization buffer, 10% Igepal, 5% sodium deoxycholate, 1% SDS, 250 mM CHAPS) were added to each sample. Samples were vortexed and kept in ice for 30 min, centrifuged for 10 min at 20,000*g* (4°C), and supernatants kept. Protein content was determined using a Bio-Rad DC Protein Assay Kit with BSA as standard. Samples were then diluted in homogenization buffer until reaching a concentration of 4 mg protein/ml, and aliquoted. Commercial Laemmli buffer (95% v/v) and β-mercaptoethanol (5% v/v) were added to each sample. Finally, all the samples were vortexed, heated at 95°C for 5 min and kept at −70°C until Western blot experiments were performed.

TABLE 1 | Demographic characteristics of postmortem human PFC samples.

Subjects with schizophrenia (n = 28) and matched controls (n = 28)				
Group	**Gender (M/F)**	**Age (years)**	**PMI (h)**	**Storage time (months)**
Schizophrenia	23M/5F	38.7 ± 2.1	17.8 ± 2.4	51.1 ± 7.7
AP-F (n = 17)	14M/3F	38.6 ± 2.4	19.2 ± 3.6	54.2 ± 11.4
AP-T (n = 11)	9M/2F	38.9 ± 3.9	16.1 ± 2.1	46.3 ± 9.1
Control group	22M/6F	38.8 ± 2.1	22.5 ± 2.1	45.8 ± 9.3

PFC, prefrontal cortex; M, male; F, female; PMI, postmortem interval; AP-F, antipsychotic-free; AP-T, antipsychotic-treated. Mean ± SEM.

Western Blot

Western blot was performed as previously described (Ibarra-Lecue et al., 2018) with minor modifications. Samples were heated (95°C), loaded (30 µg) and submitted to SDS-PAGE onto polyacrylamide gel (12%). Nitrocellulose membranes were blocked (5% non-fat dry-milk or/and 0.5% BSA) in TBS buffer followed by overnight incubation with primary antibodies (4°C). Specific antibodies against Akt, phospho-Akt(Ser473), GSK3α/β, phospho-GSK3α/β(Ser21/9), S6, phospho-S6(Ser235/236), and β-actin were used. Dilutions of primary antibody have been previously evaluated for a signal within the linear range of detection (Ibarra-Lecue et al., 2018). Incubation with fluorescent anti-IgG secondary antibodies was performed at room temperature (1 h) (further details about antibodies and dilutions are in **Supplementary Table 3**). Immunoreactivity was quantified using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Representative images of immunoblots can be found in **Supplementary Figure 1** (human) and **Supplementary Figure 2** (rat).

Data Analysis and Statistical Procedures

Data were analyzed with GraphPad Prism™ version 7.0 (GraphPad Software, San Diego, CA, USA) and InVivoStat free software. Two-group comparisons (C vs SZ) were made by unpaired Student's *t*-test. Multiple groups' comparisons (C, AP-F, AP-T) were studied by one-way analyses of variance (ANOVA), followed by Bonferroni's *post hoc* analyses. Linear regression analysis was used to assess the contribution of age, PMI, and storage time to the protein immunoreactivity values. When significant differences were observed between groups, subsequent analyses of covariance (ANCOVA) were performed to discard the effect of these potential confounding variables on the observed differences. Statistical significance was set at $p < 0.05$. Immunodensitometric values of the different target proteins were normalized to the intra-assay values obtained with anti-β-actin antibody and expressed as mean ± SEM of the percentages of an inter-assay normalization sample included in every experiment. Inter-assay normalization sample was a total homogenate from pooled brain cortical tissue of six subjects (3 C and 3 SZ) or six rats. This sample was included in every gel and β-actin corrected values of the target proteins of all the study samples are referred as a percentage from this inter-assay normalization sample. Each sample was analyzed at least in

two independent experiments. All data were subjected to a Grubbs's test to determine possible outlier values. The detected outliers were rejected for the statistical analysis.

RESULTS

The mTORC1 Pathway is Dysregulated in Schizophrenia

In the human PFC of schizophrenic subjects, Akt levels did not differ from that in matched controls (controls (C): 108% ± 5%; schizophrenic subjects (SZ): 98% ± 8%) (**Figure 1A**). When subjects were divided regarding antipsychotic presence/absence in blood, Akt immunodensity did not show significant differences between AP-F (93% ± 7%) and AP-T subjects (109% ± 18%) (**Figure 1A**). Active phosphorylated form of Akt (phospho-Akt(Ser473)) did not differ between SZ (161% ± 19%), with or without antipsychotic treatment (AP-F: 168% ± 27%; AP-T: 149% ± 25%) and matched controls (C: 128% ± 14%) (**Figure 1B**). After calculating the phospho-Akt(Ser473)/Akt ratio for each subject, a significant increase in the proportion of the active/phosphorylated form was found in the SZ group ($p < 0.05$) compared to matched controls, while no differences were found between AP-F and AP-T subjects (**Figure 1C**).

The immunodensity of GSK3β was found unaltered in schizophrenia (C: 122% ± 8%; SZ: 119% ± 10%) (**Figure 1D**), and the antipsychotic treatment did not modify significantly the content of the protein (AP-F: 134% ± 14%; AP-T: 100% ± 12%).

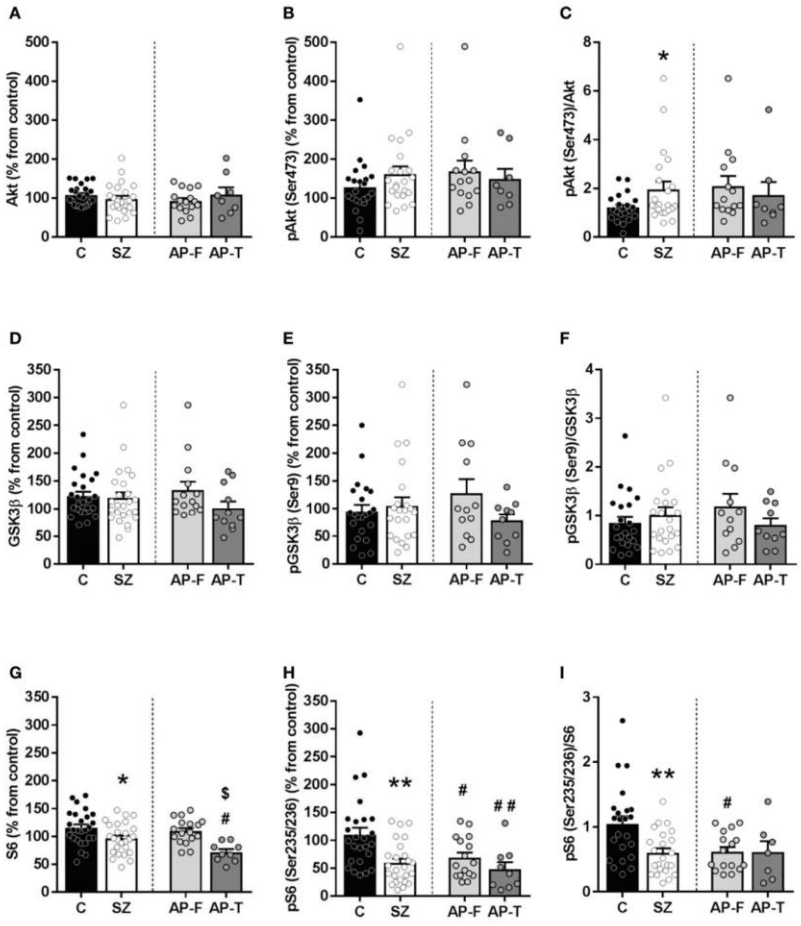


FIGURE 1 | Protein expression of signaling proteins in postmortem prefrontal cortex (PFC) from subjects with schizophrenia (SZ) [antipsychotic-treated (AP-T), or drug-free (AP-F)] and matched controls (C). Total Akt **(A)** and phospho-Akt(Ser473) protein levels **(B)**. **(C)** Phospho-Akt(Ser473)/Akt ratio. Total GSK3β **(D)** and phospho-GSK3β(Ser9) protein levels **(E)**. **(F)** Phospho-GSK3β(Ser9)/GSK3β ratio. Total S6 **(G)** and phospho-S6(Ser235/236) protein levels **(H)**. **(I)** Phospho-S6 (Ser235/236)/S6 ratio. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs* controls; unpaired Student's *t* test analysis. $^{\#}p < 0.05$ and $^{\#\#}p < 0.005$ *vs* controls and $^{\$}p < 0.05$ *vs* AP-F; one-way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test. Data represent the means ± standard errors of the mean (SEM) referred as a percentage of the inter-assay normalization sample.

The inactive/phosphorylated form (phospho-GSK3β(Ser9)) did not show differences among groups (SZ: 105% ± 15%; C: 95% ± 12%; AP-F: 128% ± 26%; AP-T: 78% ± 12%) (**Figure 1E**). In the same way, no significant differences were found in the phospho-GSK3β (Ser9)/GSK3β ratio (**Figure 1F**).

Total S6 density was significantly decreased ($p < 0.05$) in SZ compared to matched controls (SZ: 97% ± 5%; C: 115% ± 6%) (**Figure 1G**). When schizophrenia samples were divided regarding the presence of antipsychotic drugs in blood at the time of death, this decrease was only statistically significant ($p < 0.05$) in AP-T (71% ± 5%) when comparing with AP-F (109% ± 5%) and with controls (**Figure 1G**).

The density of the active phosphorylated form of the S6, phospho-S6(Ser235/236), showed a significant decrease ($p < 0.05$) in both AP-F and AP-T subjects compared to matched controls (C: 110% ± 12%; SZ: 59% ± 7%; AP-F: 68% ± 9%; AP-T: 48% ± 12%) (**Figure 1H**). Consequently, the phospho-S6 (Ser235/236)/S6 ratio showed a significant decrease in the whole population of schizophrenia samples ($p < 0.05$). This decrease was also evident in both AP-F and AP-T groups, although only in the AP-F subjects this reduction was significant ($p < 0.05$) when compared to controls (**Figure 1I**).

In order to ensure whether the differences observed in protein levels between schizophrenia and control subjects were influenced by confounding variables, the effect of age, PMI and storage time was assessed.

The phosphorylated form of S6 was significantly influenced by age but not by the PMI or storage time. Pearson's correlation analyses showed a significant negative correlation between phospho-S6(Ser235/236) levels and age at death in both controls ($p < 0.05$, $r = -0.39$) and subjects with schizophrenia ($p < 0.05$, $r = -0.43$) (**Supplementary Figure 3**). Linear regression analyses showed no significant differences in the slopes between groups. In an effort to control the confounding influence of this variable, ANCOVA analyses were performed with protein level values as the dependent variable, and age as covariate. Consistent with the previous findings, statistically significant differences between schizophrenia and control subjects were still observed [$F(1,47) = 14.26$, $p = 0.0004$].

Haloperidol and Clozapine, but not Risperidone, Modulate Akt Signaling in Rat Brain Cortex

Both haloperidol and clozapine induced a significant decrease ($p < 0.05$) in the active form phospho-Akt(Ser473) compared to saline controls (saline: 103% ± 9%; haloperidol: 66% ± 9%; risperidone: 77% ± 11%; clozapine: 71% ± 11%) while no changes were found in Akt total form (**Figures 2A, B**). A significant decrease in the phospho-Akt(Ser473)/Akt ratio was also observed in brain cortex of rats treated with haloperidol ($p < 0.05$) (**Figure 2C**).

Conversely, haloperidol induced a significant increase ($p < 0.05$) in the levels of total GSK3β (saline: 100% ± 6%; haloperidol: 126% ± 10%; risperidone: 113% ± 10%; clozapine: 102% ± 8%) (**Figure 2D**), while no changes in the phosphorylated form or the ratio were observed (**Figures 2E, F**). Clozapine did not induce any significant change in GSK3β (**Figure 2D**) or pGSK3β (Ser9) proteins (**Figure 2E**). Risperidone was not able to modulate the levels of any of the proteins evaluated. No changes were observed neither in S6 (**Figure 2G**), nor in phospho-S6 (Ser235/236) (**Figure 2H**), nor in the ratio (**Figure 2I**) in the brain cortex of rats treated with any of the antipsychotic drugs.

DISCUSSION

The main finding of our study is the strong decrease in the expression and phosphorylation of S6 in the PFC of subjects with schizophrenia. To our knowledge, this is the first report of a dysfunction in this downstream effector of mTORC1 in human brain.

Both environmental factors and extracellular stimuli previously implicated in the development of schizophrenia are known to control the mTOR cascade. The control of protein synthesis carried out by the activation of ribosomal protein S6 may underlie aspects of neurodevelopment and synaptic plasticity (Jaworski and Sheng, 2006; Ruvinsky and Meyuhas, 2006; Tang et al., 2014). This hypothesis is supported by data suggesting that dysfunctions in mTORC1/S6 pathway lead to the inhibition of oligodendrocyte precursor cells proliferation and maturation and the subsequently hypomyelination (Liu et al., 2014; Maas et al., 2017). In this context, the present results suggest a hypofunction of mTORC1 pathway in schizophrenia. This hypofunction might contribute to dysfunctions in brain connectivity and the development of the neurobiological manifestations of schizophrenia (**Figure 3**).

One of the upstream regulators of mTORC1 is Akt, an intermediator of different cellular pathways that acts in response to different extracellular stimuli. In neurons, the activation of Akt induces the phosphorylation of different substrates that, thereby, regulates a wide variety of processes such as neuronal development, morphogenesis, dendritic development, and synaptic plasticity. Growing evidence indicates that the mTORC1/S6K pathway mediates potent negative feedback loops that restrain upstream signaling. In this way, inhibition of mTORC1/S6K by specific drugs suppresses these feedback loops and causes compensatory over-activation of upstream signaling proteins, including Akt (Rozengurt et al., 2014). Thus, it has been reported that an outstanding consequence of mTORC1/S6K inhibition is the notable increase in Akt phosphorylation (O'Reilly et al., 2006; Lane and Breuleux, 2009; Rozengurt et al., 2014). Results of the present study showed a significant increase in the ratio phospho-Akt(Ser473)/Akt in cortical tissue of SZs compared with their matched controls. These data, together with the observed decrease in S6 function, point to a suppression of the feedback loops that control the PI3K/Akt/mTOR signaling.

To date, studies evaluating levels of Akt in human postmortem brains of SZs show divergent results. The first study analyzed frontal cortex samples obtained from two different brain banks (without specifying the anatomical subregions evaluated) and reported decreased Akt protein

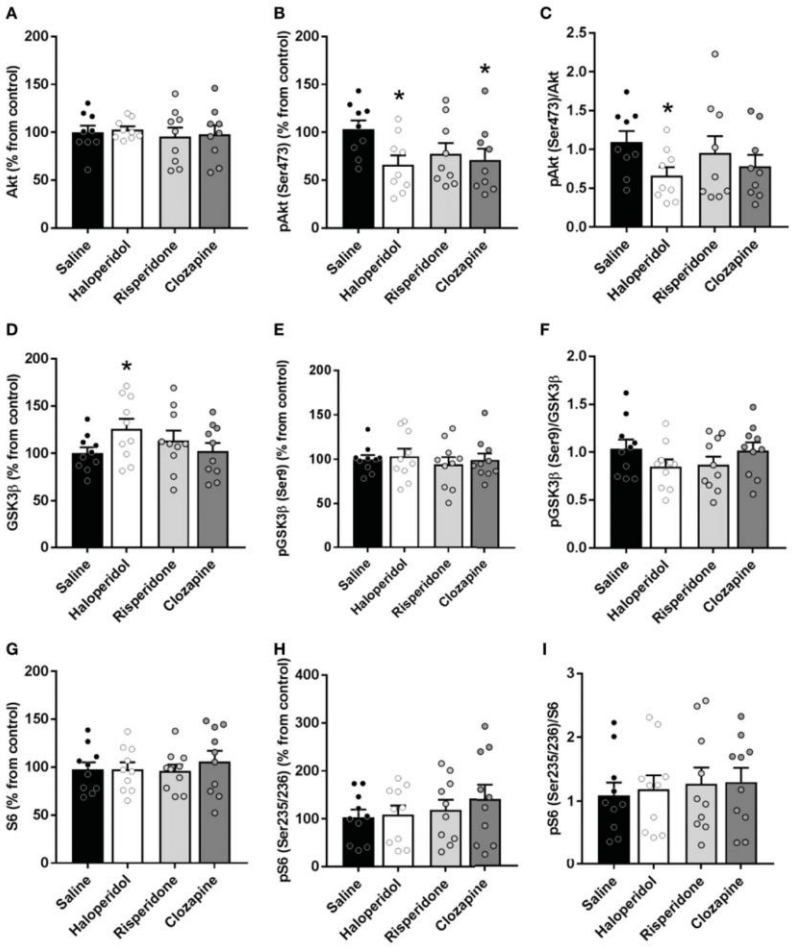


FIGURE 2 | Protein expression of signaling proteins in cortical tissue from rats treated with saline, haloperidol, risperidone, or clozapine. Total Akt **(A)** and phospho-Akt(Ser473) levels **(B)**. **(C)** Phospho-Akt(Ser473)/Akt ratio. Total GSK3β **(D)** and phospho-GSK3β(Ser9) levels **(E)**. **(F)** Phospho-GSK3β(Ser9)/GSK3β ratio. Total S6 **(G)** and phospho-S6(Ser235/236) levels **(H)**. **(I)** Phospho-S6(Ser235/236)/S6 ratio. *$p < 0.05$ *vs* controls; one-way ANOVA followed by Bonferroni *post-hoc* test. Data represent the means ± SEM, referred as a percentage of the inter-assay normalization sample.

levels (Emamian et al., 2004). Another study (Zhao et al., 2006) found a decrease in the ratio phosphorylated/total Akt in the PFC (BA46) of patients with schizophrenia. The study of Ide assessed the protein levels of Akt in frontal cortex (BA9) from the samples from the Stanley foundation (Ide et al., 2006), the same resource as one of the two used in Emamian´s study, but failed to find differences. In this study, another different cohort of samples was used (frontal cortex BA9) and no differences were found between both groups. Another study (Hino et al., 2016) found an increase in phosphorylated Akt, though the ratio of phospho/total Akt was not significantly different in postmortem tissue. On the contrary, the most recent study evaluating Akt in schizophrenia found a decrease in phospho/total Akt ratio in DLPFC (Chadha and Meador-Woodruff, 2020). These controversial results could be explained by the different cortical subregions evaluated, the different antibodies used and, mainly, by confounding factors such as age, sex, PMI, or antipsychotic treatment that have not been controlled in the vast majority of studies.

GSK3 activity is known to influence neural progenitor cell proliferation and differentiation, as well as efficient neurotransmission in differentiated adult neurons (Cole, 2012). GSK3 is indeed an important mediator of dopamine action *in vivo,* and modulation of the Akt/GSK3 pathway is thought to be relevant in dopamine-related disorders (Beaulieu et al., 2004). Recent studies have also suggested a role for this pathway in synaptic modulation and plasticity during the adult life (Hall et al., 2000). Moreover, antipsychotics regulate GSK3 (Beaulieu, 2007) thus supporting the postulate that abnormalities in dopaminergic and/or serotonergic systems in schizophrenia

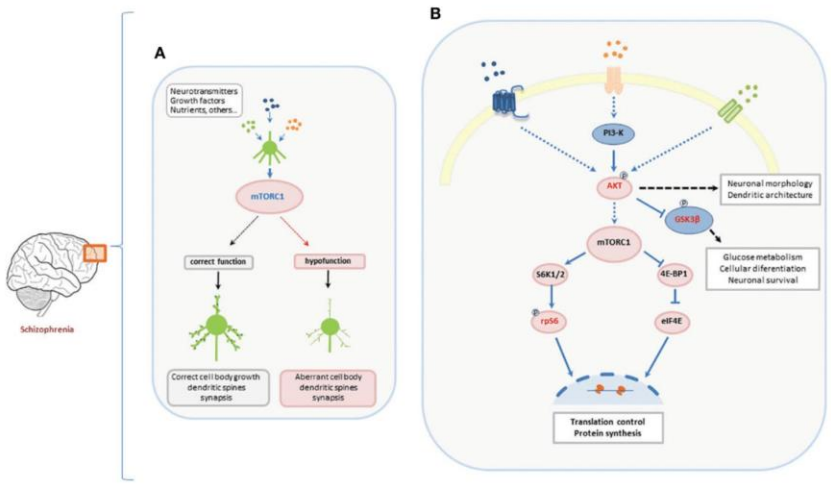


FIGURE 3 | Suggested role of mTORC1-dependent signaling pathway in the development of neurons and its implication in aberrant development of PFC in schizophrenia. **(A)** Neurotransmitters, growth factors, nutrients, and other environmental inputs coordinately activate mTORC1 leading to a correct normal neuronal development. Hypofunction of mTORC1 induces a dysregulation of the neuronal development that may be involved in the development of psychiatric disorders. **(B)** The canonical Akt/mTORC1/S6 pathway. The two main targets of mTORC1, S6K, and 4E-BP1, are involved in the translational machinery for protein synthesis. Once active, S6K regulates the activity of the ribosomal protein S6 by phosphorylating it at different sites. The phosphorylation of S6 controls translation and regulates protein synthesis in the central nervous system (CNS).

could be associated with abnormal GSK3 control (Jope and Roh, 2006). However, the direct association of GSK3 with psychiatric disorders is less clear, and studies addressing the association between GSK3 and schizophrenia show conflicting results. Previous studies have found both reduced (Kozlovsky et al., 2001; Emamian et al., 2004; Amar et al., 2008) and unchanged (Nadri et al., 2002; Ide et al., 2006) protein expression of GSK3β in postmortem PFC from subjects with schizophrenia. In this context, the different brain regions analyzed may have a putative influence in the contradictory results. Under our experimental conditions, we failed to find significant differences in the protein expression of GSK3β or its phosphorylated form phospho-GSK3β(Ser9) between SZs and matched controls.

An increasing number of studies have used S6 phosphorylation as a readout of mTOR activity and as a marker for neuronal activation in the context of synaptic plasticity or in response to therapeutic agents (Bonito-Oliva et al., 2013; Bowling et al., 2014; Biever et al., 2015; Henry et al., 2018). It has been proposed that the phosphorylation of S6 could participate in the positive regulation of global cellular translation (Ruvinsky and Meyuhas, 2006) and in dendrite development (Jaworski and Sheng, 2006; Tang et al., 2014). Thus, the inhibition of mTOR induces a substantial reduction in the number of dendritic branches and arbor shrinkage in neurons (Jaworski and Sheng, 2006). On the contrary, excessive mTORC1 activation is linked to increased dendritic spine density and aberrant synaptic pruning in autism spectrum disorders (Tang et al., 2014).

Interestingly, alterations in mTOR signaling pathway have been described in rodent models used for the study of mental disorders. In this context, a single administration of psychotomimetic drugs, such as ketamine, MK-801 and scopolamine, seems to activate mTOR signaling pathway in the rat brain cortex (Yoon et al., 2008; Li et al., 2010; Voleti et al., 2013). Whereas these drugs are being studied in relation with their fast antidepressant action, mTOR pathway activation seems to be necessary to reestablish synaptic deficits that result from exposure to stress in animal models (Duman et al., 2016). Developmental models of schizophrenia, such as repeated phencyclidine (PCP) treatment during neonatal period and post-weaning isolation have shown an increased phosphorylation of S6 specifically in the rat PFC, and not in striatum (Meffre et al., 2012). Furthermore, early postnatal treatment with the NMDA receptor antagonist MK-801 affects mTOR/p70S6K-related pathways in the frontal cortex of the adult rat brain (Kim et al., 2010). Chronic treatment with ketamine leads to downregulation of mTOR protein expression in both PFC and hippocampus of rats (Xie et al., 2020). Interestingly, chronic THC exposure during adolescence induces schizophrenia-like behaviors and decreases mTOR-p70S6K signaling pathway in the PFC of adult rat brains (Renard et al., 2016).

To our knowledge, the present is the first study exploring this mTORC1 downstream effector in postmortem PFC tissue of subjects with schizophrenia. The phosphorylation ratio of S6 is strongly reduced in schizophrenia and this reduction occurs in both AP-F and AP-T subjects suggesting that phospho-S6 (Ser235/236) expression is dysregulated as a consequence of the disorder itself. Even so, our results are also in accordance with a study that found a reduction in ribosomal proteins and in

protein synthesis in schizophrenia (English et al., 2015). In this study, authors found reduced cell area, ribosomal protein expression, and rates of protein synthesis in schizophrenia patient-derived olfactory cells. Notably, they reported a striking uniform decreased expression of 17 ribosomal proteins, including S6, although they did not evaluate the active/phosphorylated form of this ribosomal protein. Consistent with our findings, a recently published study also provides evidence that proteins associated with the AKT-mTOR signaling cascade are downregulated in schizophrenia DLPFC postmortem tissue (Chadha and Meador-Woodruff, 2020).

However, there are some limitations to consider in interpreting results from this study. The levels and phosphorylation status of these proteins may be conditioned by numerous factors that affect postmortem samples, such as sex, age, the storage time, or PMI. These variables have been taken into account in our study by matching each subject with schizophrenia with a control of the same sex and with similar age, and PMI and were performed correlation analyses to assess associations between protein expression/phosphorylation and age, PMI, and storage time. However, there are additional confounders such as exercise, diet, or tobacco smoking that we are not able to control. In addition, and unfortunately, the size of the groups hinders to extract conclusions regarding gender effects.

On the other hand, the treatment-controlled design allows us to study the putative influence of chronic antipsychotic drugs on protein density and phosphorylation.

Studies about the effects of antipsychotics in Akt/mTOR pathway are contradictory showing both increases, decreases, or no changes in the expression and functional levels of Akt, GSK3 and/or S6 (Alimohamad et al., 2005; Li et al., 2007; Bonito-Oliva et al., 2013; Pereira et al., 2013; Bowling et al., 2014; Mas et al., 2016).

In our study, both chronic haloperidol and clozapine decreased the phospho-(Ser473)Akt levels in rats, but only chronic haloperidol showed a significant decrease in the phospho-(Ser473)Akt/Akt ratio. It has been proposed that depending on its duration, activation of D2 receptors (D2R) differentially regulates Akt-dependent pathways. In this context, acute activation of D2R triggers an inactivation of Akt (Beaulieu et al., 2004). In line with this, acute treatments with haloperidol and clozapine, both exerting a D2R antagonist effect, increase phospho-(Ser473)Akt (Roh et al., 2007). In cell cultures, stimulation of D2R rapidly activates Akt signaling (Mannoury La Cour et al., 2011). By contrast, prolonged stimulation of D2R decreases Akt phosphorylation (Beaulieu et al., 2005). Taking all these data into account, continuous activation of D2R may be triggering a decrease in the tonic phospho-(Ser473)Akt levels observed in chronic haloperidol treated rats. Nevertheless, differences in the effect among antipsychotics due to the affinity for other receptors cannot be ruled out (Beaulieu, 2012).

In this study, chronic haloperidol was the only one exerting an effect over GSK3β, triggering a significant increase in total GSK3β expression in rat cortex. Although the literature is scarce, both acute haloperidol and clozapine seem to decrease the phosphorylation of GSK3β at Ser9 *in vitro* (Takaki et al., 2018). On the contrary, a study carried out in mice reported an increased in the phosphorylation status after acute clozapine and risperidone, with no changes with haloperidol (Li et al., 2007). Similar to our results, another study showed that both sub-chronic and chronic administration of haloperidol resulted in an increased expression of total GSK3 (Alimohamad et al., 2005).

It has been described that acute haloperidol increases rpS6 phosphorylation in rodent striatal neurons (Bonito-Oliva et al., 2013; Bowling et al., 2014). Moreover, an increase or a decrease in rpS6 phosphorylation has been reported in the striatum after acute haloperidol and risperidone treatment depending on the strain of the mice used (Mas et al., 2016). On the other hand, acute clozapine seems to decrease the phosphorylation of the S6K in both cortex and striatum (Pereira et al., 2013). However, all these studies have been carried out under acute treatments. Our results suggest no changes in the levels of rpS6 phosphorylation in the cortex of rats chronically treated with antipsychotics. Divergent results in rpS6 phosphorylation among studies could be due to differences in doses and modes of administration of antipsychotics, in brain regions used, and in the strains of animals used (Mas et al., 2016). These results suggest that the effects of antipsychotics on the Akt/mTOR pathway are different in schizophrenia patients and rodents, both because of species related differences, and because animals used here lack the pathophysiological substrates of schizophrenia. In fact, it is possible that antipsychotics in humans could have different effects in the normal brain and in schizophrenia-related neuropathology (Goff et al., 2017).

As previously stated, S6 participates in the positive regulation of global cellular translation (Ruvinsky and Meyuhas, 2006). Our findings, together with the reduced cell area, ribosomal protein expression and rates of protein synthesis previously reported (English et al., 2015), are consistent with the reduction of the neuronal body size (Rajkowska et al., 1998; Chana et al., 2003) and of the whole-brain volume in schizophrenia (Harrison and Weinberger, 2005; Cannon et al., 2015). Dysfunctions in mTORC1/S6 pathway inhibits oligodendrocyte proliferation and maturation (Liu et al., 2014; Maas et al., 2017), contributing to an alteration in white matter integrity in schizophrenia (Flynn et al., 2003; Schoonover et al., 2019). On the other hand, the inhibition of mTORC1-dependent signaling involves a substantial reduction in the number of dendritic branches and arbor shrinkage in developing neurons (Jaworski and Sheng, 2006; Kwon et al., 2006).

The downregulation of Akt/mTOR signaling proteins in postmortem PFC of SZs (Chadha and Meador-Woodruff, 2020), together with postmortem studies showing that spine density is reduced in the cortex of schizophrenia patients (Glantz and Lewis, 2000; Konopaske et al., 2014) further support our results. Altogether, these alterations in protein synthesis and dendritic architecture would finally contribute to dysfunctions in synaptic connectivity that underlay clinical manifestations of schizophrenia.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethic Committee for Animal Welfare of the University of the Basque Country, UPV/EHU (CEBA 188/2011).

AUTHOR CONTRIBUTIONS

LU and LC designed experiments. BM, LC, and JM obtained and classified postmortem human brain samples. Western blot assays were performed by II-L and RD-A. II-L and LU analyzed data and wrote the manuscript. All the contributors critically revised and approved the final version of the manuscript.

FUNDING

This study was funded by the Instituto de Salud Carlos III, FEDER (PI13/01529) and the Basque Government (IT211-19). II-L is recipient of a Predoctoral Fellowship from the Basque Government.

ACKNOWLEDGMENTS

The authors would like to thank the staff members of the Basque Institute of Legal Medicine for their cooperation in the study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00344/full#supplementary-material

REFERENCES

Alimohamad, H., Rajakumar, N., Seah, Y. H., and Rushlow, W. (2005). Antipsychotics alter the protein expression levels of beta-catenin and GSK-3 in the rat medial prefrontal cortex and striatum. *Biol. Psychiatry* 57, 533–542. doi: 10.1016/j.biopsych.2004.11.036

Amar, S., Shaltiel, G., Mann, L., Shamir, A., Dean, B., Scarr, E., et al. (2008). Possible involvement of post-dopamine D2 receptor signalling components in the pathophysiology of schizophrenia. *Int. J. Neuropsychopharmacol.* 11, 197–205. doi: 10.1017/S1461145707007948

American Psychiatric Association (2000). Diagnostic and Statistical Manual of Mental Disorders.

Antion, M. D., Merhav, M., Hoeffer, C. A., Reis, G., Kozma, S. C., Thomas, G., et al. (2008). Removal of S6K1 and S6K2 leads to divergent alterations in learning, memory, and synaptic plasticity. *Learn. Membr.* 15, 29–38. doi: 10.1101/lm.661908

Balu, D. T., Carlson, G. C., Talbot, K., Kazi, H., Hill-Smith, T. E., Easton, R. M., et al. (2012). Akt1 deficiency in schizophrenia and impairment of hippocampal plasticity and function. *Hippocampus* 22, 230–240. doi: 10.1002/hipo.20887

Beaulieu, J. M., Sotnikova, T. D., Yao, W. D., Kockeritz, L., Woodgett, J. R., Gainetdinov, R. R., et al. (2004). Lithium antagonizes dopamine-dependent behaviors mediated by an AKT/glycogen synthase kinase 3 signaling cascade. *Proc. Natl. Acad. Sci. U. S. A* 101, 5099–5104. doi: 10.1073/pnas.0307921101

Beaulieu, J. M., Sotnikova, T. D., Marion, S., Lefkowitz, R. J., Gainetdinov, R. R., and Caron, M. G. (2005). An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. *Cell* 122, 261–273. doi: 10.1016/j.cell.2005.05.012

Beaulieu, J. M. (2007). Not only lithium: regulation of glycogen synthase kinase-3 by antipsychotics and serotonergic drugs. *Int. J. Neuropsychopharmacol.* 10, 3–6. doi: 10.1017/S1461145706006857

Beaulieu, J. M. (2012). A role for Akt and glycogen synthase kinase-3 as integrators of dopamine and serotonin neurotransmission in mental health. *J. Psychiatry Neurosci.* 37, 7–16. doi: 10.1503/jpn.110011

Biever, A., Valjent, E., and Puighermanal, E. (2015). Ribosomal Protein S6 Phosphorylation in the Nervous System: From Regulation to Function. *Front. Mol. Neurosci.* 8, 75. doi: 10.3389/fnmol.2015.00075

Bonito-Oliva, A., Pallottino, S., Bertran-Gonzalez, J., Girault, J. A., Valjent, E., and Fisone, G. (2013). Haloperidol promotes mTORC1-dependent phosphorylation of ribosomal protein S6 via dopamine- and cAMP-regulated phosphoprotein of 32 kDa and inhibition of protein phosphatase-1. *Neuropharmacology* 72, 197–203. doi: 10.1016/j.neuropharm.2013.04.043

Bowling, H., Zhang, G., Bhattacharya, A., Perez-Cuesta, L. M., Deinhardt, K., Hoeffer, C. A., et al. (2014). Antipsychotics activate mTORC1-dependent translation to enhance neuronal morphological complexity. *Sci. Signal* 7, ra4. doi: 10.1126/scisignal.2004331

Burgering, B. M., and Coffer, P. J. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* 376, 599–602. doi: 10.1038/376599a0

Cannon, T. D., Chung, Y., He, G., Sun, D., Jacobson, A., Van Erp, T. G., et al. (2015). Progressive reduction in cortical thickness as psychosis develops: a multisite longitudinal neuroimaging study of youth at elevated clinical risk. *Biol. Psychiatry* 77, 147–157. doi: 10.1016/j.biopsych.2014.05.023

Chadha, R., and Meador-Woodruff, J. H. (2020). Downregulated AKT-mTOR signaling pathway proteins in dorsolateral prefrontal cortex in Schizophrenia. *Neuropsychopharmacology*. doi: 10.1038/s41386-020-0614-2

Chana, G., Landau, S., Beasley, C., Everall, I. P., and Cotter, D. (2003). Two-dimensional assessment of cytoarchitecture in the anterior cingulate cortex in major depressive disorder, bipolar disorder, and schizophrenia: evidence for decreased neuronal somal size and increased neuronal density. *Biol. Psychiatry* 53, 1086–1098. doi: 10.1016/S0006-3223(03)00114-8

Cole, A. R. (2012). GSK3 as a Sensor Determining Cell Fate in the Brain. *Front. Mol. Neurosci.* 5, 4. doi: 10.3389/fnmol.2012.00004

Costa-Mattioli, M., and Monteggia, L. M. (2013). mTOR complexes in neurodevelopmental and neuropsychiatric disorders. *Nat. Neurosci.* 16, 1537–1543. doi: 10.1038/nn.3546

Duman, R. S., Aghajanian, G. K., Sanacora, G., and Krystal, J. H. (2016). Synaptic plasticity and depression: new insights from stress and rapid-acting antidepressants. *Nat. Med.* 22, 238–249. doi: 10.1038/nm.4050

Emamian, E. S., Hall, D., Birnbaum, M. J., Karayiorgou, M., and Gogos, J. A. (2004). Convergent evidence for impaired AKT1-GSK3beta signaling in schizophrenia. *Nat. Genet.* 36, 131–137. doi: 10.1038/ng1296

English, J. A., Fan, Y., Focking, M., Lopez, L. M., Hryniewiecka, M., Wynne, K., et al. (2015). Reduced protein synthesis in schizophrenia patient-derived olfactory cells. *Transl. Psychiatry* 5, e663. doi: 10.1038/tp.2015.119

Flynn, S. W., Lang, D. J., Mackay, A. L., Goghari, V., Vavasour, I. M., Whittall, K. P., et al. (2003). Abnormalities of myelination in schizophrenia detected in vivo with MRI, and post-mortem with analysis of oligodendrocyte proteins. *Mol. Psychiatry* 8, 811–820. doi: 10.1038/sj.mp.4001337

Glantz, L. A., and Lewis, D. A. (2000). Decreased Dendritic Spine Density on Prefrontal Cortical Pyramidal Neurons in Schizophrenia. *Arch. Gen. Psychiatry* 57 (1), 65–73. doi: 10.1001/archpsyc.57.1.65

Goff, D. C., Falkai, P., Fleischhacker, W. W., Girgis, R. R., Kahn, R. M., Uchida, H., et al. (2017). The Long-Term Effects of Antipsychotic Medication on Clinical Course in Schizophrenia. *Am. J. Psychiatry* 174, 840–849. doi: 10.1176/appi.ajp.2017.16091016

Gururajan, A., and Van Den Buuse, M. (2014). Is the mTOR-signalling cascade disrupted in Schizophrenia? *J. Neurochem.* 129, 377–387. doi: 10.1111/jnc.12622

Hall, A. C., Lucas, F. R., and Salinas, P. C. (2000). Axonal remodeling and synaptic differentiation in the cerebellum is regulated by WNT-7a signaling. *Cell* 100, 525–535. doi: 10.1016/S0092-8674(00)80689-3

Harrison, P. J., and Weinberger, D. R. (2005). Schizophrenia genes, gene expression, and neuropathology: on the matter of their convergence. *Mol. Psychiatry* 10, 40–68 image 45. doi: 10.1038/sj.mp.4001558

Henry, F. E., Wang, X., Serrano, D., Perez, A. S., Carruthers, C. J. L., Stuenkel, E. L., et al. (2018). A Unique Homeostatic Signaling Pathway Links Synaptic Inactivity to Postsynaptic mTORC1. *J. Neurosci.* 38, 2207–2225. doi: 10.1523/JNEUROSCI.1843-17.2017

Hino, M., Kunii, Y., Matsumoto, J., Wada, A., Nagaoka, A., Niwa, S., et al. (2016). Decreased VEGFR2 expression and increased phosphorylated Akt1 in the prefrontal cortex of individuals with schizophrenia. *J. Psychiatr. Res.* 82, 100–108. doi: 10.1016/j.jpsychires.2016.07.018

Ibarra-Lecue, I., Mollinedo-Gajate, I., Meana, J. J., Callado, L. F., Diez-Alarcia, R., and Uriguen, L. (2018). Chronic cannabis promotes pro-hallucinogenic signaling of 5-HT2A receptors through Akt/mTOR pathway. *Neuropsychopharmacology* 43, 2028–2035. doi: 10.1038/s41386-018-0076-y

Ide, M., Ohnishi, T., Murayama, M., Matsumoto, I., Yamada, K., Iwayama, Y., et al. (2006). Failure to support a genetic contribution of AKT1 polymorphisms and altered AKT signaling in schizophrenia. *J. Neurochem.* 99, 277–287. doi: 10.1111/j.1471-4159.2006.04033.x

Ikeda, M., Iwata, N., Suzuki, T., Kitajima, T., Yamanouchi, Y., Kinoshita, Y., et al. (2004). Association of AKT1 with schizophrenia confirmed in a Japanese population. *Biol. Psychiatry* 56, 698–700. doi: 10.1016/j.biopsych.2004.07.023

Jaworski, J., and Sheng, M. (2006). The growing role of mTOR in neuronal development and plasticity. *Mol. Neurobiol.* 34, 205–219. doi: 10.1385/MN:34:3:205

Jope, R. S., and Roh, M. S. (2006). Glycogen synthase kinase-3 (GSK3) in psychiatric diseases and therapeutic interventions. *Curr. Drug Targets* 7, 1421–1434. doi: 10.2174/1389450110607011421

Kapur, S., Vanderspek, S. C., Brownlee, B. A., and Nobrega, J. N. (2003). Antipsychotic dosing in preclinical models is often unrepresentative of the clinical condition: a suggested solution based on in vivo occupancy. *J. Pharmacol. Exp. Ther.* 305, 625–631. doi: 10.1124/jpet.102.046987

Kennedy, S. G., Wagner, A. J., Conzen, S. D., Jordan, J., Bellacosa, A., Tsichlis, P. N., et al. (1997). The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. *Genes Dev.* 11, 701–713. doi: 10.1101/gad.11.6.701

Kim, S. H., Park, H. G., Kim, H. S., Ahn, Y. M., and Kim, Y. S. (2010). Effects of neonatal MK-801 treatment on p70S6K-S6/eIF4B signal pathways and protein translation in the frontal cortex of the developing rat brain. *Int. J. Neuropsychopharmacol.* 13, 1233–1246. doi: 10.1017/S1461145709991192

Kitagishi, Y., Kobayashi, M., Kikuta, K., and Matsuda, S. (2012). Roles of PI3K/AKT/GSK3/mTOR Pathway in Cell Signaling of Mental Illnesses. *Depress Res. Treat* 2012, 752563. doi: 10.1155/2012/752563

Konopaske, G. T., Lange, N., Coyle, J. T., and Benes, F. M. (2014). Prefrontal Cortical Dendritic Spine Pathology in Schizophrenia and Bipolar Disorder. *JAMA Psychiatry* 71, 1323–1331. doi: 10.1001/jamapsychiatry.2014.1582

Kozlovsky, N., Belmaker, R. H., and Agam, G. (2001). Low GSK-3 activity in frontal cortex of schizophrenic patients. *Schizophr. Res.* 52, 101–105. doi: 10.1016/S0920-9964(00)00174-2

Kwon, C. H., Luikart, B. W., Powell, C. M., Zhou, J., Matheny, S. A., Zhang, W., et al. (2006). Pten regulates neuronal arborization and social interaction in mice. *Neuron* 50, 377–388. doi: 10.1016/j.neuron.2006.03.023

Lai, W. S., Xu, B., Westphal, K. G., Paterlini, M., Olivier, B., Pavlidis, P., et al. (2006). Akt1 deficiency affects neuronal morphology and predisposes to abnormalities in prefrontal cortex functioning. *Proc. Natl. Acad. Sci. U. S. A* 103, 16906–16911. doi: 10.1073/pnas.0604994103

Lane, H. A., and Breuleux, M. (2009). Optimal targeting of the mTORC1 kinase in human cancer. *Curr. Opin. Cell Biol.* 21, 219–229. doi: 10.1016/j.ceb.2009.01.016

Li, X., Rosborough, K. M., Friedman, A. B., Zhu, W., and Roth, K. A. (2007). Regulation of mouse brain glycogen synthase kinase-3 by atypical antipsychotics. *Int. J. Neuropsychopharmacol.* 10, 7–19. doi: 10.1017/S1461145706006547

Li, N., Lee, B., Liu, R. J., Banasr, M., Dwyer, J. M., Iwata, M., et al. (2010). mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. *Science* 329, 959–964. doi: 10.1126/science.1190287

Liu, B., Chen, X., Wang, Z. Q., and Tong, W. M. (2014). DNA damage and oxidative injury are associated with hypomyelination in the corpus callosum of newborn Nbn(CNS-del) mice. *J. Neurosci. Res.* 92, 254–266. doi: 10.1002/jnr.23313

Maas, D. A., Valles, A., and Martens, G. J. M. (2017). Oxidative stress, prefrontal cortex hypomyelination and cognitive symptoms in schizophrenia. *Transl. Psychiatry* 7, e1171. doi: 10.1038/tp.2017.138

Magdalon, J., Sanchez-Sanchez, S. M., Griesi-Oliveira, K., and Sertie, A. L. (2017). Dysfunctional mTORC1 Signaling: A Convergent Mechanism between Syndromic and Nonsyndromic Forms of Autism Spectrum Disorder? *Int. J. Mol. Sci.* 18. doi: 10.3390/ijms18030659

Manning, B. D., and Cantley, L. C. (2007). AKT/PKB signaling: navigating downstream. *Cell* 129, 1261–1274. doi: 10.1016/j.cell.2007.06.009

Mannoury La Cour, C., Salles, M. J., Pasteau, V., and Millan, M. J. (2011). Signaling pathways leading to phosphorylation of Akt and GSK-3beta by activation of cloned human and rat cerebral D(2)and D(3) receptors. *Mol. Pharmacol.* 79, 91–105. doi: 10.1124/mol.110.065409

Mas, S., Gasso, P., Boloc, D., Rodriguez, N., Marmol, F., Sanchez, J., et al. (2016). Network analysis of gene expression in mice provides new evidence of involvement of the mTOR pathway in antipsychotic-induced extrapyramidal symptoms. *Pharmacogenomics J.* 16, 293–300. doi: 10.1038/tpj.2015.48

Mathur, A., Law, M. H., Megson, I. L., Shaw, D. J., and Wei, J. (2010). Genetic association of the AKT1 gene with schizophrenia in a British population. *Psychiatr. Genet.* 20, 118–122. doi: 10.1097/YPG.0b013e32833a2234

McGuire, J. L., Depasquale, E. A., Funk, A. J., O'donnovan, S. M., Hasselfeld, K., Marwaha, S., et al. (2017). Abnormalities of signal transduction networks in chronic schizophrenia. *NPJ Schizophr.* 3, 30. doi: 10.1038/s41537-017-0032-6

Meffre, J., Chaumont-Dubel, S., Mannoury La Cour, C., Loiseau, F., Watson, D. J., Dekeyne, A., et al. (2012). 5-HT(6) receptor recruitment of mTOR as a mechanism for perturbed cognition in schizophrenia. *EMBO Mol. Med.* 4, 1043–1056. doi: 10.1002/emmm.201201410

Meyuhas, O. (2015). Ribosomal Protein S6 Phosphorylation: Four Decades of Research. *Int. Rev. Cell Mol. Biol.* 320, 41–73. doi: 10.1016/bs.ircmb.2015.07.006

Murga, C., Laguinge, L., Wetzker, R., Cuadrado, A., and Gutkind, J. S. (1998). Activation of Akt/protein kinase B by G protein-coupled receptors. A role for alpha and beta gamma subunits of heterotrimeric G proteins acting through phosphatidylinositol-3-OH kinasegamma. *J. Biol. Chem.* 273, 19080–19085. doi: 10.1074/jbc.273.30.19080

Nadri, C., Kozlovsky, N., Agam, G., and Bersudsky, Y. (2002). GSK-3 parameters in lymphocytes of schizophrenic patients. *Psychiatry Res.* 112, 51–57. doi: 10.1016/S0165-1781(02)00191-9

O'Reilly, K. E., Rojo, F., She, Q. B., Solit, D., Mills, G. B., Smith, D., et al. (2006). mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res.* 66, 1500–1508. doi: 10.1158/0008-5472.CAN-05-2925

Parikh, V., Khan, M. M., Terry, A., and Mahadik, S. P. (2004). Differential effects of typical and atypical antipsychotics on nerve growth factor and choline acetyltransferase expression in the cortex and nucleus basalis of rats. *J. Psychiatr. Res.* 38, 521–529. doi: 10.1016/j.jpsychires.2004.03.008

Pereira, A., Zhang, B., Malcolm, P., and Sundram, S. (2013). Clozapine regulation of p90RSK and c-Fos signaling via the ErbB1-ERK pathway is distinct from olanzapine and haloperidol in mouse cortex and striatum. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 40, 353–363. doi: 10.1016/j.pnpbp.2012.10.025

Pham, X., Song, G., Lao, S., Goff, L., Zhu, H., Valle, D., et al. (2016). The DPYSL2 gene connects mTOR and schizophrenia. *Transl. Psychiatry* 6, e933. doi: 10.1038/tp.2016.204

Pietilainen, O. P., Paunio, T., Loukola, A., Tuulio-Henriksson, A., Kieseppa, T., Thompson, P., et al. (2009). Association of AKT1 with verbal learning, verbal memory, and regional cortical gray matter density in twins. *Am. J. Med. Genet. B. Neuropsychiatr. Genet.* 150b, 683–692. doi: 10.1002/ajmg.b.30890

Pirbhoy, P. S., Farris, S., and Steward, O. (2016). Synaptic activation of ribosomal protein S6 phosphorylation occurs locally in activated dendritic domains. *Learn. Membr.* 23, 255–269. doi: 10.1101/lm.041947.116

Pirbhoy, P. S., Farris, S., and Steward, O. (2017). Synaptically driven phosphorylation of ribosomal protein S6 is differentially regulated at active synapses versus dendrites and cell bodies by MAPK and PI3K/mTOR signaling pathways. *Learn. Membr.* 24, 341–357. doi: 10.1101/lm.044974.117

Puighermanal, E., Biever, A., Pascoli, V., Melser, S., Pratlong, M., Cutando, L., et al. (2017). Ribosomal Protein S6 Phosphorylation Is Involved in Novelty-Induced Locomotion, Synaptic Plasticity and mRNA Translation. *Front. Mol. Neurosci.* 10, 419. doi: 10.3389/fnmol.2017.00419

Qin, X., Jiang, B., and Zhang, Y. (2016). 4E-BP1, a multifactor regulated multifunctional protein. *Cell Cycle* 15, 781–786. doi: 10.1080/15384101.2016.1151581

Rajkowska, G., and Goldman-Rakic, P. S. (1995). Cytoarchitectonic definition of prefrontal areas in the normal human cortex: I. Remapping of areas 9 and 46 using quantitative criteria. *Cereb. Cortex* 5, 307–322. doi: 10.1093/cercor/5.4.307

Rajkowska, G., Selemon, L. D., and Goldman-Rakic, P. S. (1998). Neuronal and glial somal size in the prefrontal cortex: a postmortem morphometric study of schizophrenia and Huntington disease. *Arch. Gen. Psychiatry* 55, 215–224. doi: 10.1001/archpsyc.55.3.215

Renard, J., Loureiro, M., Rosen, L. G., Zunder, J., De Oliveira, C., Schmid, S., et al. (2016). Cannabidiol Counteracts Amphetamine-Induced Neuronal and Behavioral Sensitization of the Mesolimbic Dopamine Pathway through a Novel mTOR/p70S6 Kinase Signaling Pathway. *J. Neurosci.* 36, 5160–5169. doi: 10.1523/JNEUROSCI.3387-15.2016

Roh, M. S., Seo, M. S., Kim, Y., Kim, S. H., Jeon, W. J., Ahn, Y. M., et al. (2007). Haloperidol and clozapine differentially regulate signals upstream of glycogen synthase kinase 3 in the rat frontal cortex. *Exp. Mol. Med.* 39, 353–360. doi: 10.1038/emm.2007.39

Rozengurt, E., Soares, H. P., and Sinnet-Smith, J. (2014). Suppression of feedback loops mediated by PI3K/mTOR induces multiple overactivation of compensatory pathways: an unintended consequence leading to drug resistance. *Mol. Cancer Ther.* 13, 2477–2488. doi: 10.1158/1535-7163.MCT-14-0330

Ruvinsky, I., and Meyuhas, O. (2006). Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. *Trends Biochem. Sci.* 31, 342–348. doi: 10.1016/j.tibs.2006.04.003

Schoonover, K. E., Farmer, C. B., Cash, A. E., and Roberts, R. C. (2019). Pathology of white matter integrity in three major white matter fasciculi: a post-mortem study of schizophrenia and treatment status. *Br. J. Pharmacol.* 176, 1143–1155. doi: 10.1111/bph.14612

Schwab, S. G., Hoefgen, B., Hanses, C., Hassenbach, M. B., Albus, M., Lerer, B., et al. (2005). Further evidence for association of variants in the AKT1 gene with schizophrenia in a sample of European sib-pair families. *Biol. Psychiatry* 58, 446–450. doi: 10.1016/j.biopsych.2005.05.005

Takaki, M., Kodama, M., Mizuki, Y., Kawai, H., Yoshimura, B., Kishimoto, M., et al. (2018). Effects of the antipsychotics haloperidol, clozapine, and aripiprazole on the dendritic spine. *Eur. Neuropsychopharmacol.* 28, 610–619. doi: 10.1016/j.euroneuro.2018.03.004

Tang, G., Gudsnuk, K., Kuo, S. H., Cotrina, M. L., Rosoklija, G., Sosunov, A., et al. (2014). Loss of mTOR-dependent macroautophagy causes autistic-like synaptic pruning deficits. *Neuron* 83, 1131–1143. doi: 10.1016/j.neuron.2014.07.040

Thiselton, D. L., Vladimirov, V. I., Kuo, P. H., Mcclay, J., Wormley, B., Fanous, A., et al. (2008). AKT1 is associated with schizophrenia across multiple symptom dimensions in the Irish study of high density schizophrenia families. *Biol. Psychiatry* 63, 449–457. doi: 10.1016/j.biopsych.2007.06.005

Urigüen, L., Garcia-Fuster, M. J., Callado, L. F., Morentin, B., La Harpe, R., Casado, V., et al. (2009). Immunodensity and mRNA expression of A2A adenosine, D2 dopamine, and CB1 cannabinoid receptors in postmortem frontal cortex of subjects with schizophrenia: effect of antipsychotic treatment. *Psychopharmacol. (Berl.)* 206, 313–324. doi: 10.1007/s00213-009-1608-2

Voleti, B., Navarria, A., Liu, R. J., Banasr, M., Li, N., Terwilliger, R., et al. (2013). Scopolamine rapidly increases mammalian target of rapamycin complex 1 signaling, synaptogenesis, and antidepressant behavioral responses. *Biol. Psychiatry* 74, 742–749. doi: 10.1016/j.biopsych.2013.04.025

Xie, R., Xie, J., Ye, Y., Wang, X., Chen, F., Yang, L., et al. (2020). mTOR Expression in Hippocampus and Prefrontal Cortex Is Downregulated in a Rat Model of Schizophrenia Induced by Chronic Administration of Ketamine. *J. Mol. Neurosci.* 70, 269–275. doi: 10.1007/s12031-019-01476-9

Yoon, S. C., Seo, M. S., Kim, S. H., Jeon, W. J., Ahn, Y. M., Kang, U. G., et al. (2008). The effect of MK-801 on mTOR/p70S6K and translation-related proteins in rat frontal cortex. *Neurosci. Lett.* 434, 23–28. doi: 10.1016/j.neulet.2008.01.020

Zhao, Z., Ksiezak-Reding, H., Riggio, S., Haroutunian, V., and Pasinetti, G. M. (2006). Insulin receptor deficits in schizophrenia and in cellular and animal models of insulin receptor dysfunction. *Schizophr. Res.* 84, 1–14. doi: 10.1016/j.schres.2006.02.009

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY MATERIAL

Ribosomal protein S6 hypofunction in postmortem human brain links mTORC1-dependent signaling and schizophrenia

Inés Ibarra-Lecue[1], Rebeca Díez-Alarcia[1,2], Benito Morentin[3], J Javier Meana[1,2], Luis F Callado[1,2], Leyre Urigüen[1,2]*

[1]*Department of Pharmacology, University of the Basque Country UPV/EHU and Centro de Investigación Biomédica en Red de Salud Mental CIBERSAM, Spain.*

[2]*Biocruces Health Research Institute, Bizkaia, Spain.*

[3]*Section of Forensic Pathology, Basque Institute of Legal Medicine, Bilbao, Bizkaia, Spain.*

Content:

Supplementary Table 1.

Demographic characteristics, *postmortem* interval (PMI), cause of death and toxicological study of antipsychotic-free case subjects and matched control subjects.

Case	Gender	Age (years)	PMI (hours)	Storage time (months)	Cause of death	Psychiatric diagnosis	APs in blood
SCH1	M	21	24	175	Suicide/Jumping	Schizophrenia	-
C1	M	21	30	135	Accident/Traffic	Control	
SCH2	M	30	51	144	Suicide/Jumping	Schizophrenia	-
C2	M	29	18	32	Accident/Falling	Control	
SCH3	M	29	6	79	Suicide/Asphyxia	Schizophrenia	-
C3	M	29	36	137	Accident/Traffic	Control	
SCH4	M	31	14	78	Suicide/Jumping	Schizophrenia	-
C4	M	32	28	78	Accident/Traffic	Control	
SCH5	M	48	20	74	Suicide/Train	Schizophrenia	-
C5	M	47	18	94	Natural/CRF	Control	
SCH6	M	33	14	64	Suicide/Hanging	Schizophrenia	-
C6	M	33	4	52	Accident/Traffic	Control	
SCH7	M	45	3	60	Suicide/Gun	Schizophrenia	-
C7	M	44	21	46	Accident/Traffic	Control	
SCH8	M	27	24	60	Suicide/Overdose	Schizophrenia	-
C8	M	28	30	132	Homicide/Knife	Control	
SCH9	F	37	58	44	Suicide/Jumping	Schizophrenia	-
C9	F	36	38	168	Natural/MI	Control	
SCH10	M	46	22	35	Suicide/Jumping	Schizophrenia	-
C10	M	46	24	23	Accident/Traffic	Control	
SCH11	F	37	26	9	Suicide/Jumping	Schizophrenia	-
C11	F	38	22	6	Accident/Traffic	Control	
SCH12	M	48	11	9	Suicide/Jumping	Schizophrenia	-
C12	M	49	8	3	Natural/CRF	Control	
SCH13	M	35	5	10	Suicide/Hanging	Schizophrenia	-
C13	M	38	33	165	Accident/Traffic	Control	
SCH14	F	59	9	15	Natural/CRF	Schizophrenia	-
C14	F	58	20	136	Natural/CRF	Control	
SCH15	M	45	18	19	Suicide/Jumping	Schizophrenia	-
C15	M	47	15	4	Accident/Traffic	Control	
SCH16	M	34	15	21	Natural/CRF	Schizophrenia	-
C16	M	36	48	15	Natural/CRF	Control	
SCH17	M	52	7	26	Suicide/Jumping	Schizophrenia	-
C17	M	51	13	6	Accident/Traffic	Control	
SCH AP-F	14M/3F	38.6±2	19.2±3	54.2±11			
C	14M/3F	40.3±3	23.8±3	48.7±13			

SCH=Schizophrenia; C=Control; M=Male; F=Female; CRF=Cardiorespiratory failure; MI=Myocardial infarct; APs=Antipsychotics; AP-F=Antipsychotic-free. Mean ± SEM.

Supplementary Table 2.

Demographic characteristics, *postmortem* interval (PMI), cause of death and toxicological study of antipsychotic treated case subjects and matched control subjects.

Case	Gender	Age (years)	PMI (hours)	Storage time (months)	Cause of death	Psychiatric diagnosis	APs in blood
SCH18	M	44	7	84	Natural/CRF	Schizophrenia	CTP
C18	M	44	23	4	Accident/Traffic	Control	
SCH19	M	30	18	81	Suicide/Jumping	Schizophrenia	OLZ
C19	M	30	11	82	Accident/Electrocution	Control	
SCH20	M	32	8	77	Suicide/Hanging	Schizophrenia	QTP
C20	M	32	20	135	Accident/Crushing	Control	
SCH21	M	23	16	72	Suicide/Jumping	Schizophrenia	SLP
C21	M	23	17	45	Accident/Electrocution	Control	
SCH22	M	35	3	72	Suicide/Jumping	Schizophrenia	QTP
C22	M	36	23	45	Accident/Crushing	Control	
SCH23	M	35	11	72	Natural/CRF	Schizophrenia	CLZ
C23	M	36	18	4	Accident/Traffic	Control	
SCH24	F	60	23	24	Natural/Peritonitis	Schizophrenia	ASLP/CLZ
C24	F	60	48	94	Natural/CH	Control	
SCH25	M	56	12	14	Natural/CRF	Schizophrenia	OLZ/CLT
C25	M	54	16	20	Accident/Traffic	Control	
SCH26	F	30	17	19	Suicide/Jumping	Schizophrenia	HLP
C26	F	30	18	10	Accident/Traffic	Control	
SCH27	M	57	19	24	Suicide/Train	Schizophrenia	QTP
C27	F	57	14	17	Natural/CRF	Control	
SCH28	M	26	39	24	Suicide/Jumping	Schizophrenia	OLZ
C28	M	25	18	21	Accident/Fire	Control	
SCH AP-T	9M/2F	38.9±3	16.1±2	46.3±9			
C	8M/3F	36.6±3	20.4±2	41.4±1			

SCH=Schizophrenia; C=Control; F=Female; M=Male; CRF=Cardiorespiratory failure; CH=Cerebral hemorrhage; APs=Antipsychotics; ASLP=Amisulpiride; BIP=Biperiden; CLZ=Clozapine; CTP=Clotiapine; HLP=Haloperidol; OLZ=Olanzapine; QTP=Quetiapine; SLP=Sulpiride; AP-T=Antipsychotic-treated. Mean ± SEM.

Supplementary Table 3.

Details of the antibodies used and their dilutions for human PFC experiments.

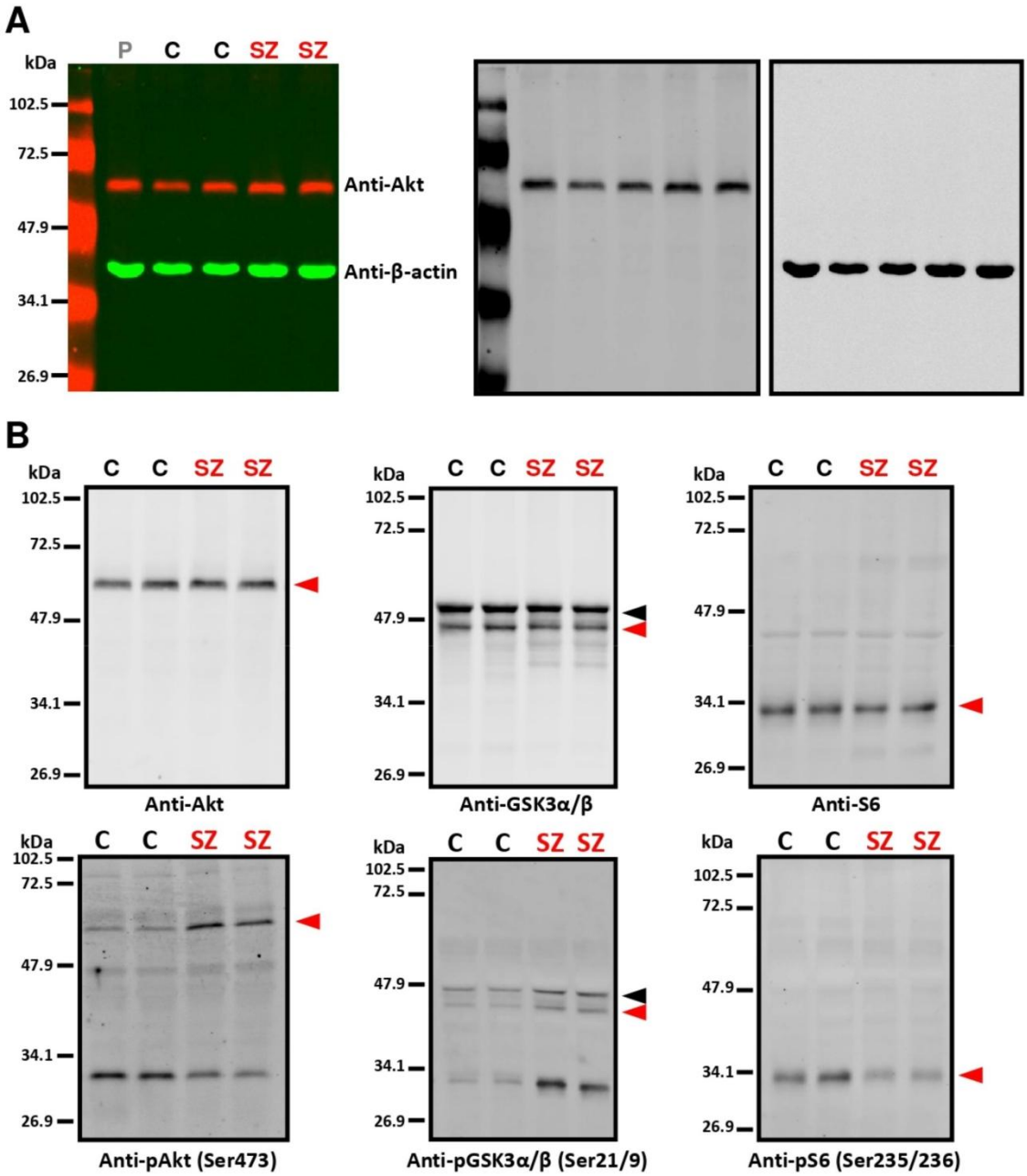
Antibody type	Clonality	Target	Host	Company	Ref. #	Dilution
Primary	Polyclonal	Akt	Rabbit	Cell Signaling	9272	1:500
Primary	Monoclonal (clone D9E)	Phospho(Ser^{473})-Akt	Rabbit	Cell Signaling	4060	1:500
Primary	Monoclonal (clone 54D2)	S6	Mouse	Cell Signaling	2317	1:250
Primary	Monoclonal (clone D57.2.2E)	Phospho($Ser^{235/236}$)-S6	Rabbit	Cell Signaling	4858	1:250
Primary	Monoclonal (clone 4G-1E)	GSK3α/β	Mouse	Merck Millipore	05-412	1:500
Primary	Polyclonal	Phospho($Ser^{21/9}$)-GSK3α/β	Rabbit	Cell Signaling	9331	1:250
Primary	Polyclonal	β-actin	Rabbit	Abcam	ab8227	1:20,000
Primary	Monoclonal (clone AC-15)	β-actin	Mouse	Sigma Aldrich	A1978	1:200,000
Secondary	Polyclonal	Alexa Fluor 680 anti-Rabbit IgG (H+L)	Goat	Thermo Fisher Sc.	A21076	1:4,000
Secondary	Polyclonal	Alexa Fluor 680 anti-Mouse IgG (H+L)	Goat	Thermo Fisher Sc.	A21057	1:4,000
Secondary	Polyclonal	IRDye 800 anti-Rabbit IgG (H+L)	Donkey	Rockland Immunoc.	611-732-127	1:5,000 - 1:20,000
Secondary	Polyclonal	IRDye 800 anti-Mouse IgG (H+L)	Donkey	Rockland Immunoc.	610-731-124	1:5,000 - 1:10,000

Details of the antibodies used and their dilutions for rat cortex experiments.

Antibody type	Clonality	Target	Host	Company	Ref. #	Dilution
Primary	Polyclonal	Akt	Rabbit	Cell Signaling	9272	1:500
Primary	Monoclonal (clone D9E)	Phospho(Ser^{473})-Akt	Rabbit	Cell Signaling	4060	1:500
Primary	Monoclonal (clone 54D2)	S6	Mouse	Cell Signaling	2317	1:500
Primary	Monoclonal (clone D57.2.2E)	Phospho($Ser^{235/236}$)-S6	Rabbit	Cell Signaling	4858	1:500
Primary	Monoclonal (clone 4G-1E)	GSK3α/β	Mouse	Merck Millipore	05-412	1:500
Primary	Polyclonal	Phospho($Ser^{21/9}$)-GSK3α/β	Rabbit	Cell Signaling	9331	1:500
Primary	Polyclonal	β-actin	Rabbit	Abcam	ab8227	1:20,000
Primary	Monoclonal (clone AC-15)	β-actin	Mouse	Sigma Aldrich	A1978	1:100,000
Secondary	Polyclonal	Alexa Fluor 680 anti-Rabbit IgG (H+L)	Goat	Thermo Fisher Sc.	A21076	1:4,000 - 1:5,000
Secondary	Polyclonal	Alexa Fluor 680 anti-Mouse IgG (H+L)	Goat	Thermo Fisher Sc.	A21057	1:4,000 - 1:5,000
Secondary	Polyclonal	Dylight 800 anti-Rabbit IgG (H+L)	Donkey	Rockland Immunoc.	611-745-127	1:5,000 - 1:20,000
Secondary	Polyclonal	Dylight 800 anti-Mouse IgG (H+L)	Donkey	Rockland Immunoc.	610-745-002	1:5,000 - 1:10,000

Ref.=Reference; IgG=Immunoglobulin G; H+L=heavy and low chains; Immunoc.=Immunochemicals; Sc.=Scientific.

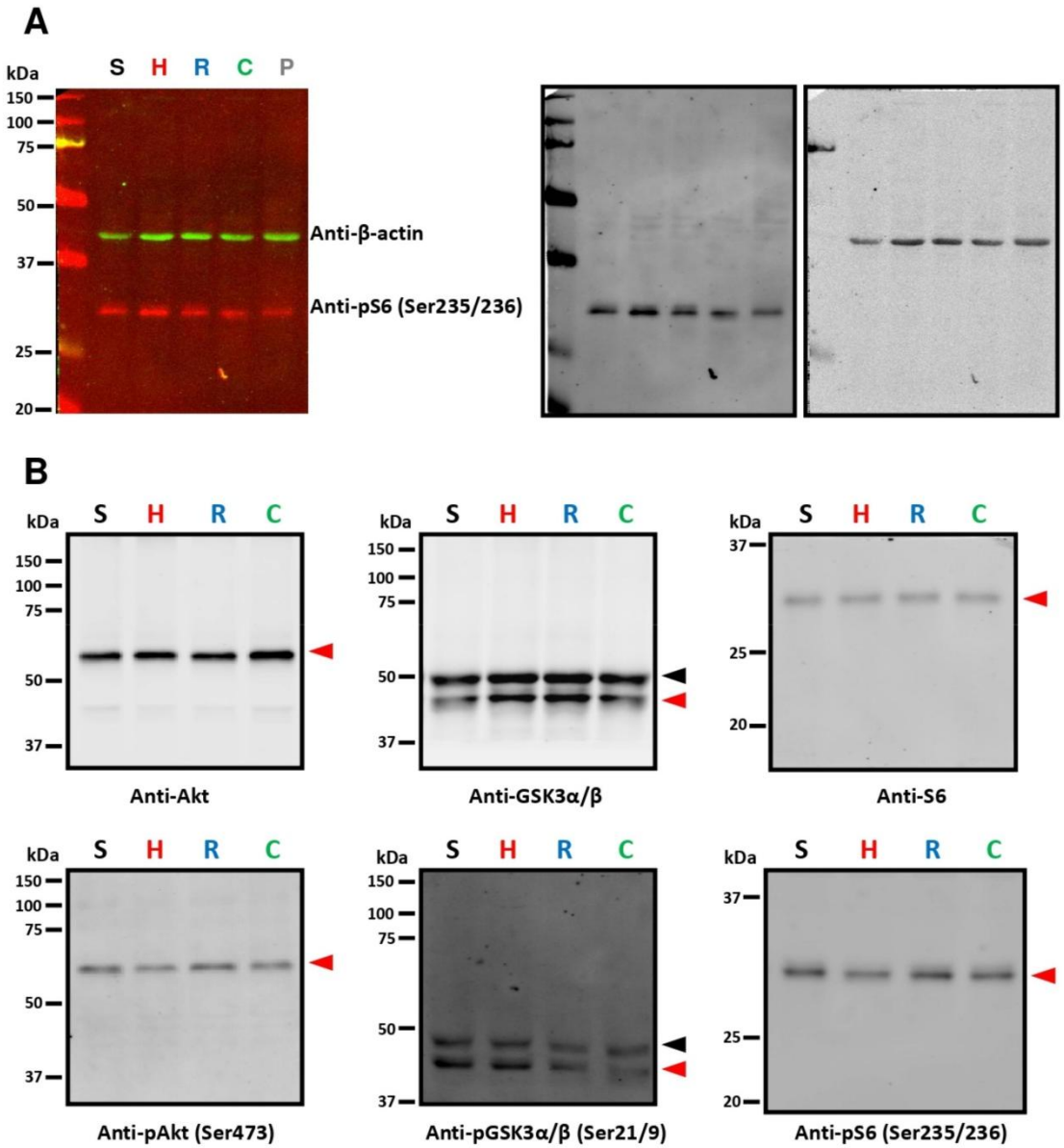
Supplementary Figure 1.



Representative images of immunoblots carried out in human PFC in the study.

A. Image of 700 and 800nm channels visualized overlaid and separately. **B.** Images of 700nm channel, where the six target proteins were visualized in the experiments. Red arrows show the bands that were analyzed. Black arrows show the α subunit of GSK3, that was not analyzed in this study. C = control; SZ = schizophrenia; P = pool/inter-experimental control; kDa = kDaltons.

Supplementary Figure 2.

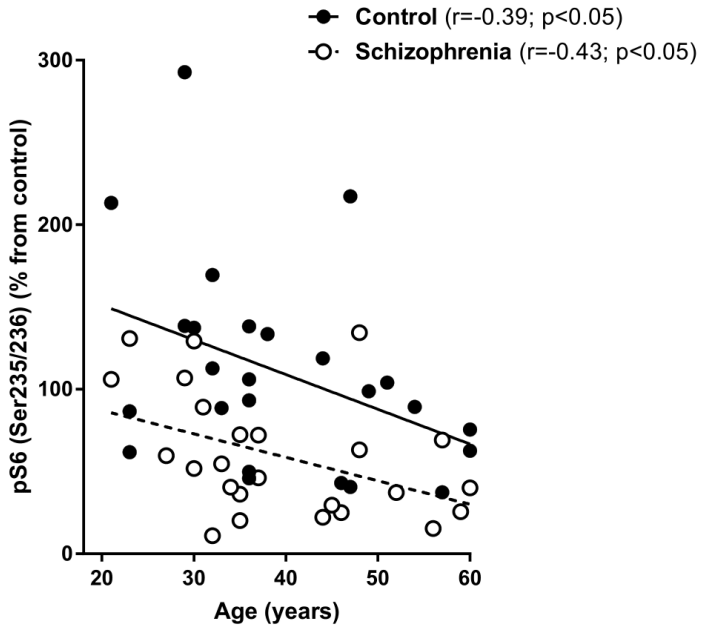


Representative images of immunoblots carried out in rat cortex in the study.

A. Image of 700 and 800nm channels visualized overlaid and separately. **B.** Images of 700nm channel, where the six target proteins were visualized in the experiments. Red arrows show the bands that were analyzed. Black arrows show the α subunit of GSK3, that was not analyzed in this study. S = saline; H = haloperidol; R = risperidone; C = clozapine; P = pool/inter-experimental control; kDa = kDaltons.

Supplementary Figure 3.

Correlations between pS6 protein levels and age at the time of death in controls (n=25) and schizophrenic subjects (n=25). Linear regressions, r and *p* values for Pearson's correlations are shown.



ARTICLE 4

Cannabis abuse differently regulates platelet 5-HT2AR and Akt in schizophrenia

Inés Ibarra-Lecue, Rocío Barrena-Barbadillo, Paula Unzueta-Larrianaga, Rebeca Díez-Alarcia, Miguel Ángel Landabaso, Begoña Mendivil, Jose Javier Meana, Luis Felipe Callado, Leyre Urigüen

ARTICLE 4

Several studies have suggested a causal relationship between long-term cannabis use and schizophrenia. Nevertheless, it seems that cannabis use may only represent a risk factor in individuals with an underlying genetic predisposition to the disease. Cannabis use disorder has been also associated with a lower age of onset of schizophrenia, and it has been suggested that patients with schizophrenia and a comorbid cannabis use disorder could represent a clinically distinct subgroup of patients with a different antipsychotic efficacy profile. However, exclusion criteria in some studies make this subpopulation understudied and reports regarding differential features among these putative patient subgroups are scarce. The aim of this study was to evaluate by western blot the protein expression and functional status of two proteins previously related with schizophrenia and cannabis psychotomimetic effects (5-HT2AR and Akt) in purified platelet homogenates of patients with a cannabis use disorder, schizophrenia, or both/dual diagnoses, compared with sex and age-matched control subjects. Patients diagnosed with a cannabis use disorder and those diagnosed with schizophrenia showed an increase in blood platelets 5-HT2AR protein expression compared to control patients mean value, while no significant changes were found in patients with a dual diagnosis. Moreover, patients with schizophrenia showed a hyperactive functional status of Akt kinase, with less total Akt protein expression, but increased phospho(Ser473)Akt, that leaded to an increased phospho(Ser473)Akt / Akt ratio compared with controls. Conversely, patients with cannabis use disorder and those with dual diagnosis failed to show substantial changes in Akt functionality. These results suggest that the groups of patients here studied present different protein expression patterns, and are in line with studies suggesting clinical divergences among them. Thus, future prospective studies in platelets of candidate proteins could be helpful for a further characterization of putative prognostic and/or predictive biomarkers in schizophrenia.

Cannabis abuse differently regulates platelet 5-HT2AR and Akt in schizophrenia

Ibarra-Lecue I.[1,3], Barrena-Barbadillo R.[2], Unzueta-Larrinaga P.[1], Díez-Alarcia R.[1,3,4], Landabaso M. A.[5], Mendivil B.[5], Meana J.J. [1,3,4], Callado L.F. [1,3,4] and Urigüen L.[1,3,4]

Departments of [1]Pharmacology and [2]Nursing, University of the Basque Country UPV/EHU, Leioa, Bizkaia, Spain. [3]Centro de Investigación Biomédica en Red de Salud Mental CIBERSAM, Spain. [4]Biocruces Bizkaia Health Research Institute, Bizkaia, Spain. [5]Osakidetza Uribe Mental Health Center, Getxo, Spain.

About one of every four subject with schizophrenia (SZ) has also a diagnosis of cannabis use disorder (CUD). Moreover, cannabis use is associated with a lower age of onset and poor prognosis of the disease. Serotonin 2A receptors (5-HT2AR) are closely involved in psychosis and, like Akt kinase they are known to be modulated by THC. Moreover, their presence in blood platelets makes them interesting proteins for the study in patients by a minimally invasive technique. The goal of the present study was to evaluate the protein expression of 5-HT2AR and the Akt functional status (total Akt / phospho(Ser473)-Akt) in platelet homogenates of subjects diagnosed with schizophrenia (SZ), cannabis use disorder (CUD), and both conditions (DUAL), compared with age- and sex-matched control (C) subjects. Results show that 5-HT2AR immunodensity is increased in platelets homogenates from CUD and SZ subjects, comparing to control values. SZ subjects also showed a decrease in total Akt immunoreactivity and an increase in phospho(Ser473)-Akt immunoreactivity, comparing with control values; resulting in a two-fold increase of phospho(Ser473)Akt/Akt ratio. Neither CUD nor DUAL subjects showed significant changes in any Akt form. Results of the present study suggest that that SZ, CUD and DUAL subjects show different protein expression patterns, and are in line with the hypothesis suggesting clinical differences among groups. Prospective studies of related proteins in platelets could be helpful for a further characterization of putative prognostic and/or predictive biomarkers in schizophrenia.

INTRODUCTION

Comorbidity rates are very high in psychiatry. In schizophrenia, up to half of the patients meet criteria for a co-occurring syndrome. Whether this is best described as comorbidity or as additional symptom dimensions of schizophrenia is uncertain, but they occur throughout all the course of the diasease and they often require specific treatment and management approaches (Buonocore et al., 2017; Siu et al., 2018). Cannabis abuse has been associated with lower age of onset of schizophrenia, more positive symptoms and higher frequency of hospitalizations (Large et al., 2011; Helle et al., 2016; Schoeler et al., 2016) compared with patients with schizophrenia (SZ) without co-morbid cannabis abuse. Nonetheless, patients with schizophrenia and psychosis are more prone to substance abuse than the general population, and 22% have a lifetime prevalence of cannabis use disorder (CUD) (Green et al., 2005). The most accepted theory is that high-potency varieties of cannabis may trigger the onset of schizophrenia in vulnerable individuals (Moore et al., 2007; Marconi et al., 2016). However, alternative explanations including self-medication and common genetic underpinnings (Dekker et al., 2009; Hartz et al., 2017; Pasman et al., 2018; Hamilton and Monaghan, 2019) are also a matter of debate.

Platelets have been extensively used as a peripheral cellular model for the analysis of molecular pathways related to neuropsychiatric disorders (Asor and Ben-Shachar, 2012; Mazereeuw et al., 2013; Dietrich-Muszalska and Wachowicz, 2016). They posses some similarities with neurons, such as serotonin (5-HT) uptake and storage mechanisms, serotonin 2A receptors (5-HT2AR), and other receptors and enzymes largely studied in relation with psychiatric disorders (Stahl, 1977; Geaney et al., 1984; de Chaffoy de Courcelles et al., 1987; Barradas and Mikhailidis, 1993). These features, along with their accessibility, turn platelets into an attractive tissue sample for the study of pathophysiological processes relevant to psychiatric disorders, including schizophrenia (Asor and Ben-Shachar, 2012).

5-HT2AR has been suggested to play a role in schizophrenia (Vollenweider et al., 1998; González-Maeso et al., 2007). It is abundantly expressed in the human brain cortex (Jakab and Goldman-Rakic, 1998), and it is also present in blood platelets, where it mediates 5-HT induced platelet aggregation (De Clerck et al., 1984). Both *postmortem* brain and platelet studies in schizophrenia patients have shown controversies regarding 5-HT2AR protein expression or density (Laruelle, 1993; Govitrapong et al., 2000; Arranz et al., 2003; González-Maeso et al., 2008; Kang et al., 2009). Most revealing results point to an increased density and functionality of this receptor in the cortex and the platelets of subjects with schizophrenia (Arora and Meltzer, 1993; Pandey et al., 1993; Govitrapong et al., 2000; Arranz et al., 2003; Muguruza et al., 2013b; García-Bea et al., 2019), while chronic antipsychotic treatment is thought to underlie cortical 5-HT2AR downregulation in these subjects (Van Oekelen et al., 2003; Roth, 2011). Interestingly, we have previously demonstrated that chronic THC increases cortical 5-HT2AR functionality in rodents (Ibarra-Lecue et al., 2018). In addition, cannabinoid receptor type 1 (CB1R) – the main target of THC in the brain – has been proposed to interact with 5-HT2AR, and it has been suggested that this 5-HT2AR/CB1 interaction may mediate some THC effects such as memory impairments (Viñals et al., 2015; Galindo et al., 2018). However, little is known about the status of this 5-HT2AR in subjects with cannabis abuse disorder.

Signaling of Akt mediates a plethora of intracellular pathways that can be activated by different extracellular stimuli including α/γ subunits of heterotrimeric G proteins coupled to GPCR (Murga et al., 1998). In neurons, the activation of Akt regulates neural survival and architecture, axonal growth or synaptic strength control (Dudek et al., 1997; Wang et al., 2003; Huang et al., 2019). Genetic studies have demonstrated an association between some polymorphisms of AKT1 gene and schizophrenia (Emamian et al., 2004b; Norton et al., 2007; Mathur et al., 2010). However, literature evaluating Akt phosphorylation status in human brain show discrepant results (Ide et al., 2006; Zhao et al., 2006; Balu et al., 2012; Hino et al., 2016; McGuire et al., 2017; Chadha and Meador-Woodruff, 2020). Moreover, an Akt polymorphism seems to moderate cannabis induced psychosis (Di Forti et al., 2012). Chronic THC is known to activate Akt in mouse brain (Ozaita et al., 2007; Ibarra-Lecue et al., 2018), and downstream effectors have been suggested to underlie THC induced cognitive impairment (Puighermanal et al., 2013) as well as modify 5-HT2AR functionality (Strachan et al., 2010).

Considering these findings, it can be proposed that cannabis use in humans may induce some effects in 5-HT2AR and Akt protein status.

Table 1.
Demographic characteristics of the subjects included in the study.

	Cannabis Use Disorder		Schizophrenia		Dual Diagnosis	
	Cases	Controls	Cases	Controls	Cases	Controls
Number of subjects	16	16	14	14	8	8
Females (%)	19	19	36	36	0	13
Age (years)	29.6±0.4	30.1±0.4	47.1±0.7	47.3±0.7	37.4±1.4	37.9±1.5

Moreover, we aimed to evaluate if these effects of cannabis occur in a different manner depending on the presence of a schizophrenia diagnosis.

MATERIALS AND METHODS

Participants

A total sample of 38 case and 38 control volunteers, aged between 18 and 64 years were used in this study. **Table 1** includes general information regarding the demographic characteristics of the groups. All the case subjects included in the study had been previously diagnosed by psychiatrists. Subjects who met inclusion criteria for schizophrenia and/or cannabis use disorder based on Structured Clinical Interview for DSM-IV or DSM-IV-TR (American Psychiatric Association, 1994, 2000) or ICD criteria (World Health Organization, 1993) were included in the study. Control subjects were recruited regarding gender and age matching criteria. Exclusion criteria for controls included any neuropsychiatric disease or any use of cannabis in the last two years prior the blood extraction. **Supplementary Tables 1-3** include further details of the diagnoses of all the cases included in the study. All the participants gave written, witnessed, informed consent for the participation in the study on the blood extraction day.

Blood collection and platelets isolation

Blood samples (~15 ml) were extracted by venipuncture in ACD solution A vacutainer® citrate blood collection tubes (Becton Dickinson & Company, Franklin Lakes, NJ, USA) by nurses in the Drug Addiction unit of the Uribe Mental Health Centre, (Getxo, Spain) belonging to the Basque Health Service; or in the Faculty of Medicine of the University of the Basque Country (UPV/EHU) (Leioa, Spain). Samples were immediately transported to the laboratory facilities at the University of the Basque Country for the extraction and purification of platelets.

The isolation of platelets was carried out by a gradient density centrifugation with Optiprep™ (60% w/v iodixanol) Density Barrier Medium (Sigma-Aldrich, St Louis, MO). Preparation of the density barrier was performed as previously described (Bagamery et al., 2005) with minor modifications. OptiPrep™ (5 vol.) was diluted with 0.85% (w/v) NaCl, 20 mM HEPES (free acid), 1 mM EDTA (Na_2O $2H_2O$) (22 vol.), to produce a 1.063 g/ml solution, pH 7.4. In 15 ml Falcon tubes (3 for each subject), 5 ml of blood was carefully layered over 5 ml of the density barrier solution and centrifuged at 350xg for 15 minutes at 20°C in a swinging-bucket rotor and a deceleration-without-brake mode, in order to avoid disturbance to the phases. The platelet-containing band, comprised by a turbid yellowish phase just below the clear brilliant yellow autologous plasma layer, and above a turbid white buffy band (leukocytes), was harvested.

Autologous plasma, leukocytes, density barrier and red blood cells pellet were discarded. In order to pellet the platelets, platelet-containing phase was subjected to a second centrifugation at 600xg, in 2 ml eppendorf tubes, for 5 minutes at 20ºC, in a deceleration-without-brake mode. Plasma was discarded and the pellet was stored at -70ºC until assays.

Preparation of total homogenates of human platelets

Platelet pellets were washed twice with a buffer containing 0.85% (w/v) NaCl, 20 mM HEPES (free acid), 1 mM EDTA ($Na_2 \cdot 2H_2O$) in order to exclude the remaining plasma in the samples. 80 µl of homogenation buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7,4; 5 µl/ml Protease Inhibitor Cocktail (Sigma), 10 µg/ml antipain, 10 µg/ml chymostatin, 1 mM Na_3VO_4, 1 mM NaF) was added and the samples were homogenized using a miniPotter (around 20 strokes). Immediately after, a buffer containing detergents (50 mM Tris-HCl, 150 mM NaCl, pH 7,4; 10% Igepal, 120 mM sodium deoxycholate, 30 mM SDS, 240 mM CHAPS) was added (1:30 ratio). Samples were kept on ice during 30 minutes, while subjected to vortex each 10 minutes. Samples were then centrifuged at 22000xg, 4ºC, 10 minutes, keeping supernatants and discarding pellets. Protein content was determined using DC method (BioRad), using bovine seroalbumin as a standard. Commercial laemmli (20% v/v) and β-mercaptoetanol (2,5% v/v) were added to 65µg protein aliquots and samples were stored at -70ºC until western blot experiment.

Western blot

Western blot experiments were performed with total homogenate fractions (S1) from platelets. Samples (30 µg) were heated (5 minutes at 95 °C), loaded onto polyacrylamide gel (10%) and submitted to SDS-PAGE. After transference on ice, nitrocellulose membranes were blocked (5% non-fat dry-milk or/and 0.5% BSA) in Tris-Buffered Saline (TBS) buffer followed by overnight incubation with primary antibodies (4 °C). Antibodies against 5-HT2AR, Akt, phospho(Ser473)-Akt, and β-actin were used (1:10.00-1:100.000 range). Incubation with fluorescent anti-IgG secondary antibodies was performed at RT (2 h) (1:7.500-1:15.000 range). Immunoreactivity was quantified using an LI-COR Odyssey® Infrared Imaging System. Immunodensity values of each target protein were normalized with both their corresponding b-actin (these values did not differ among groups, see **Supplementary Figure 1**) and an inter-experimental standard value.

Data analysis and statistical procedure

Experiments were performed under a paired design where each case and the respective matched control were always processed simultaneously. The immunoreactivity values of the target proteins were corrected by the corresponding value of β-actin; and calculated as the percentage of a standard sample loaded in every single gel to control for inter-assay variability. Data were standardized considering the mean value of the all the control samples as 100%. For every western blot data analysis, data were inspected for outliers using Grubb's test (GraphPad Software, www.graphpad.com/quickcalcs/grubbs1.cfm). Outlier values from subjects (between 0 and 1 per group) were discarded from the analysis along with its paired value. Statistical comparisons were made by two-way analysis of variance (ANOVA) to evaluate separately the effects of cannabis use disorder and schizophrenia, and the potential interaction between both in the different immunoreactivies.

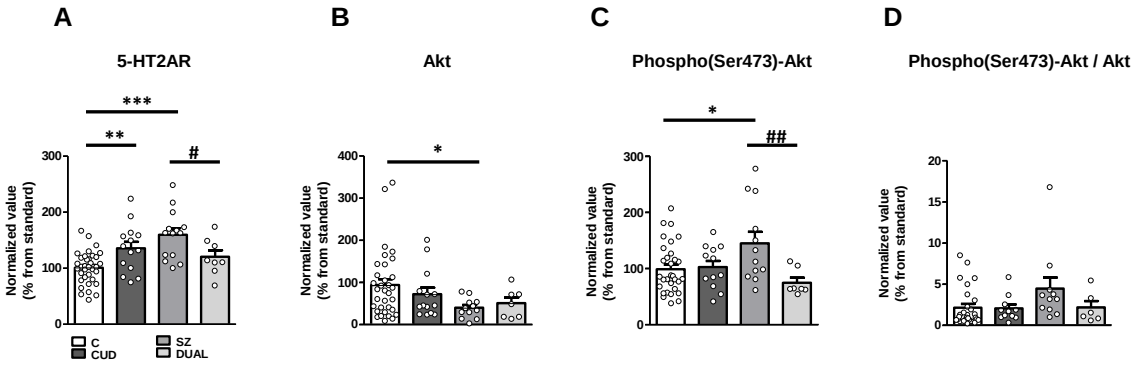


Figure 1. Cannabis use disorder affects differently to platelet 5-HT2AR and Akt protein status in patients with schizophrenia. (**A**) 5-HT2AR immunodensity in platelets homogenates of case patients with a cannabis use disorder (CUD), schizophrenia patients (SZ), patients with both diagnoses (DUAL) and control (C) values (two-way ANOVA, interaction, $F(1,68)=14.83$, $p < 0.001$; n=8-36). (**B**) Akt immunoreactivity in platelets homogenates of case patients with a CUD, SZ patients, and DUAL patients comparing to C values (two-way ANOVA, SZ effect, $F(1,62)=4.24$, $p < 0.05$; n=7-33). (**C**) Phospho(Ser473)Akt immunoreactivity in platelets homogenates of case patients with a CUD, SZ patients, DUAL patients and C values. comparing with C values (two-way ANOVA, interaction, $F(1,58)=6.85$, $p < 0.05$; n=6-28). (**D**) Phospho(Ser473)Akt/Akt ratio in platelets homogenates of case patients with a CUD, SZ patients, DUAL patients and C values (n=6-28). Bonferroni's *post hoc* comparisons: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. C. $\#p < 0.05$, $\#\#p < 0.01$ vs. SZ. Bars represent mean±SEM of individual values depicted in points.

When the analysis showed statistical significance for any effect or interaction between them, the Bonferroni´s test was used in *post hoc* analyses to evaluate the differences between groups. The matched control values of the three experimental groups were pooled together, and they did not differ in the mean values of any of the proteins evaluated (see **Supplementary Figure 2**).

Pearson's coefficient for simple correlation was calculated in order to test possible associations between protein immunoreactivities and age. The analyses were carried out with GraphPad Prism 5^{TM} software. Individual values are shown as scatterplots, and bars represent mean±SEM. The level of significance was chosen as $p < 0.05$.

RESULTS

In order to quantify the protein expression of 5-HT2AR, immunodetection with a specific antibody was performed. Western blot assays showed a single band at 55 kDa. The 5-HT2AR protein immunoreactivity in platelets homogenates showed a significant increase in subjects with CUD (+35±12% *vs.* controls, **p<0.01), as well as in those of subjects with SZ (+60±12% *vs.* controls, ***p<0.001), compared with C values. However, no significant changes were observed in platelets of subjects with both conditions (DUAL) (**Figure 1A**).

We also evaluated the Akt protein expression in both its total form and in its phosphorylated phospho(Ser473)-Akt form with specific antibodies. Platelet homogenates from SZ subjects showed a decrease in total Akt immunoreactivity (-60±7% *vs.* controls, *p<0.05) (**Figure 1B**) and an increase in phospho(Ser473)-Akt immunoreactivity (+45±21% *vs.* controls, *p<0.05) (**Figure 1C**), comparing with C values; resulting in a two-fold increase of phospho(Ser473)-Akt/Akt ratio (*p<0.05) (**Figure 1D**). However, neither CUD nor DUAL subjects showed significant changes in the

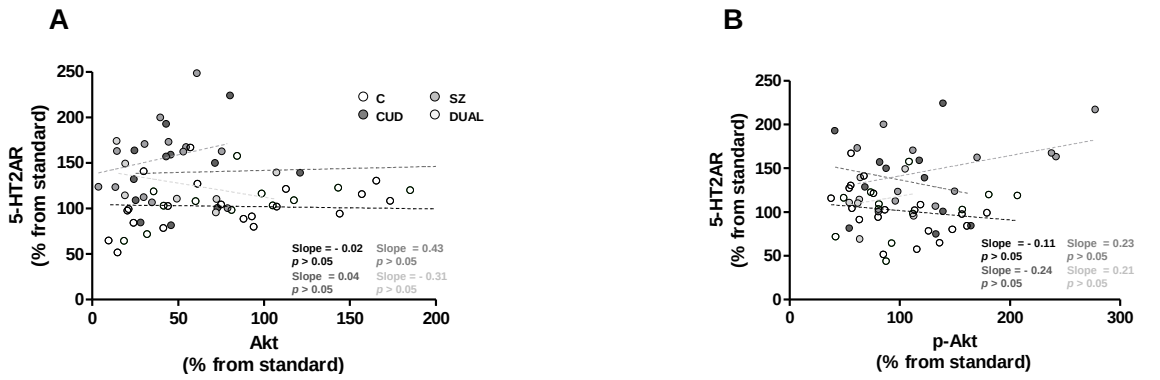


Figure 2. Neither total Akt nor phospho(Ser473)Akt immunodensities correlate with 5-HT2AR immunodensity. (**A**) Correlations between platelet 5-HT2AR and total Akt immunoreactivities among patients with a cannabis use disorder (CUD), schizophrenia patients (SZ), patients with both diagnoses (DUAL) and control (C) values. (**B**) Correlations between platelet 5-HT2AR and phospho(Ser473)Akt immunoreactivities among patients with a CUD, SZ patients, DUAL patients and C values. Individual data are depicted by points. Lines represent linear regressions of the different groups'.values.

immunoreactivity of total Akt or phospho(Ser473)-Akt.

In order to evaluate a putative association between 5-HT2AR and Akt immunoreactivity, we evaluated the correlation between the GPCR protein expression and total Akt immunoreactivity as well as between 5-HT2AR and phospho(Ser473)-Akt protein expression, although no correlations were found (**Figures 2A** and **2B**). The potential confounding factor, age (**Supplementary Figure 3**) and storage time (**Supplementary Figure 4**) were also evaluated and no correlation was found for any of the proteins studied.

DISCUSSION

Our results show for the first time, that platelets from patients with CUD have an increased 5-HT2AR protein expression. This observation is in line with the only previous study carried out in humans, which examined 5-HT2AR and cannabis abuse. In this study, enhanced number of CB1R-5-HT2AR heteromers was found in olfactory neuroepithelial cells from cannabis users (Galindo et al., 2018). Furthermore, the percentage of cells with these heteromers correlated positively with the amount of the THC metabolite THC-COOH in plasma. While this study did not evaluate 5-HT2AR protein expression, it can be understood as an indirect evidence of an increased number of 5-HT2AR resulting in more heteromers. Our study, together with this previous one, points out that THC is probably the compound responsible for the 5-HT2AR protein expression increase found in the platelets of patients with CUD. Moreover, it has been described in rats that subchronic treatment with a cannabinoid agonist, such as CP55,940, enhance 5-HT2AR protein expression in rat brain (Franklin and Carrasco, 2012). In platelets, this receptor plays a key role in coagulation processes, and the upregulation of platelets 5-HT2AR in patients with CUD shown in this study is in line with some case reports showing prothrombotic adverse effects with chronic THC intake (Mittleman et al., 2001; Marchetti et al., 2016). Whereas 5-HT2AR upregulation has not been reported in any other tissue in humans, if confirmed also in the brain,

it could be a relevant target for the treatment of CUD.

Platelets from patients with schizophrenia also showed an enhanced 5-HT2AR protein expression. This observation agrees with the vast majority of previous literature carried out in platelets (Schachter et al., 1985; Arora and Meltzer, 1993; Pandey et al., 1993; Govitrapong et al., 2000; Arranz et al., 2003) as well as some studies in *postmortem* brain of subjects with schizophrenia (González-Maeso et al., 2008; Muguruza et al., 2013b). Importantly, the major proportion of the subjects with schizophrenia included in the study (both SZ and DUAL) were treated with antipsychotics, and the pharmacological treatment is supposed to be one of the main confounding factor in studies addressing 5-HT2AR in schizophrenia (Dean, 2003). In this sense, the effect of chronic treatment with antipsychotics in the density of these receptors is controversial. Studies in platelets have suggested that either an increase (Schachter et al., 1985; Arranz et al., 2003), a decrease (Govitrapong et al., 2000) or no changes (Arora and Meltzer, 1993; Pandey et al., 1993) occurs after chronic treatment with antipsychotics of different nature.

Despite this controversy, studies addressing 5-HT2AR density in drug-free subjects with schizophrenia have demonstrated that this upregulation occurs in both platelets and brain cortex independently of antipsychotic treatment (Arranz et al., 2003; Muguruza et al., 2013b). These observations suggest that platelets may reflect similar alterations than those occurring in the brain of subjects with schizophrenia, while antipsychotics might affect platelets and brain 5-HT2AR differently. Future longitudinal studies addressing the effect of different antipsychotic treatments in platelet 5-HT2AR expression would help to ascertain whether our observations are due to schizophrenia pathology or pharmacological treatment.

An interesting finding of this study was that, while platelets from subjects with SZ and subjects with a CUD both show an increase in 5-HT2AR protein expression, subjects with both diagnoses do not show this increase. Whereas the mechanisms through which these upregulations occur in the platelets are unknown, a plausible hypothesis is that increased platelet 5-HT2AR underlying SZ occurs through a mechanism that differs from the one responsible for the increased platelet 5-HT2AR seen in CUD subjects without SZ. The first mechanisms would be sensitive upon chronic cannabis use, so as in subjects with SZ, cannabis use could be able to mitigate some alterations naturally occurring in the disease. This hypothesis is in line with some lines of evidence that shows that cannabinoid agonists exert different – sometimes opposite – effects in animal models of schizophrenia-like behavior (Levin et al., 2014) and with human evidence showing that selected compounds modulating the endocannabinoid system may be effective in schizophrenia (Leweke et al., 2016). Indeed, a recent study has also shown that regular cannabis use exerts opposite effects regarding lipid and protein composition and DNA methylation in olfactory neuroepithelial cells of patients with schizophrenia (Saladrigas-Manjón et al., 2020). It should be noted that the number of samples included in the DUAL group is substantially smaller, so as that this fact could also explain the lack of difference in this group.

Results from this study demonstrated that platelets from patients with schizophrenia show a decreased total Akt protein expression, and an increased expression of phospho(Ser473)-Akt. As far as we know, this is the first study addressing

Akt status in platelets from patients with schizophrenia. Interestingly, literature evaluating Akt status in human brain from subjects with schizophrenia have also reported decreased Akt protein expression (Zhao et al., 2006; Chadha and Meador-Woodruff, 2020), and some studies agree with our observations regarding the upregulation of phospho(Ser473)-Akt in subjects with schizophrenia (Hino et al., 2016; Callado et al., 2019). Evidence from gene-deletion and Akt inhibition studies in mice, and human genetic association studies all suggest that Akt regulates platelet function (Reséndiz et al., 2007; Jones et al., 2009; Woulfe, 2010). Moreover, the inhibition of its upstream regulator PI3K inhibits platelet activation and thrombus formation (Yi et al., 2014). Whereas the role of Akt in platelets differs from that in the brain, our findings suggest that this kinase could show a similar status in both tissues. If replicated in more studies, it would represent an interesting target in order to develop longitudinal studies addressing Akt functionality along different stages of the disease as well as putative modulations with pharmacological treatment.

Of note is that, like 5-HT2AR, phospho(Ser473)-Akt did not show any alteration when SZ patients were also diagnosed with CUD. Indeed, platelets of subjects with only CUD had similar phospho(Ser473)-Akt immunoreactivity compared with C, and the cannabis abuse seemed to normalize phospho(Ser473)-Akt expression in SZ patients. In this regard, acute administration of THC increases the phosphorylation of Akt and several downstream kinases in multiple brain areas, including cortex (Ozaita et al., 2007). Moreover, the activation of this pathway in the brain cortex has been involved in chronic THC-induced memory impairment and 5-HT2AR signaling modulation in animals (Puighermanal et al., 2009; Ibarra-Lecue et al., 2018). Additionally, a polymorphism variant of AKT1 gene is known to moderate both short-term psychotomimetic effects of cannabis use in high-risk population and the risk of being diagnosed with a psychotic disorder when having used cannabis (van Winkel and (GROUP), 2011; Di Forti et al., 2012). Furthermore, acute psychotomimetic symptoms of cannabis use are predicted by that AKT1 polymorphism in healthy young cannabis smokers (Morgan et al., 2016). All of these evidences clearly point out that Akt is an important mediator of cannabis deleterious effects. However, no human study has studied Akt protein expression with cannabis abuse. Our results suggest that cannabis abuse only modulates this kinase in patients with SZ, where it is in a hyperfunctional status. Whereas these observations do not have to correlate exactly with the ones occurring in the human brain, it is an interesting modulation that suggests that cannabis abuse may exert different effects regarding the physiological status of the organism.

These is preliminary evidence in cells where a downstream substrate of Akt pathway, ribosomal S6 kinase 2 (S6K2) interacts with 5-HT2AR and it is involved in the modulation of the receptor's functionality (Sheffler et al., 2006; Strachan et al., 2009, 2010). Indeed, neuronal ablation of phospho(Ser473)-Akt has been associated with alterations in 5-HT2AR expression and functionality (Saunders et al., 2014) and our research group demonstrated that the inhibition of Akt and downstream kinases phosphorilation avoided the modulation of 5-HT2AR functionality induced by chronic THC (Ibarra-Lecue et al., 2018). In our study neither Akt nor phospho(Ser473)-Akt immunoreactivity correlated with 5-HT2AR protein expression. Future human studies assessing 5-HT2AR functionality would be interesting in order to

ascertain whether Akt pathway is directly involved in these modulations *in vivo*.

In summary, our present work demonstrates that platelets from CUD subjects exhibit an enhanced 5-HT2AR protein expression. Interestingly, cannabis abuse seems to exert a modulatory effect in this receptor as well as in Akt phosphorilation status in the platelets from patients with SZ. These preliminary results point out that these two target and related proteins could be interesting targets for future studies assessing their role on cannabis abuse effects in relation with SZ neurobiology.

ACKNOWLEDGEMENTS

The authors would like to thank Mara Diaz for collaborating in the blood sample collection, Virginia Luque for their assistance in the experiments, as well as all the subjects for kindly participating in the study.

AUTHOR CONTRIBUTION

L.U and L.F.C conceived and designed the experiments. M.A.L and B.M recruited the cases, and R.B-B collected control subjects´ blood samples. I.I-L, R.D-A and P.U performed the experiments. L.U, I.I-L and R.D-A analyzed the data, and I.I-L and L.U wrote the manuscript. All the co-authors gave their approval to the final version of this manuscript.

BIBLIOGRAPHY

American Psychiatric Association (1994) Diagnostic and Statistical Manual of Mental Disorders, 4th Ed.

American Psychiatric Association (2000) Diagnostic and Statistical Manual of Mental Disorders, 4th Ed. DSM-IV-TR.

Arora RC, Meltzer HY (1993) Serotonin2 receptor binding in blood platelets of schizophrenic patients. Psychiatry Res 47:111–119.

Arranz B, Rosel P, Sarró S, Ramirez N, Dueñas R, Cano R, Sanchez J., San L (2003) Altered platelet serotonin 5-HT2A receptor density but not second messenger inositol trisphosphate levels in drug-free schizophrenic patients. Psychiatry Res 118:165–174.

Asor E, Ben-Shachar D (2012) Platelets: A possible glance into brain biological processes in schizophrenia. World J Psychiatry 2:124–133.

Bagamery K, Kvell K, Barnet M, Landau R, Graham J (2005) Are platelets activated after a rapid, one-step density gradient centrifugation? Evidence from flow cytometric analysis. Clin Lab Haematol 27:75–77.

Balu D., Carlson G., Talbot K, Kazi H, Hill-Smith T., Easton R, Birnbaum M., Lucki I (2012) Akt1 deficiency in schizophrenia and impairment of hippocampal plasticity and function. Hippocampus 22:230–240.

Barradas M., Mikhailidis D. (1993) The use of platelets as models for neurons: possible applications to the investigation of eating disorders. Biomed Pharmacother 47:11–18.

Buonocore M, Bosia M, Bechi M, Spangaro M, Cavedoni S, Cocchi F, Bianchi L, Guglielmino C, Mastromatteo A., Cavallaro R (2017) Targeting anxiety to improve quality of life in patients with schizophrenia. Eur Psychiatry 45:129–135.

Callado LF, Ibarra-Lecue I, Diez-Alarcia R, Meana JJ, Urigüen L (2019) Decreased ribosomal protein S6 phosphorylation in post-mortem prefrontal cortex of subjects with schizophrenia: Effects of antipsychotic treatment. Eur Neuropsychopharmacol 29:S321.

Chadha R, Meador-Woodruff JH (2020) Downregulated AKT-mTOR signaling pathway proteins in dorsolateral prefrontal cortex in Schizophrenia. Neuropsychopharmacology [Epub ahead of print].

de Chaffoy de Courcelles D, Roevens P, Wynants J, Van Belle H (1987) Serotonin-induced alterations in inositol phospholipid metabolism in human platelets. Biochim Biophys Acta 927:291–302.

De Clerck F, Xhonneux B, Leysen J, Janssen PAJ (1984) Evidence for functional 5-HT2 receptor sites on human blood platelets. Biochem Pharmacol 33:2807–2811.

Dean B (2003) The cortical serotonin2A receptor and the pathology of schizophrenia: A likely accomplice. J Neurochem 85:1–13.

Dekker N, Linszen DH, De Haan L (2009) Reasons for cannabis use and effects of cannabis use as reported by patients with psychotic disorders. Psychopathology 42:350–360.

Di Forti M, Iyegbe C, Sallis H, Kolliakou A, Falcone MA, Paparelli A, Sirianni M, La Cascia C, Stilo SA, Marques TR, Handley R, Mondelli V, Dazzan P, Pariante C, David AS, Morgan C, Powell J, Murray RM (2012) Confirmation that the AKT1 (rs2494732) genotype influences the risk of psychosis in cannabis users. Biol Psychiatry 72:811–816.

Dietrich-Muszalska A, Wachowicz B (2017) Platelet haemostatic function in psychiatric disorders: Effects of antidepressants and antipsychotic drugs. World J Biol Psychiatry 18:564–574.

Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME (1997) Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 275:661–665.

Emamian ES, Hall D, Birnbaum MJ, Karayiorgou M, Gogos JA (2004) Convergent evidence for impaired AKT1-GSK3β signaling in schizophrenia. Nat Genet 36:131–137.

Franklin JM, Carrasco GA (2012) Cannabinoid-induced enhanced interaction and protein levels of serotonin 5-HT(2A) and dopamine D(2) receptors in rat prefrontal cortex. J Psychopharmacol 26:1333–1347.

Galindo L, Moreno E, López-Armenta F, Guinart D, Cuenca-Royo A, Izquierdo-Serra M, Xicota L, Fernandez C, Menoyo E, Fernández-Fernández JM, Benítez-King G, Canela EI, Casadó V, Pérez V, de la Torre R, Robledo P (2018) Cannabis Users Show Enhanced Expression of CB1 -5HT2A Receptor Heteromers in Olfactory Neuroepithelium Cells. Mol Neurobiol 55:6347–6361.

García-Bea A, Miranda-Azpiazu P, Muguruza C, Marmolejo-Martínez-Artesero S, Díez-Alarcia R, Gabilondo AM, Callado LF, Morentin B, González-Maeso J, Meana JJ (2019) Serotonin 5-HT 2A receptor expression and functionality in postmortem frontal cortex of subjects with schizophrenia : Selective biased agonism via G α i1 -proteins. Eur Neuropsychopharmacol 29:1453–1463.

Geaney DP, Schächter M, Elliot JM, Grahame-Smith DG (1984) Characterisation of [3H]lysergic acid diethylamide binding to a 5-hydroxytryptamine receptor on human platelet membranes. Eur J Pharmacol 97:87–93.

Gonzalez-Maeso J, Ang RL, Yuen T, Chan P, Weisstaub N V, Lopez-Gimenez JF, Zhou M, Okawa Y, Callado LF, Milligan G, Gingrich JA, Filizola M, Meana JJ, Sealfon SC (2008)

Identification of a serotonin/glutamate receptor complex implicated in psychosis. Nature 452:93–97.

González-Maeso J, Weisstaub N V, Zhou M, Chan P, Ivic L, Ang R, Lira A, Bradley-Moore M, Ge Y, Zhou Q, Sealfon SC, Gingrich JA (2007) Hallucinogens Recruit Specific Cortical 5-HT2A Receptor-Mediated Signaling Pathways to Affect Behavior. Neuron 53:439–452.

Govitrapong P, Chagkutip J, Turakitwanakan W, Srikiatkhachorn A (2000) Platelet 5-HT(2A) receptors in schizophrenic patients with and without neuroleptic treatment. Psychiatry Res 96:41–50.

Green B, Young R, Kavanagh D (2005) Cannabis use and misuse prevalence among people with psychosis. Br J Psychiatry 187:306–313.

Hamilton I, Monaghan M (2019) Cannabis and Psychosis: Are We any Closer to Understanding the Relationship? Curr Psychiatry Rep 21:48.

Hartz SM, Horton AC, Oehlert M, Carey CE, Agrawal A, Bogdan R, Chen L-S, Hancock DB, Johnson EO, Pato CN, Pato MT, Rice JP, Bierut LJ (2017) Association Between Substance Use Disorder and Polygenic Liability to Schizophrenia. Biol Psychiatry 82:709–715.

Helle S, Ringen PA, Melle I, Larsen T-K, Gjestad R, Johnsen E, Lagerberg TV, Andreassen OA, Kroken RA, Joa I, ten Velden Hegelstad W, Løberg E-M (2016) Cannabis use is associated with 3 years earlier onset of schizophrenia spectrum disorder in a naturalistic, multi-site sample (N = 1119). Schizophr Res 170:217–221.

Hino M, Kunii Y, Matsumoto J, Wada A, Nagaoka A, Niwa S ichi, Takahashi H, Kakita A, Akatsu H, Hashizume Y, Yamamoto S, Yabe H (2016) Decreased VEGFR2 expression and increased phosphorylated Akt1 in the prefrontal cortex of individuals with schizophrenia. J Psychiatr Res 82:100–108.

Huang H, Miao L, Yang L, Liang F, Wang Q, Zhuang P, Sun Y, Hu Y (2019) AKT-dependent and -independent pathways mediate PTEN deletion-induced CNS axon regeneration. Cell Death Dis 10:203.

Ibarra-Lecue I, Mollinedo-Gajate I, Meana JJ, Callado LF, Diez-Alarcia R, Urigüen L (2018) Chronic cannabis promotes pro-hallucinogenic signaling of 5-HT2A receptors through Akt/mTOR pathway. Neuropsychopharmacology 43:2028–2035.

Ide M, Ohnishi T, Murayama M, Matsumoto I, Yamada K, Iwayama Y, Dedova I, Toyota T, Asada T, Takashima A, Yoshikawa T (2006) Failure to support a genetic contribution of AKT1 polymorphisms and altered AKT signaling in schizophrenia. J Neurochem 99:277–287.

Jakab RL, Goldman-Rakic PS (1998) 5-Hydroxytryptamine2A serotonin receptors in the primate cerebral cortex: Possible site of action of hallucinogenic and antipsychotic drugs in pyramidal cell apical dendrites. Proc Natl Acad Sci U S A 95:735–740.

Jones CI et al. (2009) A functional genomics approach reveals novel quantitative trait loci associated with platelet signaling pathways. Blood 114:1405–1416.

Kang K, Huang X-F, Wang Q, Deng C (2009) Decreased density of serotonin 2A receptors in the superior temporal gyrus in schizophrenia-a postmortem study. Prog Neuro-Psychopharmacology Biol Psychiatry 33:867–871.

Large M, Sharma S, Compton MT, Slade T, Nielssen O (2011) Cannabis Use and Earlier

Onset of Psychosis: a systematic meta-analysis. Arch Gen Psychiatry 68:555–561.

Laruelle M (1993) Selective Abnormalities of Prefrontal Serotonergic Receptors in Schizophrenia. A postmortem study. Arch Gen Psychiatry 50:810–818.

Levin R, Peres FF, Almeida V, Calzavara MB, Zuardi AW, Hallak JEC, Crippa JAS, Abílio VC (2014) Effects of cannabinoid drugs on the deficit of prepulse inhibition of startle in an animal model of schizophrenia: the SHR strain. Front Pharmacol 5:10.

Leweke FM, Mueller JK, Lange B, Rohleder C (2016) Therapeutic Potential of Cannabinoids in Psychosis. Biol Psychiatry 79:604–612.

Marchetti D, Spagnolo A, De Matteis V, Filograna L, De Giovanni N (2016) Coronary thrombosis and marijuana smoking: a case report and narrative review of the literature. Drug Test Anal 8:56–62.

Marconi A, Di Forti M, Lewis CM, Murray RM, Vassos E (2016) Meta-analysis of the Association Between the Level of Cannabis Use and Risk of Psychosis. Schizophr Bull 42:1262–1269.

Mathur A, Law MH, Megson IL, Shaw DJ, Wei J (2010) Genetic association of the AKT1 gene with schizophrenia in a British population. Psychiatr Genet 20:118–122.

Mazereeuw G, Herrmann N, Bennett SAL, Swardfager W, Xu H, Valenzuela N, Fai S, Lanctôt KL (2013) Platelet activating factors in depression and coronary artery disease: A potential biomarker related to inflammatory mechanisms and neurodegeneration. Neurosci Biobehav Rev 37:1611–1621.

McGuire JL, Depasquale EA, Funk AJ, O'Donnovan SM, Hasselfeld K, Marwaha S, Hammond JH, Hartounian V, Meador-Woodruff JH, Meller J, McCullumsmith RE (2017) Abnormalities of signal transduction networks in chronic schizophrenia. npj Schizophr 3:30.

Mittleman MA, Lewis RA, Maclure M, Sherwood JB, Muller JE (2001) Triggering Myocardial Infarction by Marijuana. Circulation 103:2805–2809.

Moore TH, Zammit S, Lingford-Hughes A, Barnes TR, Jones PB, Burke M, Lewis G (2007) Cannabis use and risk of psychotic or affective mental health outcomes: a systematic review. Lancet 370:319–328.

Morgan CJA, Freeman TP, Powell J, Curran H V (2016) AKT1 genotype moderates the acute psychotomimetic effects of naturalistically smoked cannabis in young cannabis smokers. Transl Psychiatry 6:e738.

Muguruza C, Moreno JL, Umali A, Callado LF, Meana JJ, González-Maeso J (2013b) Dysregulated 5-HT2A receptor binding in postmortem frontal cortex of schizophrenic subjects. Eur Neuropsychopharmacol 23:852–864.

Murga C, Laguinge L, Wetzker R, Cuadrado A, Gutkind JS (1998) Activation of Akt/Protein Kinase B by G Protein-coupled Receptors. J Biol Chem 273:19080–19085.

Norton N, Williams HJ, Dwyer S, Carroll L, Peirce T, Moskvina V, Segurado R, Nikolov I, Williams NM, Ikeda M, Iwata N, Owen MJ, O'Donovan MC (2007) Association analysis of AKT1 and schizophrenia in a UK case control sample. Schizophr Res 93:58–65.

Ozaita A, Puighermanal E, Maldonado R (2007) Regulation of PI3K/Akt/GSK-3 pathway by cannabinoids in the brain. J Neurochem

102:1105–1114.

Pandey SC, Sharma RP, Janicak PG, Marks RC, Davis JM, Pandey GN (1993) Platelet serotonin-2 receptors in schizophrenia: Effects of illness and neuroleptic treatment. Psychiatry Res 48:57–68.

Pasman JA et al. (2018) GWAS of lifetime cannabis use reveals new risk loci, genetic overlap with psychiatric traits, and a causal influence of schizophrenia. Nat Neurosci 21:1161–1170.

Puighermanal E, Busquets-Garcia A, Gomis-González M, Marsicano G, Maldonado R, Ozaita A (2013) Dissociation of the Pharmacological Effects of THC by mTOR Blockade. Neuropsychopharmacology 38:1334–1343.

Puighermanal E, Marsicano G, Busquets-Garcia A, Lutz B, Maldonado R, Ozaita A (2009) Cannabinoid modulation of hippocampal long-term memory is mediated by mTOR signaling. Nat Neurosci 12:1152–1158.

Reséndiz JC, Kroll MH, Lassila R (2007) Protease-activated receptor-induced Akt activation - Regulation and possible function. J Thromb Haemost 5:2484–2493.

Roth BL (2011) Irving Page Lecture: 5-HT2A serotonin receptor biology: Interacting proteins, kinases and paradoxical regulation. Neuropharmacology 61:348–354.

Saladrigas-Manjón S, Dučić T, Galindo L, Fernández-Avilés C, Pérez V, de la Torre R, Robledo P (2020) Effects of Cannabis Use on the Protein and Lipid Profile of Olfactory Neuroepithelium Cells from Schizophrenia Patients Studied by Synchrotron-Based FTIR Spectroscopy. Biomolecules 10:329.

Saunders C, Siuta M, Robertson SD, Davis AR, Sauer J, Matthies HJG, Gresch PJ, Airey DC, Lindsley CW, Schetz JA, Niswender KD, Veenstra-Vanderweele JM, Galli A (2014) Neuronal ablation of p-Akt at Ser473 leads to altered 5-HT1A/2A receptor function. Neurochem Int 73:113–121.

Schachter M, Geaney D, Grahame-Smith D, Cowen P, Elliott J (1985) Increased platelet membrane [3H]-LSD binding in patients on chronic neuroleptic treatment. Br J Clin Pharmacol 19:453–457.

Schoeler T, Petros N, Forti M Di, Pingault JB, Klamerus E, Foglia E, Small A, Murray R, Bhattacharyya S (2016) Association between continued cannabis use and risk of relapse in first-episode psychosis a quasi-experimental investigation within an observational study. JAMA Psychiatry 73:1173–1179.

Sheffler DJ, Kroeze WK, Garcia BG, Deutch AY, Hufeisen SJ, Leahy P, Bruning JC, Roth BL (2006) p90 ribosomal S6 kinase 2 exerts a tonic brake on G protein-coupled receptor signaling. Proc Natl Acad Sci 103:4717–4722.

Siu M, Chong CS, Lo WT (2018) Prevalence and clinicians' awareness of psychiatric comorbidities among first–episode schizophrenia. Early Interv Psychiatry 12:1128–1136.

Stahl SM (1977) The Human Platelet. A diagnostic and research tool for the study of biogenic amines in psychiatric and neurologic disorders. Arch Gen Psychiatry 34:509–516.

Strachan RT, Sciaky N, Cronan MR, Kroeze WK, Roth BL (2010) Genetic Deletion of p90 Ribosomal S6 Kinase 2 Alters Patterns of 5-Hydroxytryptamine 2A Serotonin Receptor Functional Selectivity. Mol Pharmacol 77:327–338.

Strachan RT, Sheffler DJ, Willard B, Kinter M,

Kiselar JG, Roth BL (2009) Ribosomal S6 Kinase 2 Directly Phosphorylates the 5-Hydroxytryptamine 2A (5-HT 2A) Serotonin Receptor, Thereby Modulating 5-HT 2A Signaling. J Biol Chem 284:5557–5573.

Van Oekelen D, Luyten WHML, Leysen JE (2003) 5-HT2A and 5-HT2C receptors and their atypical regulation properties. Life Sci 72:2429–2449.

van Winkel R, (GROUP) GR and O of P (2011) Family-Based Analysis of Genetic Variation Underlying Psychosis-Inducing Effects of Cannabis: Sibling Analysis and Proband Follow-up. Arch Gen Psychiatry 68:148–157.

Viñals X, Moreno E, Lanfumey L, Cordomí A, Pastor A, de La Torre R, Gasperini P, Navarro G, Howell LA, Pardo L, Lluís C, Canela EI, McCormick PJ, Maldonado R, Robledo P (2015) Cognitive Impairment Induced by Delta9-tetrahydrocannabinol Occurs through Heteromers between Cannabinoid CB1 and Serotonin 5-HT2A Receptors Nestler EJ, ed. PLOS Biol 13:e1002194.

Vollenweider FX, Vollenweider-Scherpenhuyzen MFI, Bäbler A, Vogel H, Hell D (1998) Psilocybin induces schizophrenia-like psychosis in humans via a serotonin-2 agonist action. Neuroreport 9:3897–3902.

Wang Q, Liu L, Pei L, Ju W, Ahmadian G, Lu J, Wang Y, Liu F, Wang YT (2003) Control of Synaptic Strength, a Novel Function of Akt. Neuron 38:915–928.

World Health Organization (1993) The ICD-10 classification of mental and behavioural disorders: Diagnostic criteria for research.

Woulfe DS (2010) Akt signaling in platelets and thrombosis. Expert Rev Hematol 3:81–91.

Yi W, Li Q, Shen J, Ren L, Liu X, Wang Q, He S, Wu Q, Hu H, Mao X, Zhu L (2014) Modulation of Platelet Activation and Thrombus Formation Using a Pan-PI3K Inhibitor S14161 Li Z, ed. PLoS One 9:e102394.

Zhao Z, Ksiezak-Reding H, Riggio S, Haroutunian V, Pasinetti GM (2006) Insulin receptor deficits in schizophrenia and in cellular and animal models of insulin receptor dysfunction. Schizophr Res 84:1–14.

SUPPLEMENTARY MATERIAL

Cannabis use differently regulates 5-HT2AR and Akt in schizophrenia

Ibarra-Lecue I.[1,3], Barrena-Barbadillo R.[2], Unzueta-Larrinaga P.[1], Díez-Alarcia R.[1,3,4], Landabaso M.A.[5], Mendibil B.[5], Meana J.J.[1,3,4], Callado L.F.[1,3,4] and Urigüen L.[1,3,4]

[1]Departments of Pharmacology and [2]Nursing, University of the Basque Country UPV/EHU, Leioa, Bizkaia, Spain

[3]Centro de Investigación Biomédica en Red de Salud Mental CIBERSAM, Spain.

[4]Biocruces Health Research Institute, Bizkaia, Spain.

[5]Osakidetza Uribe Mental Health Center, Getxo, Spain.

Content:

Supplementary Table 1.

Demographic characteristics and pharmacological prescription of subjects with cannabis use disorder and matched control subjects.

	Patients with Cannabis Use Disorder (CUD)					Controls (C)	
Pair number	**Gender**	**Age (years)**	**Diagnosis of interest**	**AP prescribed**	**Other treatments prescribed**	**Gender**	**Age (years)**
1	Male	19	CUD	-	Metilphenidate	Male	18
2	Male	24	CUD	-	Atomoxetine	Male	23
3	Male	23	CUD	-	Agomelatine	Male	23
4	Male	40	CUD	Pimozide	-	Male	38
5	Male	34	CUD	-	Escitalopram	Male	35
6	Male	22	CUD	-	Paroxetine	Male	22
7	Male	24	CUD	-	Agomelatine	Male	27
8	Male	36	CUD	-	Agomelatine	Male	36
9	Female	24	CUD	-	-	Female	24
10	Female	28	CUD	-	Oxcarbazepin, escitalopram	Female	29
11	Male	37	CUD	-	Duloxetin	Male	39
12	Male	41	CUD	-	Pregabaline	Male	42
13	Male	32	CUD	-	Disulfiram	Male	35
14	Male	30	CUD	-	Atomoxetine	Male	30
15	Male	33	CUD	-	Atomoxetine	Male	35
16	Female	27	CUD	-	Agomelatine, pregabalin	Female	25
Mean		29,6					30,1
SEM		0,4					0,4

AP=antipsychotics

Supplementary Table 2.

Demographic characteristics and pharmacological prescription of subjects with schizophrenia and matched control subjects.

	Patients with Schizophrenia (SZ)					Controls (C)	
Pair number	**Gender**	**Age (years)**	**Diagnosis of interest**	**AP prescribed**	**Other treatments prescribed**	**Gender**	**Age (years)**
1	Female	55	SZ	Olanzapine	-	Female	55
2	Female	44	SZ	Aripiprazol	Bupropion	Female	49
3	Male	36	SZ	Aripiprazol	-	Male	36
4	Female	29	SZ	Clozapine	-	Female	29
5	Male	41	SZ	Clozapine, aripiprazol	Lamotrigine	Male	42
6	Male	43	SZ	Risperidone	-	Male	41
7	Female	42	SZ	Olanzapine, aripiprazol	Lamotrigine, escitalopram	Female	42
8	Male	49	SZ	Paliperidone	Vortioxetine	Male	50
9	Male	63	SZ	Aripiprazol	-	Male	61
10	Female	49	SZ	Risperidone	-	Female	49
11	Male	45	SZ	Olanzapine, aripiprazol	-	Male	45
12	Male	52	SZ	Clozapine	Lithium, valproic, escitalopram	Male	50
13	Male	64	SZ	Olanzapine	Fluoxetine	Male	64
14	Male	48	SZ	Zuclopenthixol, quetiapine, asenapine	-	Male	49
Mean		47,1					47,3
SEM		0,7					0,7

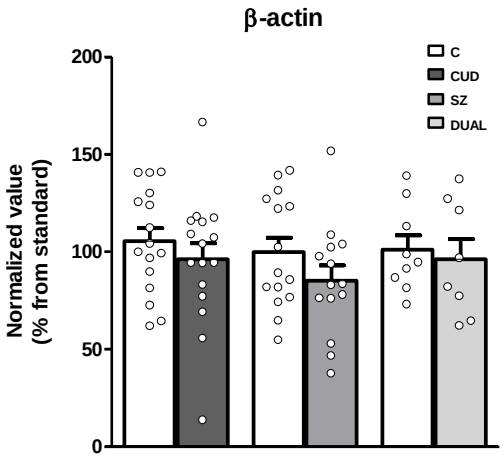
Supplementary Table 3.

Demographic characteristics and pharmacological prescription of subjects with cannabis use disorder and schizophrenia, and matched control subjects.

	Patients with Dual Diagnosis (DUAL)					Controls (C)	
Pair number	**Gender**	**Age (years)**	**Diagnosis of interest**	**AP prescribed**	**Other treatments prescribed**	**Gender**	**Age (years)**
1	Male	56	SZ, CUD	Aripiprazol	-	Male	57
2	Male	22	SZ, CUD	Quetiapine	-	Male	22
3	Male	31	SZ, CUD	Olanzapine, aripiprazol	Lithium	Male	36
4	Female	32	SZ, CUD	Risperidone	-	Male	32
5	Male	50	SZ, CUD	Olanzapine, quetiapine	Disulfiram, valproic	Male	50
6	Male	30	SZ, CUD	Aripiprazol, olanzapine	-	Male	28
7	Male	35	SZ, CUD	Clozapine, aripiprazol	-	Male	34
8	Male	43	SZ, CUD	Aripiprazol	-	Male	44
Mean		37,4					37,9
SEM		1,4					1,5

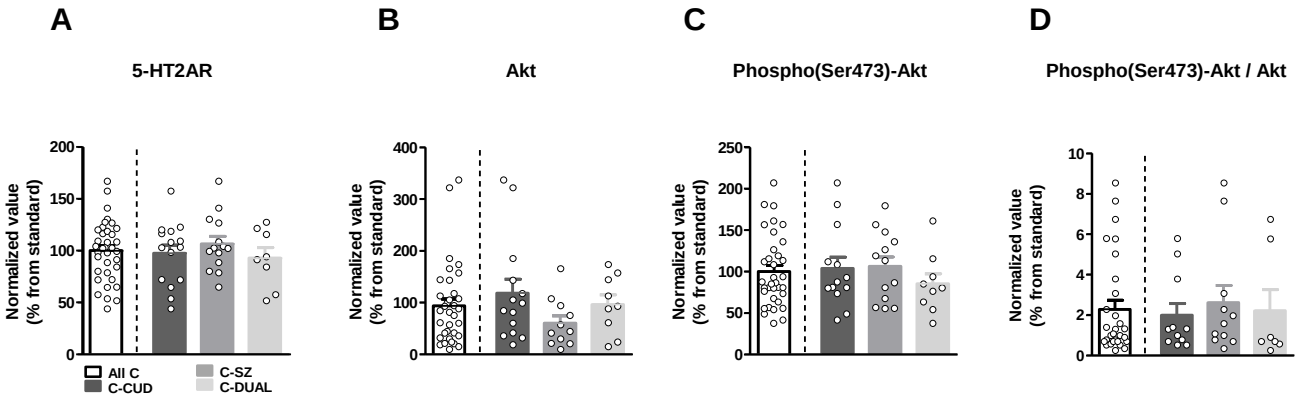
Supplementary Figure 1.

β-actin immunoreactivity in platelets homogenates of subjects with cannabis use disorder (CUD), subjects with schizophrenia (SZ), subjects with both diagnoses (DUAL), and their matched control subjects (C).



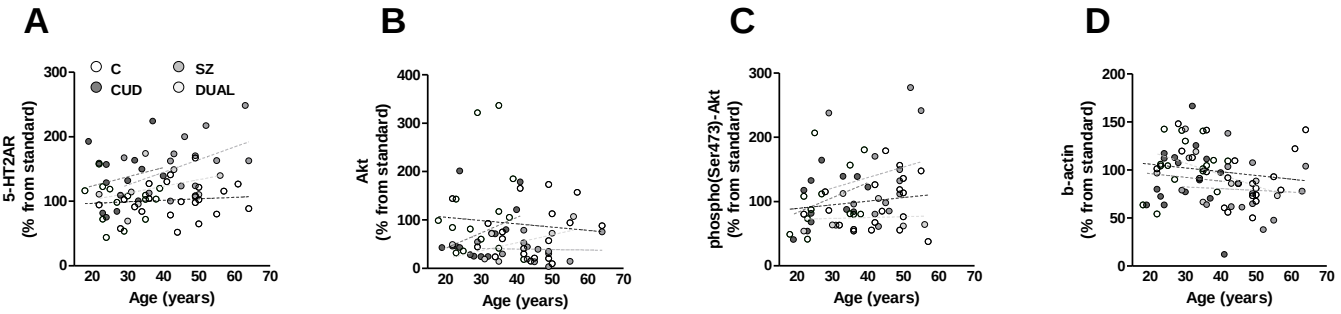
Supplementary Figure 2.

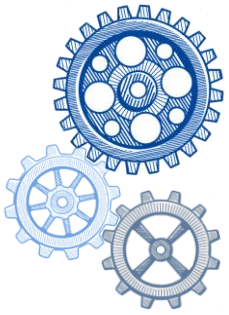
Immunoreactivity of (**A**) 5-HT2AR, (**B**) total Akt, (**C**) phospho(Ser473)Akt, and (**D**) phospho(Ser473)Akt/Akt ratio in platelets homogenates of matched conrtrol subjects of each group separated (C-CUD, C,SZ, C-DUAL) and pooled (All C).



Supplementary Figure 3.

Correlations between (**A**) 5-HT2AR, (**B**) total Akt, (**C**) phospho(Ser473)Akt and (**D**) β-actin immunoreactivity in platelets homogenates of subjects with cannabis use disorder (CUD), subjects with schizophrenia (SZ), subjects with both diagnoses (DUAL), and their matched conrtrol subjects (C).





GENERAL DISCUSSION

The following section of this Doctoral Thesis provides a general discussion of the findings of this work.

Differences in Gα protein signaling pattern induced by cannabinoid agonists

The results arisen from the characterization of the Gα protein subunits activation with three different cannabinoid agonists shown in the present Doctoral Thesis (**Results - Article 1**) raise some valuable concerns involving both endocannabinoid system and the impact of cannabis use on CNS. Most significant findings of this study include (1) WIN55212-2, ACEA and THC activate not only inhibitory Gαi/o protein subtypes, but also other Gα protein subtypes, such as Gαz, Gαq/11 and Gα12/13 in mouse cortex, (2) WIN55212-2 shows a higher activation in all the Gα protein subtypes studied comparing with THC and ACEA in this system and, (3) some Gα protein subtypes activated by cannabinoid agonists in mouse cortex seem to be mediated by CB2R.

Cannabinoid receptors are broadly expressed in the organism, and they are primarily responsible for the cannabis effects in both humans and rodents. Substantial scientific evidence suggests that these ligands trigger similar, but not identical behavioral and molecular responses. They are known to have different affinities and potencies for cannabinoid receptors, which are class A GPCR that once activated, trigger Gα subunit activation followed by different intracellular responses. Since the discovery of CB1R and CB2R, the development of synthetic ligands has allowed the study of the involvement of the endocannabinoid system in many physiological functions such as memory and learning, reward processing, appetite, energy metabolism, gastrointestinal motility or immune modulation. These synthetic cannabinoid ligands have different affinities for cannabinoid receptors and can activate them with different potencies. These two features, the affinity and the potency, have been extensively used for explaining the observed differences in the effects exerted by these compounds in animals and humans (D'Amico et al., 2004; Cannizzaro et al., 2006).

GPCRs are complex systems that can activate several Gα protein subtypes, triggering distinct intracellular signaling cascades and leading to distinct physiological effects. These effects may depend on both the ligand used and the GPCR conformation(s) that it stabilizes. Additionally, the receptor location, oligomerization processes and pathological conditions affect GPCR signaling. This idea, known as functional selectivity, is recent for cannabinoid receptors (Bosier et al., 2010; Busquets-Garcia et al., 2018), and was firstly posited due to observations in other GPCR, such as muscarinic receptors (Kenakin, 1988).

The characterization study of the Gα protein subunits activation with three different cannabinoid agonists (**Results - Article 1**) demonstrates that cannabinoid agonists show

functional selectivity in native mouse brain tissue, and provides further evidence supporting the idea that cannabinoid ligands can trigger differential intracellular effects in CNS that may be relevant in the context of pathological conditions and therapeutics.

Existing literature aiming to study the Gα protein subtypes activated by CB1R and CB2R in the brain are often based on techniques that usually apply to cells selectively expressing cannabinoid receptors, and measure intracellular cAMP or Ca^{2+}, ERK, Akt and MAPK phosphorylation (Glass and Felder, 1997; Glass and Northup, 1999). These technical approaches are based on indirect and likely unspecific measures of different Gα protein activation. Methodology applied in **Article 1** allows to directly measuring the activity of each Gα protein subtype after the receptor activation. This method avoids the uncertainty regarding the Gα protein subtype responsible for general responses such as, in the case of cannabinoid agonists, the widely reported decrease in cAMP.

Regarding the activation of Gα protein subtypes different from Gαi/o exerted by WIN55212-2, ACEA and THC in mouse cortex, only two studies have been carried out under comparable conditions. One study was carried out in rat brain (Prather et al., 2000) and another one in human brain cortex (Erdozain et al., 2012). In the study in rat brain, it was described that WIN55,212-2 activates 6 subtypes of Gα proteins, all belonging to the Gαi/o family. There is evidence on different cell lines showing that cannabinoid agonists may also exert Gαs, Gαq/11 or Gα12/13 mediated effects under certain conditions (McIntosh et al., 2009; Dalton et al., 2013; Laprairie et al., 2014; Roland et al., 2014; Presley et al., 2016). To our knowledge, this is the first study in which activation of other Gα protein subtypes apart from the classic Gαi/o proteins is described in native mouse brain tissue. In this regard, it is important to note that, in human brain cortex, WIN55,212-2 activates only inhibitory Gαi/o protein subtypes, and not Gαq/11 or Gαz (Erdozain et al., 2012). Whereas it seems clear that this receptor displays Gα protein coupling promiscuity, we have to be aware of important inter-species differences, most of which are not elucidated yet.

As stated before, WIN55212-2 showed a higher activation in all the Gα protein subtypes studied comparing with THC and ACEA in mouse cortex. These findings are in line with previous [^{35}S]GTPγS binding studies in rat brain tissue showing that WIN55,212-2 is a full-agonist, whereas THC is a weak partial agonist (Breivogel et al., 1998). In relation to this, extensive literature shows that WIN55,212-2 and other synthetic cannabinoids are more potent than cannabis in several aspects, such as reinforcing effects and abuse liability in animal models (Zanda and Fattore, 2018). These synthetic cannabinoids are also known to exert more psychotomimetic effects and display a higher potential for abuse in humans (Cohen and Weinstein, 2018). This pharmacological characterization in rodents' cortex tissue contributes to understand the differences between natural and synthetic cannabinoids.

Perhaps one of the most surprising finding of this study was the CB2R involvement in some of the Gα protein subtype activation exerted with the three cannabinoids in mouse brain cortex. Thus, Gαo, Gαz and Gα12/13 subunits activation by cannabinoids seems to be mediated by CB2R in this tissue. Of these three Gα protein subtypes, Gαo activation occurs also in human cortex tissue with the full-agonist WIN55,212-2. In contrast, Gαz is not activated in human cortex, while no data exists for Gα12/13 (Erdozain et al., 2012). CB2R expression seems to be residual and restricted to glial cells in the human brain (Golech et al., 2004; Núñez et al., 2004). However, upregulation of this receptor may occur under certain pathological conditions involving neuroinflammation, where its functional role require further study (Benito et al., 2008).

The endocannabinoid system is involved in the modulation of neurotransmitter systems, and there is an increased interest in the study of cannabis effects in relation to several disorders. Animal studies are valuable tools for providing hypothesis for mechanisms of action of cannabis and cannabinoid ligands, as they can be manipulated through several techniques to address specific hypothesis, some of which are difficult to evaluate in human brain nowadays. However, it is important to be aware of some limitations, such as the previously mentioned differences in the signaling of cannabinoid receptors in humans compared to that of rodents.

Apart from the inter-species differences, of special interest is the different patterns of coupling that WIN55,212-2 and THC exert in mouse brain cortex. An enormous amount of literature showing conclusions over cannabis effects are based on findings carried out with WIN55,212-2 (Trezza and Vanderschuren, 2008; Dong et al., 2019). Whereas WIN55,212-2 is a valuable drug for the pharmacological characterization of cannabinoid receptors, its effects may be far from comparable to the ones exerted by the THC present in cannabis. Thus, conclusions regarding cannabis effects based on this drug should be discouraged.

This study in native animal cortical tissue provides important findings about the Gα protein coupling of cannabinoid receptors, demonstrating a functional selectivity that may be involved in the different physiological effects of cannabinoid ligands. Results of the present study may help to dissect the specific signaling pathways involved in the different pharmacological actions of cannabinoids that could lead to the design of biased cannabinoid drugs with therapeutic effects and a reduced range of adverse psychotomimetic effects.

Effects of THC and other cannabinoid agonists on cortical and peripheral 5-HT2AR

The main aim of this Doctoral Thesis was the study of the molecular mechanisms underlying the relationship that exists between cannabis abuse in adolescence and the increased risk of developing schizophrenia. The studies carried out in mice chronically treated with THC

(**Results - Article 2**) and in subjects with a cannabis use disorder (**Results - Article 4**) converged in a finding that strengthens the hypothesis of this Doctoral Thesis. The finding is the modulation of 5-HT2AR associated with chronic exposure to THC. The relevance of this finding lies mainly on the involvement of 5-HT2AR in both schizophrenia and the pharmacological treatment of the disease (Dean, 2003).

The increased 5-HT2AR expression in platelets of patients with schizophrenia without cannabis use reported in our study is in line with the vast majority of previous literature (Schachter et al., 1985; Arora and Meltzer, 1993; Pandey et al., 1993; Govitrapong et al., 2000; Arranz et al., 2003), thus reinforcing the reliability of our results.

Literature evaluating long-term cannabis effect on either central or peripheral 5-HT2AR in humans is scarce. There are, however, some findings in animals suggesting that oleamide, a compound that interferes with AEA hydrolysis and seems to act as a CB1R agonist (Leggett et al., 2004), can modulate the signal transduction of several 5-HT receptor subtypes (Thomas et al., 1997; Boger et al., 1998) and enhance 5-HT affinity for cortical 5-HT2AR in rats (Cheer et al., 1999). Moreover, cannabinoid agonists, such as THC, WIN55,212-2 or CP55,940, strongly inhibit 5-HT2AR mediated behavioral effects, such as head-twitch and ear-scratch responses (Darmani, 2001). Likewise, both AEA (Egashira et al., 2004) and blockade of endocannabinoid reuptake seems to inhibit central 5-HT2AR mediated responses, whereas CB1R blockade enhances this effect (Gorzalka et al., 2005).

On the other hand, 5-HT2AR activation has been shown to trigger 2-AG release in cells and rat cerebellar neurons (Best and Regehr, 2008). This 2-AG release and its binding to CB1R has been proposed to exert the previously mentioned negative regulatory effect of 5-HT2AR activity (Parrish and Nichols, 2006).

However, experiments with rodents have shown that the lack of CB1R decreases 5-HT2AR mediated head twitches (Mato et al., 2007), whereas the lack of 5-HT2AR decreases THC induced amnesic effects (Viñals et al., 2015). These two observations suggest that both GPCR could modulate the others' function by direct interactions between them. Indeed, a functional CB1R-5-HT2AR heteromer has been recently proposed to appear in mouse brain (Viñals et al., 2015).

Independently of the mechanisms underlying these phenomena, all these observations are in line with ours, in which cannabinoids, mainly the ones acting on CB1R, are able to exert a modulation of 5-HT2AR expression or functionality. We have demonstrated that long-term THC treatment in mice or regular cannabis consumption in humans modulates this receptor in the brain cortex and also in platelets. Interestingly, the study carried out in cortical tissue of mice after chronic THC treatment demonstrate that the increase in 5-HT2AR functionality

is selective to the non-canonical signaling towards inhibitory Gαi/o protein subtypes, a observation that also applies to *postmortem* cortex of subjects with schizophrenia (García-Bea et al., 2019).

A possible explanation for these observations could be that chronic THC treatment modulates CB1R-5-HT2AR heteromers in the mouse cortex, directly leading to the 5-HT2AR signaling modulation. Further experiments would be needed in order to test this hypothesis.

The study carried out in platelets of subjects with a cannabis use disorder was restricted to protein expression. Future studies addressing 5-HT2AR functionality would ascertain whether the enhancement of protein expression that was observed translates into a higher functionality, as already demonstrated in mice after chronic THC treatment and in the brain of subjects with schizophrenia (García-Bea et al., 2019). Likewise, similar findings have been shown in blood platelets of patients with schizophrenia (Pandey et al., 1993; Arranz et al., 2003). In this regard, 5-HT2AR binding studies seem to show the same characteristics as in the brain cortex (Leysen et al., 1983) and its functionality can be measured in this tissue (De Clerck et al., 1984), so as it is thought to be a valid proxy to study 5-HT system in peripheral tissue. Studies have consistently shown an apparent increase in 5-HT2AR in platelets from non-treated patients with schizophrenia. Besides, the effect of chronic treatment with antipsychotics in the density of these receptors is more controversial, and have reported an increase, a decrease and even no changes after antipsychotic treatment (Arora and Meltzer, 1993; Pandey et al., 1993; Govitrapong et al., 2000; Arranz et al., 2003). Despite this controversy, our study in patients with cannabis use disorder is not confounded by this factor, as they have not been prescribed with any antipsychotic. Thus, it can be hypothesized that the enhanced receptor protein expression is likely due to cannabis abuse.

To our knowledge, this is the first report of an increased density of 5-HT2AR in patients with cannabis use disorder. Our observations are in line with previous animal studies, in which subchronic administration of potent synthetic cannabinoids enhance 5-HT2AR mediated wet-dog shakes (Hill et al., 2006) and prolactin and corticosterone responses, as well as 5-HT2AR protein expression in paraventricular nucleus (Franklin et al., 2013) and frontal cortex of rats (Franklin and Carrasco, 2012).

As previously mentioned, animal and cell studies suggest that both endocannabinoids and THC, at least acutely, exert a regulatory effect on 5-HT2AR (Darmani, 2001; Egashira et al., 2004; Gorzalka et al., 2005). Thus, chronic cannabis could be leading to a compensatory modulation of 5-HT2AR translation in bone marrow megakaryocytes, thus increasing 5-HT2AR protein expression in the platelets. Interestingly, platelets are now known to contain both a pool of megakaryocyte-derived mRNAs and miRNA, and the complete machinery for

de novo protein synthesis (Nassa et al., 2018). In this context, dynamic modifications of protein expression in mature platelets cannot be ruled out. An important aspect to take into account in the platelets study is that cannabis used by these subjects do not only contains THC, but also CBD and many other cannabinoids, most of which remain understudied. Further studies would be necessary to clarify which is the compound responsible for this 5-HT2AR modulation.

Studies in this area have suggested that both 2-AG and 5-HT can reciprocally reinforce their binding on platelet surface (Maccarrone et al., 2003). It was suggested that 5-HT-induced platelet activation was partly due to the stimulation of 2-AG release and its binding to a cannabinoid-type receptor (Maccarrone et al., 2001), thus interacting with other platelet activators and enhancing the increase in IP3 and the decrease in cAMP within platelets (Maccarrone et al., 2003). There is evidence that platelets express CB1R and CB2R (Deusch et al., 2004; Catani et al., 2010) as well as GPR55 (Baldassarri et al., 2008), although studies evaluating their implication in 2-AG effects show conflicting results (Baldassarri et al., 2008; Kargl et al., 2013). 2-AG alone has also been reported to induce platelet formation in human megakaryoblasts (Gasperi et al., 2014). Although these effects were proposed to be mediated by cannabinoid receptors, it was clarified that in the case of 2-AG, it involves its degradation by MAGL, leading to the formation of AA that triggers the mentioned effects (Brantl et al., 2014).

Additionally, high doses of both THC and AEA are also able to induce platelet activation processes (Maccarrone et al., 1999; Deusch et al., 2004), leading to shape change and aggregation (Braud et al., 2000).

Both endocannabinoids and THC are able to activate CB1R and CB2R, whereas CBD is a negative allosteric modulator of CB1R (Laprairie et al., 2015) and act as an antagonist of GPR55 (Ryberg et al., 2009). Taken all the observations mentioned above, it could be hypothesized that chronic exposure to cannabis could be either downregulating these receptors in the platelets, or modulating endocannabinoid levels in plasma. If the first hypothesis was true, a subsequent upregulation of 5-HT2AR protein expression could be a mechanism to counterbalance the loss of cannabinoid-sensitive receptors, in order to preserve normal 5-HT effectiveness on platelet aggregation. If the second hypothesis was true, then less plasma 2-AG levels could be occurring as a result of chronic cannabis consumption. This would lead to decreased AA levels, and the upregulation of 5-HT2AR protein expression would be again, a mechanism to counterbalance the deficiency of this procoagulant compound. In line with this hypothesis, a human study shows that acute THC injection modulates AEA and 2-AG plasma levels (Thieme et al., 2014). Further studies

regarding plasma endocannabinoid levels after chronic cannabis use would be useful in order to understand the mechanisms underlying 5-HT2AR expression modulation.

Irrespective of how this occurs, the upregulation of platelets 5-HT2AR in patients with cannabis use disorder shown in this Doctoral Thesis is in line with the only previous study which examined 5-HT2AR in regular cannabis users. In this study, an enhanced number of CB1R-5-HT2AR heteromers was found in olfactory neuroepithelial cells from these subjects (Galindo et al., 2018). Whereas 5-HT2AR upregulation has not been reported in any other tissue in humans, if confirmed also in the brain, it could be a relevant target for the treatment of cannabis use disorder.

Another interesting finding of this Doctoral Thesis is that, while platelets from subjects with schizophrenia and subjects with a cannabis use disorder both show an increase in 5-HT2AR protein expression, subjects with both diagnoses do not show this increase. As previously mentioned, mechanisms through which increased 5-HT2AR protein expression occurs in the platelets of subjects with schizophrenia are unknown. Regular cannabis use also seems to upregulate this receptor. However, both phenomena do not exert a cumulative effect. A plausible explanation for this, is that increased platelet 5-HT2AR underlying schizophrenia pathology occurs through a mechanism that differs from the one responsible for the increased platelet 5-HT2AR seen in regular cannabis use in subjects without the disease. The first mechanisms would be sensitive upon chronic cannabis use, so as in subjects with schizophrenia, cannabis use could be able to mitigate some alterations naturally occurring in the disease. This hypothesis is in line with some lines of evidence that shows that cannabinoid agonists exert different – sometimes opposite – effects in animal models of schizophrenia-like behavior (Levin et al., 2014) and with human evidence showing that selected compounds modulating the endocannabinoid system may be effective in schizophrenia (Leweke et al., 2016).

Akt/mTOR pathway alterations in schizophrenia and its implication in chronic THC effects

The study carried out in *postmortem* brain of patients with schizophrenia (**Results – Article 3**) led us to conclude that (1) Akt kinase is hyperactive in PFC of subjects with schizophrenia, while (2) ribosomal protein S6 is hypofunctional in the same area. Chronic antipsychotic treatment does not seem to produce these effects in rat cortex, suggesting that hypofunctional S6 could play a role in the pathogenesis of the disease. Moreover, hyperactive Akt is also found in platelets from subjects with schizophrenia (**Results – Article 4**), thus pointing to a similar modulation in both tissues in schizophrenia disease.

Regarding finding (1) the hyperactive status of Akt in PFC of subjects with schizophrenia, Akt was firstly reported to be altered in schizophrenia in 2004 (Emamian et al., 2004a).

Nowadays, genetic studies have demonstrated an association between some polymorphisms of AKT1 gene and schizophrenia in several populations (Emamian et al., 2004a; Norton et al., 2007; Mathur et al., 2010). However, literature evaluating Akt phosphorylation status in human brain is limited and show discrepant results. Total Akt1 has been showed to be decreased (Emamian et al., 2004a) and increased (Hino et al., 2016) in cortical tissue of patients with schizophrenia, whereas other studies have failed to find any change in this protein expression (Ide et al., 2006). Studies evaluating total Akt have shown decreases (Zhao et al., 2006; Chadha and Meador-Woodruff, 2020) or no changes (Ide et al., 2006; Balu et al., 2012; McGuire et al., 2017), and the same discrepancy is found regarding phospho(Ser473)-Akt1 and total phospho(Ser473)-Akt (Ide et al., 2006; Zhao et al., 2006; Balu et al., 2012; Hino et al., 2016; McGuire et al., 2017; Chadha and Meador-Woodruff, 2020). The only study that has evaluated the three isoforms of Akt – Akt1, Akt2 and Akt3 – in the PFC of patients with schizophrenia found a mRNA decrease exclusively for Akt1, without changes in Akt2 and Akt3 isoforms (Thiselton et al., 2008). In the present work, total Akt protein expression was found to be unaltered in prefrontal cortical tissue of subjects with schizophrenia. Although the individual role of the two most highly expressed isoforms in the brain, Akt1 and Akt3, are still poorly understood (Lee et al., 2011), changes in individual isoforms should not be discarded.

While total Akt protein expression was not altered in our study in PFC, a significant increase was found in phospho(Ser473)-Akt/Akt ratio in subjects with schizophrenia. A similar finding was reported in a recent study (Hino et al., 2016), where increased phospho(Ser473)-Akt1 was found in brain cortical tissue of subjects with schizophrenia. Despite the results of the Akt ratio, the increased phospho(Ser473)-Akt protein expression was not statistically significant in our study. Similarly as with the evaluation of Akt protein expression, we evaluated total phospho(Ser473)-Akt, so it could be hypothesized that if an exclusive increased expression of the phosphorylated form of Akt1 occurs in our experimental group, it could be masked by the expression of other isoforms in our measure. Future studies addressing protein expression of each isoform separately would be interesting in order to clarify these discrepancies.

Another finding of this study was that chronic treatment with haloperidol decreases both phospho(Ser473)-Akt and phospho(Ser473)-Akt/Akt ratio in rat cortex. A decreased phosphorylation of this kinase was found in the major proportion of the studies, so it should be noted that this decrease could be occurring as a result of the treatment, rather than naturally occurring in schizophrenia.

Moreover, the same increased phospho(Ser473)-Akt/Akt ratio was also found in platelets from subjects with schizophrenia. Evidence from gene-deletion studies in mice, incubation

with Akt inhibitors and human genetic association studies suggest that Akt regulates platelet function (Reséndiz et al., 2007; Jones et al., 2009; Woulfe, 2010), and inhibition of its upstream regulator PI3K inhibits platelet activation and thrombus formation (Yi et al., 2014). Whereas the role of Akt in platelets may differ from that in the brain, our findings are in line with studies suggesting that adenosine diphosphate (ADP)-induced platelet aggregation is increased in schizophrenia subjects (Dietrich-Muszalska and Olas, 2009).

To our knowledge, this is the first study reporting a ribosomal protein S6 hypofunction in *postmortem* brain tissue of subjects with schizophrenia. This downstream effector of mTORC1 is regulated by several environmental factors and extracellular stimuli, and in the CNS it seems to play a key role in the regulation of cognitive processes, such as learning and memory (Meyuhas, 2015; Pirbhoy et al., 2016, 2017). Although the protein expression of this kinase has not been studied in schizophrenia before, studies in animals show that mTOR pathway regulates diverse biological processes important for cell growth, ribosome biogenesis and protein translation (Ruvinsky and Meyuhas, 2006). In the CNS, mTOR pathway plays a key role in axon growth (Park et al., 2008), and mTOR-dependent protein synthesis is also involved in the regulation of dendritic arborization (Jaworski and Sheng, 2006). In line with our results, a general deregulation of protein synthesis in olfactory neurosphere derived cells from subjects with schizophrenia has been demonstrated, involving not only ribosomal protein S6, but many other ribosomal proteins (English et al., 2015). Furthermore, deficiency in the phosphorylation of ribosomal protein S6 affects the translation efficiency of a subset of mRNAs in some brain areas of mice (Puighermanal et al., 2017), and excessive mTOR activation is linked to synaptic pruning deficits in autism spectrum disorders (Tang et al., 2014). The observed hypofunction of S6 in schizophrenia subjects, together with *postmortem* studies showing that spine density is reduced in the cortex of schizophrenia subjects (Moyer et al., 2015) further support the hypothesis that alterations in protein synthesis and dendritic architecture would finally contribute to dysfunctions in synaptic connectivity that underlie clinical manifestations of schizophrenia.

A correct regulation of mTOR pathway activity is also thought to be essential for olygodendrocyte progenitor cells differentiation into mature olygodendrocytes (Zou et al., 2014) and myelination processes (Liu et al., 2014). Our results suggest that the pathway is hypofunctional at downstream level, and further supports the hypothesis that hypomyelination is another relevant phenomenon involved in schizophrenia (Takahashi et al., 2011). Studies demonstrating that white matter integrity is altered in schizophrenia (Samartzis et al., 2014), even in prodromal stages (Witthaus et al., 2008); as well as studies with animal models showing delayed differentiation of olygodendrocytes (Lindahl et al., 2008) are also in line with this theory. Interestingly, it has been hypothesized recently that cortex hypomyelination in schizophrenia could be underlying cognitive deficits associated

with this disease (Maas et al., 2017). Moreover, the significant increase in the phospho-Akt(Ser473)/Akt ratio, together with the observed decrease in S6 function in *postmortem* brain cortex of subjects with schizophrenia points to a suppression of the negative feedback loops that mTOR/S6K exerts, that restrains upstream kinases signaling and has been reported previously in other tissues (O'Reilly et al., 2006; Rozengurt et al., 2014).

Aside from the study of the Akt/mTOR pathway status in schizophrenia disease, results derived from the study carried out in mice (**Results – Article 2**) demonstrated that chronic treatment with THC leads to a hyperfunctional Akt and S6 status in brain cortex. Acute administration of THC is known to increase the phosphorylation of Akt and several downstream kinases in multiple brain areas, including cortex (Ozaita et al., 2007), and the activation of this pathway has been involved in THC-induced memory impairment in animals (Puighermanal et al., 2009). Both short-term psychotomimetic effects of cannabis use in high-risk population and an increasing risk of being diagnosed with a psychotic disorder when having used cannabis is moderated by a polymorphism variant of AKT1 gene (van Winkel and (GROUP), 2011; Di Forti et al., 2012). Moreover, AKT1 genotype seems to modulate the THC effect on striatal function (Bhattacharyya et al., 2012) and acute psychotomimetic symptoms of cannabis use are predicted by that same variation in healthy young cannabis smokers (Morgan et al., 2016).

Apart from the THC effects on Akt kinase, subchronic THC has been demonstrated to increase the phosphorylation of the hippocampal mTOR downstream target S6K, and produce cognitive deleterious effects in mice, whereas mTOR inhibition prevented both THC effects (Puighermanal et al., 2013). These results are in line with our observations, which show an enduring hyperactive status of cortical Akt/mTOR pathway after chronic THC. Moreover, mTOR inhibition in our study prevented chronic THC induced modulation of 5-HT2AR signaling. Interestingly, the mTOR downstream effector S6K2, whose target substrate is S6, has been shown to interact with 5-HT2AR and modulate its signaling by phosphorylation of specific serine residue, and seems to play a role in 5-HT2AR desensitization (Sheffler et al., 2006; Strachan et al., 2009). Moreover, ablation of the kinase elicit profound changes in patterns of agonist functional selectivity (Strachan et al., 2010). Neuronal ablation of phospho(Ser473)-Akt also led to alteration in 5-HT2AR expression and functionality (Saunders et al., 2014).

As far as we know, our study is the first one evaluating Akt phosphorylation in platelets in relation with cannabis abuse. There are some seminal studies that demonstrate that endocannabinoids, such as 2-AG or AEA, triggers downstream events, including Ak phosphorylation, decrease of intracellular cAMP, increase intracellular Ca^{2+}, and unleash actin polymerization, platelet aggregation and platelet survival (Catani et al., 2010; Grazia

Signorello et al., 2011; Signorello and Leoncini, 2014, 2016). Whereas the receptor involved in these effects and the Akt downstream substrates responsible for these effects are still unclear, it could be hypothesized that chronic cannabis consume could exert a differential modulation of either circulating endocannabinoid levels or cannabinoid receptor expression or functionality in subjects with and without schizophrenia. In subjects without schizophrenia it would not affect Akt phosphorylation and in those suffering schizophrenia, cannabis abuse would decrease the phosphorylated Akt that is present within platelets of these subjects.

Although further experiments would be needed to test all of the hypotheses previously mentioned in this section, results derived from this Doctoral Thesis suggest, as a whole, that cannabis abuse and schizophrenia are pathologies whose underlying molecular mechanisms interact, and shed light to molecular pathways that may have a prognostic value in schizophrenia disease.

Strengths and limitations on the present study

The results of the present Doctoral Thesis show several strengths that should be contextualized in the context of their own limitations. Strengths of this study involve the use of three different systems – mouse cortical tissue, human cortical tissue, human blood platelets – to face the hypothesis. Each system has its own limitation and it has been useful in addressing distinct objectives of this study. Whereas inherent differences regarding species and tissues should not be overlooked, common observations among the experimental systems, understood as reproducible in more than one system, strength the reliability of the conclusions that can be extracted.

However, it is important to be aware of the limitations present in this Doctoral Thesis. A brief description of these limitations is presented below:

Firstly, a cause-effect relationship between chronic THC and alterations in both cortical 5-HT2AR and Akt/mTOR signaling pathway has been demonstrated exclusively in mice. These results, together with extensive literature reviewed in this Doctoral Thesis led us to hypothesize that the alterations observed in patients with cannabis use disorder are induced by chronic cannabis exposure. However, these alterations could also underlie addictive pathology. Studies addressing cortical 5-HT2AR and Akt/mTOR signaling pathway in other addictive pathologies that do not include cannabis use would shed light on this issue.

It should not be overlooked the fact that animal models are far from properly modeling the whole symptomatology of a psychiatric disease. They can be suitable in order to test certain hypotheses that require a high control of possible confounding factors, but identical effects

of drugs in rodents and humans should not be assumed. This aspect applies to both the effects of chronic THC in mice and the effects of chronic antipsychotic treatment in rats.

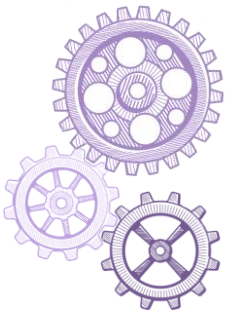
In relation to this, THC use in humans is known to be highly heterogeneous. It would also be interesting to evaluate if intermittent treatment with different doses of THC would lead to similar alterations in mouse brain. Other crucial factors that have not been addressed in this study involve that cannabis used by humans contain many other compounds apart from THC, and that humans consume it mainly by inhalation, and voluntarily. Regarding the first factor, CBD contained in cannabis seems to have a potential antipsychotic effect (Iseger and Bossong, 2015; Leweke et al., 2016; McGuire et al., 2018; O'Neill et al., 2020). Further studies will be needed to ascertain whether this or other compounds present in cannabis plant could prevent or decrease the effects that chronic THC exert on 5-HT2AR and Akt/mTOR signaling pathway. Regarding the second factor, efforts are still being made in order to develop self-administered THC models, as well as inhaled THC delivery devices for animal studies (Nguyen et al., 2016). It will also be interesting to evaluate whether these administration routes exert similar effects.

In the context of schizophrenia research, studies in human tissue, and especially *postmortem* brain studies, provide valuable information about molecular alterations underlying the neurobiology of the disease. However, these studies posses their own inherent difficulties. One of the main ones is the high interindividual variability. Whereas animal studies are often carried out with rodents with identical genetic background and environmental conditions, subjects included in the *postmortem* brain study are much more heterogeneous. In relation to this, both the chronicity of the disease and the antipsychotic treatment are two factors that may confound the observations. In this sense, studies with treatment-naïve patients would be valuable in order to test whether the alterations in Akt/mTOR pathway are present independently of any pharmacological treatment. Studies replicating the results present in this Doctoral Thesis would also help in this sense.

Finally, another limitation of this study is the exclusive use of male mice for all the animal studies. There is an increasing amount of evidence in scientific literature showing sex-dimorphic effects of many drugs, including THC (de Salas-Quiroga et al., 2020). While hormonal fluctuations during estrous cycles as putative confounding factor has been commonly used in order to justify studies only in males, scientific literature is moving towards studies assessing their hypotheses in both genders. Further studies in female mice will be needed to evaluate whether the alterations showed after chronic THC treatment can be generalized to both genders.

To conclude, there is wide evidence in the literature which has been the basis of the hypothesis of this study, which supports cannabis consume to be a risk factor for schizophrenia. Although further work is needed in order to understand the differences in susceptibility for schizophrenia onset in relation with cannabis and, specifically, THC abuse, this work provides valuable molecular targets for more in-deep clinical studies that may address this hypothesis longitudinally.

The schizophrenia x cannabis abuse interaction regarding both 5-HT2AR protein expression and Akt phosphorylation is, in our opinion, an interesting finding that agrees with the hypothesis that these two proteins are elements of an interacting mechanism involving chronic THC pharmacological effect and schizophrenia pathogenesis.



CONCLUSIONS

The main conclusions derived from this Doctoral Thesis are:

1. The cannabinoid agonists Δ^9-THC, WIN55,212-2 and ACEA selectively activate different inhibitory and non-inhibitory Gα protein subtypes through the activation of CB1 and/or CB2 receptors in mouse cortex.

2. Δ^9-THC activates Gαi1 and Gαq/11 through CB1 receptor, while Δ^9-THC-induced Gαo and Gαz activations is mediated by CB2 receptors in mouse cortex.

3. Chronic Δ^9-THC treatment in mice triggers a supersensitivity to prepulse inhibition disruption induced by the 5-HT2AR agonist (±)DOI, and modulates cortical 5-HT2AR signaling pattern towards inhibitory Gαi1, Gαi3, Gαo and Gαz protein subtypes.

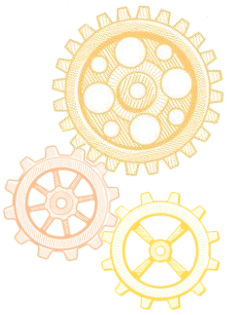
4. Chronic Δ^9-THC treatment evokes a hyperactive state of Akt/mTOR signaling pathway in mouse cortex.

5. Chronic mTOR complex inhibition prevents the Δ^9-THC-induced hyperactive Akt/mTOR signaling, together with the supersensitivity to prepulse inhibition disruption and the modulation of 5-HT2AR signaling pattern.

6. Akt kinase is in a hyperactive status in the prefrontal cortex and in blood platelets from subjects with schizophrenia. However, the downstream ribosomal protein S6 is found hypoactive in prefrontal cortex of these subjects, regardless of antipsychotic presence at the time of death.

7. Chronic treatment with haloperidol diminishes Akt phosphorylation in rat cortex, while chronic risperidone or clozapine treatments do not exert any effect. None of the three antipsychotics elicit a significant modulation of either GSK3β or S6 activation status in rat cortex.

8. 5-HT2AR protein expression is increased in blood platelets of patients with cannabis use disorder, as well as in those of patients with schizophrenia. However, patients with both clinical diagnoses do not show a significant 5-HT2AR protein expression modulation.



REFERENCES

Aas M, Melle I, Bettella F, Djurovic S, Le Hellard S, Bjella T, Ringen PA, Lagerberg T V., Smeland OB, Agartz I, Andreassen OA, Tesli M (2018) Psychotic patients who used cannabis frequently before illness onset have higher genetic predisposition to schizophrenia than those who did not. Psychol Med 48:43–49.

Abbas A, Roth BL (2008) Arresting serotonin. Proc Natl Acad Sci 105:831–832.

Abbas AI, Yadav PN, Yao W-D, Arbuckle MI, Grant SGN, Caron MG, Roth BL (2009) PSD-95 Is Essential for Hallucinogen and Atypical Antipsychotic Drug Actions at Serotonin Receptors. J Neurosci 29:7124–7136.

Abbott A (2016) The mental-health crisis among migrants. Nature 538:158–171.

Abdallah CG, De Feyter HM, Averill LA, Jiang L, Averill CL, Chowdhury GMI, Purohit P, de Graaf RA, Esterlis I, Juchem C, Pittman BP, Krystal JH, Rothman DL, Sanacora G, Mason GF (2018) The effects of ketamine on prefrontal glutamate neurotransmission in healthy and depressed subjects. Neuropsychopharmacology 43:2154–2160.

Abdallah CG, Sanacora G, Duman RS, Krystal JH (2015) Ketamine and Rapid-Acting Antidepressants: A Window into a New Neurobiology for Mood Disorder Therapeutics. Annu Rev Med 66:509–523.

Abi-Dargham A (2007) Alterations of Serotonin Transmission in Schizophrenia. In: International Review of Neurobiology, pp 133–164.

Achim AM, Maziade M, Raymond É, Olivier D, Mérette C, Roy M. (2011) How prevalent are anxiety disorders in schizophrenia? a meta-analysis and critical review on a significant association. Schizophr Bull 37:811–821.

Adams R, Baker BR, Wearn RB (1940) Structure of Cannabinol. III. Synthesis of Cannabinol, 1-Hydroxy-3-n-amyl-6,6,9-trimethyl-6-dibenzopyran. J Am Chem Soc 62:2204–2207.

Agirregoitia E, Carracedo A, Subirán N, Valdivia A, Agirregoitia N, Peralta L, Velasco G, Irazusta J (2010) The CB2 cannabinoid receptor regulates human sperm cell motility. Fertil Steril 93:1378–1387.

Aikawa S, Kobayashi H, Nemoto T, Matsuo S, Wada Y, Mamiya N, Yamaguchi T, Katagiri N, Tsujino N, Mizuno M (2018) Social anxiety and risk factors in patients with schizophrenia: Relationship with duration of untreated psychosis. Psychiatry Res 263:94–100.

Akbarian S, Viñuela A, Kim JJ, Potkin SG, Bunney WE, Jones EG (1993) Distorted Distribution of Nicotinamide-Adenine Dinucleotide Phosphate—Diaphorase Neurons in Temporal Lobe of Schizophrenics Implies Anomalous Cortical Development. Arch Gen Psychiatry 50:178–

187.

American Psychiatric Association (1994) Diagnostic and Statistical Manual of Mental Disorders, 4th Ed.

American Psychiatric Association (2000) Diagnostic and Statistical Manual of Mental Disorders, 4th Ed. DSM-IV-TR.

Andreasen NC, Nopoulos P, Magnotta V, Pierson R, Ziebell S, Ho B. (2011) Progressive brain change in schizophrenia: A prospective longitudinal study of first-episode schizophrenia. Biol Psychiatry 70:672–679.

Andréasson S, Allebeck P, Engström A, Rydberg U (1987) Cannabis and schizophrenia. A longitudinal study of Swedish conscripts. Lancet 2:1483–1486.

Angrist B, Peselov E, Rubinstein M, Wolkin A, Rotrosen J (1985) Amphetamine response and relapse risk after depot neuroleptic discontinuation. Psychopharmacology (Berl) 85:277–283.

Anis NA, Berry SC, Burton NR, Lodge D (1983) The dissociative anaesthetics, ketamine and phencyclidine, selectively reduce excitation of central mammalian neurones by N-methyl-aspartate. Br J Pharmacol 79:565–575.

Arora RC, Meltzer HY (1993) Serotonin2 receptor binding in blood platelets of schizophrenic patients. Psychiatry Res 47:111–119.

Arranz B, Rosel P, Sarró S, Ramirez N, Dueñas R, Cano R, Sanchez J., San L (2003) Altered platelet serotonin 5-HT2A receptor density but not second messenger inositol trisphosphate levels in drug-free schizophrenic patients. Psychiatry Res 118:165–174.

Arseneault L, Cannon M, Poulton R, Murray R, Caspi A, Moffitt T. (2002) Cannabis use in adolescence and risk for adult psychosis: Longitudinal prospective study. Br Med J 325:1212–1213.

Ashtari M, Avants B, Cyckowski L, Cervellione K., Roofeh D, Cook P, Gee J, Sevy S, Kumra S (2011) Medial temporal structures and memory functions in adolescents with heavy cannabis use. J Psychiatr Res 45:1055–1066.

Asor E, Ben-Shachar D (2012) Platelets: A possible glance into brain biological processes in schizophrenia. World J Psychiatry 2:124–133.

Auclair N, Otani S, Soubrie P, Crepel F (2000) Cannabinoids modulate synaptic strength and plasticity at glutamatergic synapses of rat prefrontal cortex pyramidal neurons. J Neurophysiol 83:3287–3293.

Bagamery K, Kvell K, Barnet M, Landau R, Graham J (2005) Are platelets activated after a rapid, one-step density gradient centrifugation? Evidence from flow cytometric analysis. Clin Lab Haematol 27:75–77.

Baier PC, Koch JM, Seeck-Hirschner M, Ohlmeyer K, Wilms S, Aldenhoff JB, Hinze-Selch D (2009) A flow-cytometric method to investigate glutamate-receptor-sensitivity in whole blood platelets – Results from healthy controls and patients with schizophrenia. J Psychiatr Res 43:585–591.

Baldassarri S, Bertoni A, Bagarotti A, Sarasso C, Zanfa M, Catani M V., Avigliano L, Maccarrone M, Torti M, Sinigaglia F (2008) The endocannabinoid 2-arachidonoylglycerol activates human platelets through non-CB 1 /CB 2 receptors. J Thromb Haemost 6:1772–1779.

Balster R., Prescott W. (1992) Δ9-Tetrahydrocannabinol discrimination in rats as a model for cannabis intoxication. Neurosci Biobehav Rev 16:55–62.

Balu D., Carlson G., Talbot K, Kazi H, Hill-Smith T., Easton R, Birnbaum M., Lucki I (2012) Akt1 deficiency in schizophrenia and impairment of hippocampal plasticity and function. Hippocampus 22:230–240.

Barradas M., Mikhailidis D. (1993) The use of platelets as models for neurons: possible applications to the investigation of eating disorders. Biomed Pharmacother 47:11–18.

Bassett A., Scherer S., Brzustowicz L. (2010) Copy number variations in schizophrenia: Critical review and new perspectives on concepts of genetics and disease. Am J Psychiatry 167:899–914.

Bell D (1965) Comparison of Amphetamine Psychosis and Schizophrenia. Br J Psychiatry 111:701–707.

Ben-Shabat S, Fride E, Sheskin T, Tamiri T, Rhee M., Vogel Z, Bisogno T, De Petrocellis L, Di Marzo V, Mechoulam R (1998) An entourage effect: inactive endogenous fatty acid glycerol esters enhance 2-arachidonoyl-glycerol cannabinoid activity. Eur J Pharmacol 353:23–31.

Bénard G et al. (2012) Mitochondrial CB 1 receptors regulate neuronal energy metabolism. Nat Neurosci 15:558–564.

Benito C, Núñez E, Tolón RM, Carrier EJ, Rábano A, Hillard CJ, Romero J (2003) Cannabinoid CB2 Receptors and Fatty Acid Amide Hydrolase Are Selectively Overexpressed in Neuritic Plaque-Associated Glia in Alzheimer's Disease Brains. J Neurosci 23:11136–11141.

Benito C, Tolón R., Pazos M., Núñez E, Castillo A., Romero J (2008) Cannabinoid CB 2

receptors in human brain inflammation. Br J Pharmacol 153:277–285.

Berdenis van Berlekom A, Muflihah CH, Snijders GJLJ, MacGillavry HD, Middeldorp J, Hol EM, Kahn RS, de Witte LD (2020) Synapse Pathology in Schizophrenia: A Meta-analysis of Postsynaptic Elements in Postmortem Brain Studies. Schizophr Bull 46:374–386.

Best A., Regehr W. (2008) Serotonin evokes endocannabinoid release and retrogradely suppresses excitatory synapses. J Neurosci 28:6508–6515.

Bhatnagar A, Sheffler D., Kroeze W., Compton-Toth B, Roth B. (2004) Caveolin-1 interacts with 5-HT2A serotonin receptors and profoundly modulates the signaling of selected Gαq-coupled protein receptors. J Biol Chem 279:34614–34623.

Bhattacharyya S, Atakan Z, Martin-Santos R, Crippa J., Kambeitz J, Prata D, Williams S, Brammer M, Collier D., McGuire P. (2012) Preliminary report of biological basis of sensitivity to the effects of cannabis on psychosis: AKT1 and DAT1 genotype modulates the effects of δ 9-tetrahydrocannabinol on midbrain and striatal function. Mol Psychiatry 17:1155–1157.

Bisogno T, Hanuš L, De Petrocellis L, Tchilibon S, Ponde D., Brandi I, Moriello A., Davis J., Mechoulam R, Di Marzo V (2001) Molecular targets for cannabidiol and its synthetic analogues: Effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. Br J Pharmacol 134:845–852.

Bisogno T, Howell F, Williams G, Minassi A, Cascio M, Ligresti A, Matias I, Schiano-Moriello A, Paul P, Williams E., Gangadbaran U, Hobbs C, Di Marzo V, Doherty P (2003) Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. J Cell Biol 163:463–468.

Bisogno T, Sepe N, Melck D, Maurelli S, De Petrocellis L, Di Marzo V (1997) Biosynthesis, release and degradation of the novel endogenous cannabimimetic metabolite 2-arachidonoylglycerol in mouse neuroblastoma cells. Biochem J 322:671–677.

Bitter I, Lieberman JA, Gaudoux F, Sokoloff P, Groc M, Chavda R, Delsol C, Barthe L, Brunner V, Fabre C, Fagard M, Montagne A, Tonner F (2019) Randomized, double-blind, placebo-controlled study of F17464, a preferential D3 antagonist, in the treatment of acute exacerbation of schizophrenia. Neuropsychopharmacology 44:1917–1924.

Blankman J., Simon G., Cravatt B. (2007) A Comprehensive Profile of Brain Enzymes that Hydrolyze the Endocannabinoid 2-Arachidonoylglycerol. Chem Biol 14:1347–1356.

Blasco-Benito S, Seijo-Vila M, Caro-Villalobos M, Tundidor I, Andradas C, García-Taboada E, Wade J, Smith S, Guzmán M, Pérez-Gómez E, Gordon M, Sánchez C (2018) Appraising the

"entourage effect": Antitumor action of a pure cannabinoid versus a botanical drug preparation in preclinical models of breast cancer. Biochem Pharmacol 157:285–293.

Blasi G, Napolitano F, Ursini G, Taurisano P, Romano R, Caforio G, Fazio L, Gelao B, Di Giorgio A, Iacovelli L, Sinibaldi L, Popolizio T, Usiello A, Bertolino A (2011) DRD2/AKT1 interaction on D2 c-AMP independent signaling, attentional processing, and response to olanzapine treatment in schizophrenia. Proc Natl Acad Sci U S A 108:1158–1163.

Bluett R., Báldi R, Haymer A, Gaulden A., Hartley N., Parrish W., Baechle J, Marcus D., Mardam-Bey R, Shonesy B., Uddin J, Marnett L., Mackie K, Colbran R., Winder D., Patel S (2017) Endocannabinoid signalling modulates susceptibility to traumatic stress exposure. Nat Commun 8:14782.

Boger D., Fecik R., Patterson J., Miyauchi H, Patricelli M., Cravatt B. (2000) Fatty acid amide hydrolase substrate specificity. Bioorganic Med Chem Lett 10:2613–2616.

Boger D., Patterson J., Jin Q (1998) Structural requirements for 5-HT2A and 5-HT1A serotonin receptor potentiation by the biologically active lipid oleamide. Proc Natl Acad Sci U S A 95:4102–4107.

Boos HBM, Aleman A, Cahn W, Hulshoff Pol H, Kahn RS (2007) Brain volumes in relatives of patients with schizophrenia: A meta-analysis. Arch Gen Psychiatry 64:297–304.

Bosier B, Muccioli GG, Hermans E, Lambert DM (2010) Functionally selective cannabinoid receptor signalling: Therapeutic implications and opportunities. Biochem Pharmacol 80:1–12.

Bourque F, Van Der Ven E, Malla A (2011) A meta-analysis of the risk for psychotic disorders among first- and second-generation immigrants. Psychol Med 41:897–910.

Bowling H, Zhang G, Bhattacharya A, Perez-Cuesta LM, Deinhardt K, Hoeffer CA, Neubert TA, Gan W., Klann E, Chao M V. (2014) Antipsychotics Activate mTORC1-Dependent Translation to Enhance Neuronal Morphological Complexity. Sci Signal 7:ra4.

Brandl F, Avram M, Weise B, Shang J, Simões B, Bertram T, Hoffmann Ayala D, Penzel N, Gürsel D., Bäuml J, Wohlschläger A., Vukadinovic Z, Koutsouleris N, Leucht S, Sorg C (2019) Specific Substantial Dysconnectivity in Schizophrenia: A Transdiagnostic Multimodal Meta-analysis of Resting-State Functional and Structural Magnetic Resonance Imaging Studies. Biol Psychiatry 85:573–583.

Brantl SA, Khandoga AL, Siess W (2014) Mechanism of platelet activation induced by endocannabinoids in blood and plasma. Platelets 25:151–161.

Braud S, Bon C, Touqui L, Mounier C (2000) Activation of rabbit blood platelets by anandamide through its cleavage into arachidonic acid. FEBS Lett 471:12–16.

Breakey W., Goodell H, Lorenz P., Mchugh P. (1974) Hallucinogenic drugs as precipitants of schizophrenia'. Psychol Med 4:255–261.

Breivogel C., Selley D., Childers S. (1998) Cannabinoid receptor agonist efficacy for stimulating [35S]GTPγS binding to rat cerebellar membranes correlates with agonist-induced decreases in GDP affinity. J Biol Chem 27:16865–16873.

Brittis P., Silver J, Walsh F., Doherty P (1996) Fibroblast growth factor receptor function is required for the orderly projection of ganglion cell axons in the developing mammalian retina. Mol Cell Neurosci 8:120–128.

Brown A., Derkits EJ (2010) Prenatal infection and schizophrenia: A review of epidemiologic and translational studies. Am J Psychiatry 167:261–280.

Buckley N., McCoy K., Mezey É, Bonner T, Zimmer A, Felder C., Glass M, Zimmer A (2000) Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB2 receptor. Eur J Pharmacol 396:141–149.

Buckley P., Miller B., Lehrer D., Castle D. (2009) Psychiatric comorbidities and schizophrenia. Schizophr Bull 35:383–402.

Bunzow J., Van Tol HH., Grandy D., Albert P, Salon J, MacDonald C, Machida C., Neve K., Civelli O (1988) Cloning and expression of a rat D2 dopamine receptor cDNA. Nature 336:783–787.

Buonocore M, Bosia M, Bechi M, Spangaro M, Cavedoni S, Cocchi F, Bianchi L, Guglielmino C, Mastromatteo A., Cavallaro R (2017) Targeting anxiety to improve quality of life in patients with schizophrenia. Eur Psychiatry 45:129–135.

Burgering BMT, Coffer PJ (1995) Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature:599–602.

Burnet PWJ, Eastwood S., Harrison P. (1996) 5-HT(1A) 5-HT(2A) receptor mRNAs and binding site densities are differentially altered in schizophrenia. Neuropsychopharmacology 15:442–455.

Burt D., Creese I, Snyder S. (1976) Properties of [3H]haloperidol and [3H]dopamine binding associated with dopamine receptors in calf brain membranes. Mol Pharmacol 12:800–812.

Busquets-Garcia A, Bains J, Marsicano G (2018) CB 1 Receptor Signaling in the Brain:

Extracting Specificity from Ubiquity. Neuropsychopharmacology 43:4–20.

Callado LF, Ibarra-Lecue I, Diez-Alarcia R, Meana JJ, Urigüen L (2019) Decreased ribosomal protein S6 phosphorylation in post-mortem prefrontal cortex of subjects with schizophrenia: Effects of antipsychotic treatment. Eur Neuropsychopharmacol 29:S321.

Cannizzaro C, D'Amico M, Preziosi P, Martire M (2006) Presynaptic effects of anandamide and WIN55,212-2 on glutamatergic nerve endings isolated from rat hippocampus. Neurochem Int 48:159–165.

Cannon M, Jones P., Murray R. (2002) Obstetric complications and schizophrenia: Historical and meta-analytic review. Am J Psychiatry 159:1080–1092.

Cardno A., Owen M. (2014) Genetic relationships between schizophrenia, bipolar disorder, and schizoaffective disorder. Schizophr Bull 40:504–515.

Carlsson A, Lindqvist M (1963) Effect of Chlorpromazine or Haloperidol on Formation of 3-Methoxytyramine and Normetanephrine in Mouse Brain. Acta Pharmacol Toxicol (Copenh) 20:140–144.

Carr D., Cooper D., Ulrich S., Spruston N, Surmeier D. (2002) Serotonin receptor activation inhibits sodium current and dendritic excitability in prefrontal cortex via a protein kinase C-dependent mechanism. J Neurosci 22:6846–6855.

Carracedo A, Ma L, Teruya-Feldstein J, Rojo F, Salmena L, Alimonti A, Egia A, Sasaki A, Thomas G, Kozma S, Papa A, Nardella C, Cantley L, Baselga J, Pandolfi P (2008) Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. J Clin Invest 118:3065–3074.

Caspari D (1999) Cannabis and schizophrenia: Results of a follow-up study. Eur Arch Psychiatry Clin Neurosci 249:45–49.

Caspi A, Houts R., Belsky, ha S, Shalev I, Poulton R, Moffitt TED., Goldman-Mellor S., Harrington H, Israel S, Meier MH, Ramrak (2014) The p factor: One general psychopathology factor in the structure of psychiatric disorders? Clin Psychol Sci 2:119–137.

Caspi A, Moffitt T. (2018) All for one and one for all: Mental disorders in one dimension. Am J Psychiatry 175:831–844.

Caspi A, Moffitt T., Cannon M, McClay J, Murray R, Harrington H., Taylor A, Arseneault L, Williams B, Braithwaite A, Poulton R, Craig I. (2005) Moderation of the effect of adolescent-onset cannabis use on adult psychosis by a functional polymorphism in the catechol-O-methyltransferase gene: Longitudinal evidence of a gene X environment interaction. Biol

Psychiatry 57:1117–1127.

Catani M., Gasperi V, Catanzaro G, Baldassarri S, Bertoni A, Sinigaglia F, Avigliano L, Maccarrone M (2010) Human Platelets Express Authentic CB1 and CB2 Receptors. Curr Neurovasc Res 7:311–318.

Catts V., Lai Y., Weickert C., Weickert T., Catts S. (2016) A quantitative review of the postmortem evidence for decreased cortical N-methyl-d-aspartate receptor expression levels in schizophrenia: How can we link molecular abnormalities to mismatch negativity deficits? Biol Psychol 116:57–67.

Ceccarini J, De Hert M, Van Winkel R, Peuskens J, Bormans G, Kranaster L, Enning F, Koethe D, Leweke FM, Van Laere K (2013) Increased ventral striatal CB1 receptor binding is related to negative symptoms in drug-free patients with schizophrenia. Neuroimage 79:304–312.

Chadha R, Meador-Woodruff JH (2020) Downregulated AKT-mTOR signaling pathway proteins in dorsolateral prefrontal cortex in Schizophrenia. Neuropsychopharmacology:1–9.

Chandra S, Radwan M., Majumdar C., Church J., Freeman T., ElSohly M. (2019) New trends in cannabis potency in USA and Europe during the last decade (2008–2017). Eur Arch Psychiatry Clin Neurosci 269:5–15.

Chang F, Lee J., Navolanic P., Steelman L., Shelton J., Blalock W., Franklin R., McCubrey JA (2003) Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: A target for cancer chemotherapy. Leukemia 17:590–603.

Charlson F., Ferrari AJ, Santomauro D., Diminic S, Stockings E, Scott JG, McGrath J., Whiteford H. (2018) Global epidemiology and burden of schizophrenia: Findings from the global burden of disease study 2016. Schizophr Bull 44:1195–1203.

Cheer JF, Cadogan A-K, Marsden CA, Fone KCF, Kendall DA (1999) Modification of 5-HT2 receptor mediated behaviour in the rat by oleamide and the role of cannabinoid receptors. Neuropharmacology 38:533–541.

Chevaleyre V, Castillo PE (2003) Heterosynaptic LTD of hippocampal GABAergic synapses: A novel role of endocannabinoids in regulating excitability. Neuron 38:461–472.

Cloninger CR (1987) A Systematic Method for Clinical Description and Classification of Personality Variants: A Proposal. Arch Gen Psychiatry 44:573–588.

Coffer PJ, Jin J, Woodgett JR (1998) Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. Biochem J 335:1–13.

Cogolludo A, Moreno L, Lodi F, Frazziano G, Cobeño L, Tamargo J, Perez-Vizcaino F (2006) Serotonin inhibits voltage-gated K+ currents in pulmonary artery smooth muscle cells: Role of 5-HT2A receptors, caveolin-1, and KV1.5 channel internalization. Circ Res 98:931–938.

Cohen K, Weinstein AM (2018) Synthetic and Non-synthetic Cannabinoid Drugs and Their Adverse Effects-A Review From Public Health Prospective. Front Public Heal 6:162.

Conley RR, Ascher-Svanum H, Zhu B, Faries DE, Kinon BJ (2007) The burden of depressive symptoms in the long-term treatment of patients with schizophrenia. Schizophr Res 90:186–197.

Conn PJ, Sanders-Bush E (1987) Central serotonin receptors: effector systems, physiological roles and regulation. Psychopharmacology (Berl) 92:267–277.

Corssen G, Domino E. (1966) Dissociative anesthesia: further pharmacologic studies and first clinical experience with the phencyclidine derivative CI-581. Anesth Analg 45:29–40.

Cortes-Briones J, Skosnik PD, Mathalon D, Cahill J, Pittman B, Williams A, Sewell RA, Ranganathan M, Roach B, Ford J, D'Souza DC (2015) ∆9-THC Disrupts Gamma (γ)-Band Neural Oscillations in Humans. Neuropsychopharmacology 40:2124–2134.

Costa-Mattioli M, Monteggia LM (2013) mTOR complexes in neurodevelopmental and neuropsychiatric disorders. Nat Neurosci 16:1537–1543.

Covey DP, Mateo Y, Sulzer D, Cheer JF, Lovinger DM (2017) Endocannabinoid modulation of dopamine neurotransmission. Neuropharmacology 124:52–61.

Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB (1996) Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. Nature 384:83–87.

Creese I, Burt DR, Snyder SH (1976) Dopamine receptor binding predicts clinical and pharmacological potencies of antischizophrenic drugs. Science (80-) 192:481–483.

Croci T, Manara L, Aureggi G, Guagnini F, Rinaldi-Carmona M, Maffrand JP, Le Fur G, Mukenge S, Ferla G (1998) In vitro functional evidence of neuronal cannabinoid CB1 receptors in human ileum. Br J Pharmacol 125:1393–1395.

D'Amico M, Cannizzaro C, Preziosi P, Martire M (2004) Inhibition by anandamide and synthetic cannabimimetics of the release of [3H]D-aspartate and [3H]GABA from synaptosomes isolated from the rat hippocampus. Neurochem Res 29:1553–1561.

D'Souza DC, Abi-Saab WM, Madonick S, Forselius-Bielen K, Doersch A, Braley G,

Gueorguieva R, Cooper TB, Krystal JH (2005) Delta-9-tetrahydrocannabinol effects in schizophrenia: Implications for cognition, psychosis, and addiction. Biol Psychiatry 57:594–608.

D'Souza DC, Braley G, Blaise R, Vendetti M, Oliver S, Pittman B, Ranganathan M, Bhakta S, Zimolo Z, Cooper T, Perry E (2008a) Effects of haloperidol on the behavioral, subjective, cognitive, motor, and neuroendocrine effects of Δ-9-tetrahydrocannabinol in humans. Psychopharmacology (Berl) 198:587–603.

D'Souza DC, Cortes-Briones JA, Ranganathan M, Thurnauer H, Creatura G, Surti T, Planeta B, Neumeister A, Pittman B, Normandin MD, Kapinos M, Ropchan J, Huang Y, Carson RE, Skosnik PD (2016) Rapid Changes in Cannabinoid 1 Receptor Availability in Cannabis-Dependent Male Subjects after Abstinence from Cannabis. Biol Psychiatry Cogn Neurosci Neuroimaging 1:60–67.

D'Souza DC, Fridberg DJ, Skosnik PD, Williams A, Roach B, Singh N, Carbuto M, Elander J, Schnakenberg A, Pittman B, Sewell RA, Ranganathan M, Mathalon D (2012) Dose-related modulation of event-related potentials to novel and target stimuli by intravenous δ 9-THC in humans. Neuropsychopharmacology 37:1632–1646.

D'Souza DC, Perry E, MacDougall L, Ammerman Y, Cooper T, Wu Y Te, Braley G, Gueorguieva R, Krystal JH (2004) The psychotomimetic effects of intravenous delta-9-tetrahydrocannabinol in healthy individuals: Implications for psychosis. Neuropsychopharmacology 29:1558–1572.

D'Souza DC, Ranganathan M, Braley G, Gueorguieva R, Zimolo Z, Cooper T, Perry E, Krystal J (2008b) Blunted psychotomimetic and amnestic effects of Δ-9- tetrahydrocannabinol in frequent users of cannabis. Neuropsychopharmacology 33:2505–2516.

Dalton GD, Peterson LJ, Howlett AC (2013) CB 1 cannabinoid receptors promote maximal FAK catalytic activity by stimulating cooperative signaling between receptor tyrosine kinases and integrins in neuronal cells. Cell Signal 25:1665–1677.

Darmani NA (2001) Cannabinoids of diverse structure inhibit two DOI-induced 5-HT2A receptor-mediated behaviors in mice. Pharmacol Biochem Behav 68:311–317.

Davis JM, Chen N, Glick ID (2003) A meta-analysis of the efficacy of second-generation antipsychotics. Arch Gen Psychiatry 60:553–564.

Davis KL, Kahn RS, Ko G, Davidson M (1991) Dopamine in schizophrenia: A review and reconceptualization. Am J Psychiatry 148:1474–1486.

de Chaffoy de Courcelles D, Roevens P, Wynants J, Van Belle H (1987) Serotonin-induced alterations in inositol phospholipid metabolism in human platelets. Biochim Biophys Acta 927:291–302.

De Clerck F, Xhonneux B, Leysen J, Janssen PAJ (1984) Evidence for functional 5-HT2 receptor sites on human blood platelets. Biochem Pharmacol 33:2807–2811.

De Jesús ML, Hostalot C, Garibi JM, Sallés J, Meana JJ, Callado LF (2010) Opposite changes in cannabinoid CB1 and CB2 receptor expression in human gliomas. Neurochem Int 56:829–833.

De Jesús ML, Sallés J, Meana JJ, Callado LF (2006) Characterization of CB1 cannabinoid receptor immunoreactivity in postmortem human brain homogenates. Neuroscience 140:635–643.

de Jonge JC, Vinkers CH, Hulshoff Pol HE, Marsman A (2017) GABAergic Mechanisms in Schizophrenia: Linking Postmortem and In Vivo Studies. Front Psychiatry 8:118.

de Salas-Quiroga A, García-Rincón D, Gómez-Domínguez D, Valero M, Simón-Sánchez S, Paraíso-Luna J, Aguareles J, Pujadas M, Muguruza C, Callado LF, Lutz B, Guzmán M, de la Prida LM, Galve-Roperh I (2020) Long-term hippocampal interneuronopathy drives sex-dimorphic spatial memory impairment induced by prenatal THC exposure. Neuropsychopharmacology:1–10.

Deadwyler SA, Hampson RE, Mu J, Whyte A, Childers S (1995) Cannabinoids modulate voltage sensitive potassium A-current in hippocampal neurons via a cAMP-dependent process. J Pharmacol Exp Ther 273:734–743.

Dean B (2003) The cortical serotonin2A receptor and the pathology of schizophrenia: A likely accomplice. J Neurochem 85:1–13.

Dean B, Crossland N, Boer S, Scarr E (2008a) Evidence for altered post-receptor modulation of the serotonin 2a receptor in schizophrenia. Schizophr Res 104:185–197.

Dean B, Hayes W, Hill C, Copolov D (1998) Decreased serotonin2A receptors in Brodmann's area 9 from schizophrenic subjects. Mol Chem Neuropathol 34:133–145.

Dean B, Karl T, Pavey G, Boer S, Duffy L, Scarr E (2008b) Increased levels of serotonin2A receptors and serotonin transporter in the CNS of neuregulin 1 hypomorphic/mutant mice. Schizophr Res 99:341–349.

Dean B, Sundaram S, Hill C, Copolov DL (1996) Platelet [3H]dopamine uptake is differentially affected by neuroleptic drug treatment in schizophrenia and schizophreniform

disorder. Prog Neuro-Psychopharmacology Biol Psychiatry 20:45–55.

Dekker N, Linszen DH, De Haan L (2009) Reasons for Cannabis Use and Effects of Cannabis Use as Reported by Patients with Psychotic Disorders. Psychopathology 42:350–360.

Deng C, Han M, Huang X-F (2007) No changes in densities of cannabinoid receptors in the superior temporal gyrus in schizophrenia. Neurosci Bull 23:341–347.

Deusch E, Kress HG, Kraft B, Kozek-Langenecker SA (2004) The Procoagulatory Effects of Delta-9-Tetrahydrocannabinol in Human Platelets. Anesth Analg 99:1127–1130.

Devane WA, Dysarz 3rd FA, Johnson MR, Melvin LS, Howlett AC (1988) Determination and characterization of a cannabinoid receptor in rat brain. Mol Pharmacol 34:605–613.

Devane WA, Hanuš L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science (80-) 258:1946–1949.

Devinsky O et al. (2016) Cannabidiol in patients with treatment-resistant epilepsy: an open-label interventional trial. Lancet Neurol 15:270–278.

Di Forti M et al. (2014) Daily use, especially of high-potency cannabis, drives the earlier onset of psychosis in cannabis users. Schizophr Bull 40:1509–1517.

Di Forti M et al. (2019) The contribution of cannabis use to variation in the incidence of psychotic disorder across Europe (EU-GEI): a multicentre case-control study. The Lancet Psychiatry 6:427–436.

Di Forti M, Iyegbe C, Sallis H, Kolliakou A, Falcone MA, Paparelli A, Sirianni M, La Cascia C, Stilo SA, Marques TR, Handley R, Mondelli V, Dazzan P, Pariante C, David AS, Morgan C, Powell J, Murray RM (2012) Confirmation that the AKT1 (rs2494732) genotype influences the risk of psychosis in cannabis users. Biol Psychiatry 72:811–816.

Di Forti M, Morgan C, Dazzan P, Pariante C, Mondelli V, Marques TR, Handley R, Luzi S, Russo M, Paparelli A, Butt A, Stilo SA, Wiffen B, Powell J, Murray RM (2009) High-potency cannabis and the risk of psychosis. Br J Psychiatry 195:488–491.

Di Marzo V (2006) Endocannabinoids: Synthesis and degradation. Rev Physiol Biochem Pharmacol 160:1–24.

Di Marzo V, Fontana A, Cadas H, Schinelli S, Cimino G, Schwartz JC, Piomelli D (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. Nature 372:686–691.

Di Marzo V, Melck D, Bisogno T, De Petrocellis L (1998) Endocannabinoids: Endogenous cannabinoid receptor ligands with neuromodulatory action. Trends Neurosci 21:521–528.

Dietrich-Muszalska A, Olas B (2009) The changes of aggregability of blood platelets in schizophrenia. World J Biol Psychiatry 10:171–176.

Dietrich-Muszalska A, Wachowicz B (2016) Platelet haemostatic function in psychiatric disorders: Effects of antidepressants and antipsychotic drugs. World J Biol Psychiatry 18:564–574.

Dinh TP, Kathuria S, Piomelli D (2004) RNA interference suggests a primary role for monoacylglycerol lipase in the degradation of the endocannabinoid 2-arachidonoylglycerol. Mol Pharmacol 66:1260–1264.

Dong C, Tian Z, Zhang K, Chang L, Qu Y, Pu Y, Ren Q, Fujita Y, Ohgi Y, Futamura T, Hashimoto K (2019) Increased BDNF-TrkB signaling in the nucleus accumbens plays a role in the risk for psychosis after cannabis exposure during adolescence. Pharmacol Biochem Behav 177:61–68.

Dong D, Wang Y, Chang X, Jiang Y, Klugah-Brown B, Luo C, Yao D (2017) Shared abnormality of white matter integrity in schizophrenia and bipolar disorder: A comparative voxel-based meta-analysis. Schizophr Res 185:41–50.

Dowie MJ, Grimsey NL, Hoffman T, Faull RLM, Glass M (2014) Cannabinoid receptor CB2 is expressed on vascular cells, but not astroglial cells in the post-mortem human Huntington's disease brain. J Chem Neuroanat 59–60:62–71.

Dowie MJ, Scotter EL, Molinari E, Glass M (2010) The therapeutic potential of G-protein coupled receptors in Huntington's disease. Pharmacol Ther 128:305–323.

Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME (1997) Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science (80-) 275:661–665.

Dugré JR, Bitar N, Dumais A, Potvin S (2019) Limbic Hyperactivity in Response to Emotionally Neutral Stimuli in Schizophrenia: A Neuroimaging Meta-Analysis of the Hypervigilant Mind. Am J Psychiatry 176:1021–1029.

Dykxhoorn J, Hollander AC, Lewis G, Magnusson C, Dalman C, Kirkbride JB (2019) Risk of schizophrenia, schizoaffective, and bipolar disorders by migrant status, region of origin, and age-at-migration: A national cohort study of 1.8 million people. Psychol Med 49:2354–2363.

Eastwood SL, Burnet PWJ, Gittins R, Baker K, Harrison PJ (2001) Expression of serotonin 5-

HT2A receptors in the human cerebellum and alterations in schizophrenia. Synapse 42:104–114.

Egan CT, Herrick-Davis K, Miller K, Glennon RA, Teitler M (1998) Agonist activity of LSD and lisuride at cloned 5HT(2A) and 5HT(2C) receptors. Psychopharmacology (Berl) 136:409–414.

Egashira N, Mishima K, Uchida T, Hasebe N, Nagai H, Mizuki A, Iwasaki K, Ishii H, Nishimura R, Shoyama Y, Fujiwara M (2004) Anandamide inhibits the DOI-induced head-twitch response in mice. Psychopharmacology (Berl) 171:382–389.

Egerton A, Brugger S, Raffin M, Barker GJ, Lythgoe DJ, McGuire PK, Stone JM (2012) Anterior cingulate glutamate levels related to clinical status following treatment in first-episode schizophrenia. Neuropsychopharmacology 37:2515–2521.

Egerton A, Modinos G, Ferrera D, McGuire P (2017) Neuroimaging studies of GABA in schizophrenia: A systematic review with meta-analysis. Transl Psychiatry 7:e1147.

Egertová M, Elphick MR (2000) Localisation of cannabinoid receptors in the rat brain using antibodies to the intracellular C-terminal tail of CB1. J Comp Neurol 422:159–171.

Eggan SM, Hashimoto T, Lewis DA (2008) Reduced Cortical Cannabinoid 1 Receptor Messenger RNA and Protein Expression in Schizophrenia. Arch Gen Psychiatry 65:772.

Eggan SM, Stoyak SR, Verrico CD, Lewis DA (2010) Cannabinoid CB1 Receptor Immunoreactivity in the Prefrontal Cortex: Comparison of Schizophrenia and Major Depressive Disorder. Neuropsychopharmacology 35:2060–2071.

ElSohly MA (2007) Marijuana and the cannabinoids.

Emamian ES, Hall D, Birnbaum MJ, Karayiorgou M, Gogos JA (2004a) Convergent evidence for impaired AKT1-GSK3β signaling in schizophrenia. Nat Genet 36:131–137.

Emamian ES, Hall D, Birnbaum MJ, Karayiorgou M, Gogos JA (2004b) Convergent evidence for impaired AKT1-GSK3β signaling in schizophrenia. Nat Genet 36:131–137.

Emrich HM, Weber MM, Wendl A, Zihl J, Von Meyer L, Hanisch W (1991) Reduced binocular depth inversion as an indicator of cannabis-induced censorship impairment. Pharmacol Biochem Behav 40:689–690.

English JA, Fan Y, Föcking M, Lopez LM, Hryniewiecka M, Wynne K, Dicker P, Matigian N, Cagney G, Mackay-Sim A, Cotter DR (2015) Reduced protein synthesis in schizophrenia patient-derived olfactory cells. Transl Psychiatry 5:e663.

Erdozain AM, Diez-Alarcia R, Meana JJ, Callado LF (2012) The inverse agonist effect of

rimonabant on G protein activation is not mediated by the cannabinoid CB1 receptor: Evidence from postmortem human brain. Biochem Pharmacol 83:260–268.

Erritzoe D, Rasmussen H, Kristiansen KT, Frokjaer VG, Haugbol S, Pinborg L, Baaré W, Svarer C, Madsen J, Lublin H, Knudsen GM, Glenthoj BY (2008) Cortical and subcortical 5-HT2A receptor binding in neuroleptic-naive first-episode schizophrenic patients. Neuropsychopharmacology 33:2435–2441.

Ertugrul A, Ucar G, Basar K, Demir B, Yabanoglu S, Ulug B (2007) Influence of clozapine on platelet serotonin, monoamine oxidase and plasma serotonin levels. Psychiatry Res.

Fakhoury M (2017) Role of the Endocannabinoid System in the Pathophysiology of Schizophrenia. Mol Neurobiol 54:768–778.

Farrell MS, Werge T, Sklar P, Owen MJ, Ophoff RA, O'Donovan MC, Corvin A, Cichon S, Sullivan PF (2015) Evaluating historical candidate genes for schizophrenia. Mol Psychiatry 20:555–562.

Featherstone RE, Kapur S, Fletcher PJ (2007) The amphetamine-induced sensitized state as a model of schizophrenia. Prog Neuro-Psychopharmacology Biol Psychiatry 31:1556–1571.

Felder CC, Briley EM, Axelrod J, Simpson JT, Mackie K, Devane WA (1993) Anandamide, an endogenous cannabimimetic eicosanoid, binds to the cloned human cannabinoid receptor and stimulates receptor-mediated signal transduction. Proc Natl Acad Sci U S A 90:7656–7660.

Felder CC, Kanterman RY, Ma AL, Axelrod J (1990) Serotonin stimulates phospholipase A2 and the release of arachidonic acid in hippocampal neurons by a type 2 serotonin receptor that is independent of inositolphospholipid hydrolysis. Proc Natl Acad Sci U S A 87:2187–2191.

Fergusson DM, Horwood LJ, Ridder EM (2005) Tests of causal linkages between cannabis use and psychotic symptoms. Addiction 100:354–366.

Fergusson DM, Horwood LJ, Swain-Campbell NR (2003) Cannabis dependence and psychotic symptoms in young people. Psychol Med 33:15–21.

Fernandez-Espejo E, Viveros M-P, Núñez L, Ellenbroek BA, Rodriguez de Fonseca F (2009) Role of cannabis and endocannabinoids in the genesis of schizophrenia. Psychopharmacology (Berl) 206:531–549.

Ferraro L, Murray RM, Di Forti M, Quattrone D, Tripoli G, Sideli L, La Barbera D, La Cascia C (2019) Premorbid Adjustment and IQ in Patients With First-Episode Psychosis: A Multisite

Case-Control Study of Their Relationship With Cannabis Use. Schizophr Res 210:81–88.

Finlay DB, Cawston EE, Grimsey NL, Hunter MR, Korde A, Vemuri VK, Makriyannis A, Glass M (2017) Gαs signalling of the CB1 receptor and the influence of receptor number. Br J Pharmacol 174:2545–2562.

Fiorica-Howells E, Hen R, Gingrich J, Li Z, Gershon MD (2002) 5-HT2A receptors: Location and functional analysis in intestines of wild-type and 5-HT2A knockout mice. Am J Physiol - Gastrointest Liver Physiol 282:G877–G893.

Fitzgerald ML, Mackie K, Pickel VM (2019) Ultrastructural localization of cannabinoid CB1 and mGluR5 receptors in the prefrontal cortex and amygdala. J Comp Neurol 527:2730–2741.

Foley C, Corvin A, Nakagome S (2017) Genetics of Schizophrenia: Ready to Translate? Curr Psychiatry Rep 19:61.

Frangos E, Athanassenas G, Tsitourides S, Katsanou N, Alexandrakou P (1985) Prevalence of DSM III schizophrenia among the first-degree relatives of schizophrenic probands. Acta Psychiatr Scand 72:382–386.

Franklin JM, Carrasco GA (2012) Cannabinoid-induced enhanced interaction and protein levels of serotonin 5-HT(2A) and dopamine D(2) receptors in rat prefrontal cortex. J Psychopharmacol 26:1333–1347.

Franklin JM, Mathew M, Carrasco GA (2013) Cannabinoid-induced upregulation of serotonin 2A receptors in the hypothalamic paraventricular nucleus and anxiety-like behaviors in rats. Neurosci Lett 548:165–169.

Freedman R (2003) Schizophrenia. N Engl J Med.

Freeman AM, Petrilli K, Lees R, Hindocha C, Mokrysz C, Curran HV, Saunders R, Freeman TP (2019) How does cannabidiol (CBD) influence the acute effects of delta-9-tetrahydrocannabinol (THC) in humans? A systematic review. Neurosci Biobehav Rev 107:696–712.

French ED, Dillon K, Wu X (1997) Cannabinoids excite dopamine neurons in the ventral tegmentum and substantia nigra. Neuroreport 8:649–652.

French L et al. (2015) Early cannabis use, polygenic risk score for schizophrenia, and brain maturation in adolescence. JAMA Psychiatry 72:1002–1011.

Fride E, Mechoulam R (1993) Pharmacological activity of the cannabinoid receptor agonist, anandamide, a brain constituent. Eur J Pharmacol:313–314.

Fromer M et al. (2014) De novo mutations in schizophrenia implicate synaptic networks. Nature 506:179–184.

Fuhrmann D, Knoll LJ, Blakemore SJ (2015) Adolescence as a Sensitive Period of Brain Development. Trends Cogn Sci 19:558–566.

Gage SH (2019) Cannabis and psychosis: triangulating the evidence. The Lancet Psychiatry 6:364–365.

Gage SH, Zammit S, Hickman M (2013) Stronger evidence is needed before accepting that cannabis plays an important role in the aetiology of schizophrenia in the population. F1000 Med Rep.

Galiègue S, Mary S, Marchand J, Dussossoy D, Carrière D, Carayon P, Bouaboula M, Shire D, LE Fur G, Casellas P (1995) Expression of Central and Peripheral Cannabinoid Receptors in Human Immune Tissues and Leukocyte Subpopulations. Eur J Biochem 232:54–61.

Galindo L, Moreno E, López-Armenta F, Guinart D, Cuenca-Royo A, Izquierdo-Serra M, Xicota L, Fernandez C, Menoyo E, Fernández-Fernández JM, Benítez-King G, Canela EI, Casadó V, Pérez V, de la Torre R, Robledo P (2018) Cannabis Users Show Enhanced Expression of CB1 -5HT2A Receptor Heteromers in Olfactory Neuroepithelium Cells. Mol Neurobiol 55:6347–6361.

Galve-Roperh I, Sánchez C, Cortés ML, del Pulgar TG, Izquierdo M, Guzmán M (2000) Anti-tumoral action of cannabinoids: Involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. Nat Med 6:313–319.

Gao Y et al. (2010) Loss of retrograde endocannabinoid signaling and reduced adult neurogenesis in diacylglycerol lipase knock-out mice. J Neurosci 30:2017–2024.

Gaoni Y, Mechoulam R (1964) Isolation, Structure, and Partial Synthesis of an Active Constituent of Hashish. J Am Chem Soc 86:1646–1647.

Gaoni Y, Mechoulam R (1971) Isolation and structure of delta1- tetrahydrocannabinol and other neutral cannabinoids from hashish. J Am Chem Soc 93:217–224.

García-Bea A, Miranda-Azpiazu P, Muguruza C, Marmolejo-Martínez-Artesero S, Díez-Alarcia R, Gabilondo AM, Callado LF, Morentin B, González-Maeso J, Meana JJ (2019) Serotonin 5-HT 2A receptor expression and functionality in postmortem frontal cortex of subjects with schizophrenia : Selective biased agonism via G α i1 -proteins. Eur Neuropsychopharmacol 29:1453–1463.

Garcia-Sevilla JA, Pardro D, Giralt MT, Guimon J, Areso P (1990) α2-Adrenoceptor—

Mediated Inhibition of Platelet Adenylate Cyclase and Induction of Aggregation in Major Depression: Effect of Long-term Cyclic Antidepressant Drug Treatment. Arch Gen Psychiatry.

García-Sevilla JA, Ventayol P, Pérez V, Rubovszky G, Puigdemont D, Ferrer-Alcón M, Andreoli A, Guimón J, Alvarez E (2004) Regulation of platelet α2A-adrenoceptors, Gi proteins and receptor kinases in major depression: Effects of mirtazapine treatment. Neuropsychopharmacology 29:580–588.

Gasperi V, Avigliano L, Evangelista D, Oddi S, Chiurchiù V, Lanuti M, Maccarrone M, Catani MV (2014) 2-Arachidonoylglycerol enhances platelet formation from human megakaryoblasts. Cell Cycle 13:3938–3947.

Geaney DP, Schächter M, Elliot JM, Grahame-Smith DG (1984) Characterisation of [3H]lysergic acid diethylamide binding to a 5-hydroxytryptamine receptor on human platelet membranes. Eur J Pharmacol 97:87–93.

Gershon ES, Alliey-Rodriguez N, Liu C (2011) After GWAS: Searching for genetic risk for schizophrenia and bipolar disorder. Am J Psychiatry 168:253–256.

Giedd JN, Blumenthal J, Jeffries NO, Castellanos FX, Liu H, Zijdenbos A, Paus T, Evans AC, Rapoport JL (1999) Brain development during childhood and adolescence: A longitudinal MRI study. Nat Neurosci 2:861–863.

Glantz LA, Lewis DA (2000) Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. Arch Gen Psychiatry 57:65–73.

Glass M, Dragunow M, Faull RLM (1997) Cannabinoid receptors in the human brain: A detailed anatomical and quantitative autoradiographic study in the fetal, neonatal and adult human brain. Neuroscience 77:299–318.

Glass M, Felder CC (1997) Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: Evidence for a G(s) linkage to the CB1 receptor. J Neurosci 17:5327–5333.

Glass M, Northup JK (1999) Agonist selective regulation of G proteins by cannabinoid CB1 and CB2 receptors. Mol Pharmacol 56:1362–1369.

Glennon RA, Titeler M, McKenney JD (1984) Evidence for 5-HT2 involvement in the mechanism of action of hallucinogenic agents. Life Sci 35:2505–2511.

Gokoh M, Kishimoto S, Oka S, Metani Y, Sugiura T (2005) 2-Arachidonoylglycerol, an endogenous cannabinoid receptor ligand, enhances the adhesion of HL-60 cells differentiated into macrophage-like cells and human peripheral blood monocytes. FEBS Lett

579:6473–6478.

Goldman AL, Pezawas L, Mattay VS, Fischl B, Verchinski BA, Zoltick B, Weinberger DR, Meyer-Lindenberg A (2008) Heritability of Brain Morphology Related to Schizophrenia: A Large-Scale Automated Magnetic Resonance Imaging Segmentation Study. Biol Psychiatry 63:475–483.

Golech SA, McCarron RM, Chen Y, Bembry J, Lenz F, Mechoulam R, Shohami E, Spatz M (2004) Human brain endothelium: Coexpression and function of vanilloid and endocannabinoid receptors. Mol Brain Res 132:87–92.

Gómez-Gálvez Y, Palomo-Garo C, Fernández-Ruiz J, García C (2016) Potential of the cannabinoid CB2 receptor as a pharmacological target against inflammation in Parkinson's disease. Prog Neuro-Psychopharmacology Biol Psychiatry 64:200–208.

Gong JP, Onaivi ES, Ishiguro H, Liu QR, Tagliaferro PA, Brusco A, Uhl GR (2006) Cannabinoid CB2 receptors: Immunohistochemical localization in rat brain. Brain Res 1071:10–23.

Gonsiorek W, Lunn C, Fan X, Narula S, Lundell D, Hipkin RW (2000) Endocannabinoid 2-arachidonyl glycerol is a full agonist through human type 2 cannabinoid receptor: Antagonism by anandamide. Mol Pharmacol 57:1045–1050.

Gonzalez-Burgos G, Lewis DA (2008) GABA neurons and the mechanisms of network oscillations: Implications for understanding cortical dysfunction in schizophrenia. Schizophr Bull 34:944–961.

González-Maeso J, Ang RL, Yuen T, Chan P, Weisstaub N V, López-Giménez JF, Zhou M, Okawa Y, Callado LF, Milligan G, Gingrich JA, Filizola M, Meana JJ, Sealfon SC (2008) Identification of a serotonin/glutamate receptor complex implicated in psychosis. Nature 452:93–97.

González-Maeso J, Sealfon SC (2009) Psychedelics and schizophrenia. Trends Neurosci 32:225–232.

González-Maeso J, Weisstaub N V, Zhou M, Chan P, Ivic L, Ang R, Lira A, Bradley-Moore M, Ge Y, Zhou Q, Sealfon SC, Gingrich JA (2007) Hallucinogens Recruit Specific Cortical 5-HT2A Receptor-Mediated Signaling Pathways to Affect Behavior. Neuron 53:439–452.

Gonzalez-Maeso J, Yuen T, Ebersole BJ, Wurmbach E, Lira A, Zhou M, Weisstaub N, Hen R, Gingrich JA, Sealfon SC (2003) Transcriptome fingerprints distinguish hallucinogenic and nonhallucinogenic 5-hydroxytryptamine 2A receptor agonist effects in mouse somatosensory

cortex. J Neurosci 23:8836–8843.

Gorzalka BB, Hill MN, Sun JC (2005) Functional role of the endocannabinoid system and AMPA/kainate receptors in 5-HT2A receptor-mediated wet dog shakes. Eur J Pharmacol 516:28–33.

Gottesman II (1989) Confirming Unexpressed Genotypes for Schizophrenia. Arch Gen Psychiatry 46:867.

Gottesman II, Shields J (1972) Schizophrenia and Genetics: A Twin Study Vantage Point. Oxford, England: Academic Press.

Govitrapong P, Chagkutip J, Turakitwanakan W, Srikiatkhachorn A (2000) Platelet 5-HT(2A) receptors in schizophrenic patients with and without neuroleptic treatment. Psychiatry Res 96:41–50.

Grassin-Delyle S, Naline E, Buenestado A, Faisy C, Alvarez JC, Salvator H, Abrial C, Advenier C, Zemoura L, Devillier P (2014) Cannabinoids inhibit cholinergic contraction in human airways through prejunctional CB 1 receptors. Br J Pharmacol 171:2767–2777.

Gray JA, Roth BL (2001) Paradoxical trafficking and regulation of 5-HT2A receptors by agonists and antagonists. Brain Res Bull 56:441–451.

Grazia Signorello M, Giacobbe E, Segantin A, Avigliano L, Sinigaglia F, Maccarrone M, Leoncini G (2011) Activation of Human Platelets by 2-Arachidonoylglycerol: Role of PKC in NO/cGMP Pathway Modulation. Curr Neurovasc Res 8:200–209.

Grech A, Van Os J, Jones PB, Lewis SW, Murray RM (2005) Cannabis use and outcome of recent onset psychosis. Eur Psychiatry 20:349–353.

Green B, Young R, Kavanagh D (2005) Cannabis use and misuse prevalence among people with psychosis. Br J Psychiatry 187:306–313.

Gu L, Long J, Yan Y, Chen Q, Pan R, Xie X, Mao X, Hu X, Wei B, Su L (2013) HTR2A-1438A/G polymorphism influences the risk of schizophrenia but not bipolar disorder or major depressive disorder: A meta-analysis. J Neurosci Res 91:623–633.

Guloksuz S et al. (2019) Examining the independent and joint effects of molecular genetic liability and environmental exposures in schizophrenia: results from the EUGEI study. World Psychiatry 18:173–182.

Gulsuner S et al. (2020) Genetics of schizophrenia in the South African Xhosa. Science (80-) 367:569–573.

Gulyas AI, Cravatt BF, Bracey MH, Dinh TP, Piomelli D, Boscia F, Freund TF (2004) Segregation of two endocannabinoid-hydrolyzing enzymes into pre- and postsynaptic compartments in the rat hippocampus, cerebellum and amygdala. Eur J Neurosci 20:441–458.

Guo C, He P, Song X, Zheng X (2019) Long-term effects of prenatal exposure to earthquake on adult schizophrenia. Br J Psychiatry 15:730–735.

Guo J, Ikeda SR (2004) Endocannabinoids Modulate N-Type Calcium Channels and G-Protein-Coupled Inwardly Rectifying Potassium Channels via CB1 Cannabinoid Receptors Heterologously Expressed in Mammalian Neurons. Mol Pharmacol 65:665–674.

Gupta S, De Aquino JP, D'Souza DC, Ranganathan M (2019) Effects of haloperidol on the delta-9-tetrahydrocannabinol response in humans: a responder analysis. Psychopharmacology (Berl) 236:2635–2640.

Gurriarán X, Rodríguez-López J, Flórez G, Pereiro C, Fernández JM, Fariñas E, Estévez V, Arrojo M, Costas J (2019) Relationships between substance abuse/dependence and psychiatric disorders based on polygenic scores. Genes, Brain Behav 18:e12504.

Gururajan A, van den Buuse M (2014) Is the mTOR-signalling cascade disrupted in Schizophrenia? J Neurochem 129:377–387.

Häfner H, Löffler W, Maurer K, Hambrecht M, An Der Heiden W (1999) Depression, negative symptoms, social stagnation and social decline in the early course of schizophrenia. Acta Psychiatr Scand 100:105–118.

Halford J, Harrold J, Lawton C, Blundell J (2005) Serotonin (5-HT) Drugs: Effects on Appetite Expression and Use for the Treatment of Obesity. Curr Drug Targets 6:201–213.

Hamilton I, Monaghan M (2019) Cannabis and Psychosis: Are We any Closer to Understanding the Relationship? Curr Psychiatry Rep 21:48.

Hampson RE, Evans GJO, Mu J, Zhuang S yuan, King VC, Childers SR, Deadwyler SA (1995) Role of cyclic amp dependent protein kinase in cannabinoid receptor modulation of potassium "A-current" in cultured rat hippocampal neurons. Life Sci 56:2081–2088.

Han J, Kesner P, Metna-Laurent M, Duan T, Xu L, Georges F, Koehl M, Abrous DN, Mendizabal-Zubiaga J, Grandes P, Liu Q, Bai G, Wang W, Xiong L, Ren W, Marsicano G, Zhang X (2012) Acute cannabinoids impair working memory through astroglial CB 1 receptor modulation of hippocampal LTD. Cell 148:1039–1050.

Hartz SM, Horton AC, Oehlert M, Carey CE, Agrawal A, Bogdan R, Chen L-S, Hancock DB,

Johnson EO, Pato CN, Pato MT, Rice JP, Bierut LJ (2017) Association Between Substance Use Disorder and Polygenic Liability to Schizophrenia. Biol Psychiatry 82:709–715.

Helle S, Ringen PA, Melle I, Larsen T-K, Gjestad R, Johnsen E, Lagerberg TV, Andreassen OA, Kroken RA, Joa I, ten Velden Hegelstad W, Løberg E-M (2016) Cannabis use is associated with 3 years earlier onset of schizophrenia spectrum disorder in a naturalistic, multi-site sample (N = 1119). Schizophr Res 170:217–221.

Henquet C, Krabbendam L, Spauwen J, Kaplan C, Lieb R, Wittchen HU, Van Os J (2005) Prospective cohort study of cannabis use, predisposition for psychosis, and psychotic symptoms in young people. Br Med J 330:11–14.

Herkenham M, Lynn AB, Johnson MR, Melvin LS, De Costa BR, Rice KC (1991) Characterization and localization of cannabinoid receptors in rat brain: A quantitative in vitro autoradiographic study. J Neurosci 11:563–583.

Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, de Costa BR, Rice KC (1990) Cannabinoid receptor localization in brain. Proc Natl Acad Sci 87:1932–1936.

Heston LL (1966) Psychiatric disorders in foster home reared children of schizophrenic mothers. Br J Psychiatrysychiatry 112:819–825.

Hickman M, Vickerman P, MacLeod J, Lewis G, Zammit S, Kirkbride J, Jones P (2009) If cannabis caused schizophrenia - How many cannabis users may need to be prevented in order to prevent one case of schizophrenia? England and Wales calculations. Addiction.

Hilker R, Helenius D, Fagerlund B, Skytthe A, Christensen K, Werge TM, Nordentoft M, Glenthøj B (2018) Heritability of Schizophrenia and Schizophrenia Spectrum Based on the Nationwide Danish Twin Register. Biol Psychiatry 83:492–498.

Hill MN, Sun JC, Tse MTL, Gorzalka BB (2006) Altered responsiveness of serotonin receptor subtypes following long-term cannabinoid treatment. Int J Neuropsychopharmacol 9:277–286.

Hino M, Kunii Y, Matsumoto J, Wada A, Nagaoka A, Niwa S ichi, Takahashi H, Kakita A, Akatsu H, Hashizume Y, Yamamoto S, Yabe H (2016) Decreased VEGFR2 expression and increased phosphorylated Akt1 in the prefrontal cortex of individuals with schizophrenia. J Psychiatr Res 82:100–108.

Ho BC, Wassink TH, Ziebell S, Andreasen NC (2011) Cannabinoid receptor 1 gene polymorphisms and marijuana misuse interactions on white matter and cognitive deficits in schizophrenia. Schizophr Res 128:66–75.

Hoeffer CA, Klann E (2010) mTOR signaling: At the crossroads of plasticity, memory and disease. Trends Neurosci 33:67–75.

Hollander A-C, Dal H, Lewis G, Magnusson C, Kirkbride JB, Dalman C (2016) Refugee migration and risk of schizophrenia and other non-affective psychoses: cohort study of 1.3 million people in Sweden. BMJ 352:i1030.

Hollister LE (1974) Structure activity relationships in man of cannabis constituents, and homologs and metabolites of ∆9tetrahydrocannabinol. Pharmacology 11:3–11.

Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R, Pertwee RG (2002) International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. Pharmacol Rev 54:161–202.

Howlett AC, Qualy JM, Khachatrian LL (1986) Involvement of Gi in the inhibition of adenylate cyclase by cannabimimetic drugs. Mol Pharmacol 29:307–313.

Hoyer D, Hannon JP, Martin GR (2002) Molecular, pharmacological and functional diversity of 5-HT receptors. Pharmacol Biochem Behav 71:533–554.

Hu W, Macdonald ML, Elswick DE, Sweet RA (2015) The glutamate hypothesis of schizophrenia: Evidence from human brain tissue studies. Ann N Y Acad Sci.

Huang H, Miao L, Yang L, Liang F, Wang Q, Zhuang P, Sun Y, Hu Y (2019) AKT-dependent and -independent pathways mediate PTEN deletion-induced CNS axon regeneration. Cell Death Dis 10:203.

Hughes JR, Hatsukami DK, Mitchell JE, Dahlgren LA (1986) Prevalence of smoking among psychiatric outpatients. Am J Psychiatry 143:993–997.

Huhn M, Nikolakopoulou A, Schneider-Thoma J, Krause M, Samara M, Peter N, Arndt T, Bäckers L, Rothe P, Cipriani A, Davis J, Salanti G, Leucht S (2019) Comparative efficacy and tolerability of 32 oral antipsychotics for the acute treatment of adults with multi-episode schizophrenia: a systematic review and network meta-analysis. Lancet 394:939–951.

Ibarra-Lecue I, Mollinedo-Gajate I, Meana JJ, Callado LF, Diez-Alarcia R, Urigüen L (2018) Chronic cannabis promotes pro-hallucinogenic signaling of 5-HT2A receptors through Akt/mTOR pathway. Neuropsychopharmacology 43:2028–2035.

Ide M, Ohnishi T, Murayama M, Matsumoto I, Yamada K, Iwayama Y, Dedova I, Toyota T, Asada T, Takashima A, Yoshikawa T (2006) Failure to support a genetic contribution of AKT1 polymorphisms and altered AKT signaling in schizophrenia. J Neurochem 99:277–287.

Immonen J, Jääskeläinen E, Korpela H, Miettunen J (2017) Age at onset and the outcomes of schizophrenia: A systematic review and meta-analysis. Early Interv Psychiatry.

Inayama Y, Yoneda H, Sakai T, Ishida T, Nonomura Y, Kono Y, Takahata R-I, Koh J, Sakai J, Takai A, Inada Y, Asaba H (1996) Positive association between a DNA sequence variant in the serotonin 2A receptor gene and schizophrenia. Am J Med Genet - Semin Med Genet 67:103–105.

Ingvar DH, Franzén G (1974) Distribution of cerebral activity in chronic schizophrenia. Lancet 2:1484–1486.

Iseger TA, Bossong MG (2015) A systematic review of the antipsychotic properties of cannabidiol in humans. Schizophr Res.

Iwata Y, Nakajima S, Suzuki T, Keefe RSE, Plitman E, Chung JK, Caravaggio F, Mimura M, Graff-Guerrero A, Uchida H (2015) Effects of glutamate positive modulators on cognitive deficits in schizophrenia: A systematic review and meta-Analysis of double-blind randomized controlled trials. Mol Psychiatry 20:1151–1160.

J. Moreau (1845) Du hachisch et de l'aliénation mentale: études psychologiques. Paris.

Jaaro-Peled H (2009) Gene models of schizophrenia: DISC1 mouse models. Prog Brain Res 179:75–86.

Jakab RL, Goldman-Rakic PS (1998) 5-Hydroxytryptamine2A serotonin receptors in the primate cerebral cortex: Possible site of action of hallucinogenic and antipsychotic drugs in pyramidal cell apical dendrites. Proc Natl Acad Sci U S A 95:735–740.

James SL et al. (2018) Global, regional, and national incidence, prevalence, and years lived with disability for 354 Diseases and Injuries for 195 countries and territories, 1990-2017: A systematic analysis for the Global Burden of Disease Study 2017. Lancet.

Janssen I, Krabbendam L, Bak M, Hanssen M, Vollebergh W, Graaf R, Os J (2004) Childhood abuse as a risk factor for psychotic experiences. Acta Psychiatr Scand 109:38–45.

Jaworski J, Sheng M (2006) The Growing Role of mTOR in Neuronal Development and Plasticity. Mol Neurobiol 34:205–220.

Jenkins T, Nguyen J, Polglaze K, Bertrand P (2016) Influence of Tryptophan and Serotonin on Mood and Cognition with a Possible Role of the Gut-Brain Axis. Nutrients 8:56.

Jenko KJ, Hirvonen J, Henter ID, Anderson KB, Zoghbi SS, Hyde TM, Deep-Soboslay A, Innis RB, Kleinman JE (2012) Binding of a tritiated inverse agonist to cannabinoid CB1 receptors is

increased in patients with schizophrenia. Schizophr Res 141:185–188.

Jentsch JD, Roth RH (1999) The neuropsychopharmacology of phencyclidine: From NMDA receptor hypofunction to the dopamine hypothesis of schizophrenia. Neuropsychopharmacology 20:201–225.

Jernigan CS, Goswami DB, Austin MC, Iyo AH, Chandran A, Stockmeier CA, Karolewicz B (2011) The mTOR signaling pathway in the prefrontal cortex is compromised in major depressive disorder. Prog Neuro-Psychopharmacology Biol Psychiatry 35:1774–1779.

Jones C, Watson D, Fone K (2011) Using appropriate animal models to guide clinical drug development animal models of schizophrenia. Br J Pharmacol 164:1162–1194.

Jones CI et al. (2009) A functional genomics approach reveals novel quantitative trait loci associated with platelet signaling pathways. Blood 114:1405–1416.

Joyce JN, Shane A, Lexow N, Winokur A, Casanova MF, Kleinman JE (1993) Serotonin Uptake Sites and Serotonin Receptors Are Altered in the Limbic System of Schizophrenics. Neuropsychopharmacology 8:315–336.

Kahn RS, Linszen DH, Van Os J, Wiersma D, Bruggeman R, Cahn W, De Haan L, Krabbendam L, Myin-Germeys I (2011) Evidence that familial liability for psychosis is expressed as differential sensitivity to cannabis: An analysis of patient-sibling and sibling-control pairs. Arch Gen Psychiatry 68:138–147.

Kane J (1988) Clozapine for the Treatment-Resistant Schizophrenic. Arch Gen Psychiatry 45:789.

Kang K, Huang X-F, Wang Q, Deng C (2009) Decreased density of serotonin 2A receptors in the superior temporal gyrus in schizophrenia-a postmortem study. Prog Neuro-Psychopharmacology Biol Psychiatry 33:867–871.

Kapur S (2003) Psychosis as a State of Aberrant Salience: A Framework Linking Biology, Phenomenology, and Pharmacology in Schizophrenia. Am J Psychiatry 160:13–23.

Kapur S, Seeman P (2002) NMDA receptor antagonists ketamine and PCP have direct effects on the dopamine D2 and serotonin 5-HT2 receptors—implications for models of schizophrenia. Mol Psychiatry 7:837–844.

Kapur S, Zipursky R, Jones C, Remington G, Houle S (2000) Relationship between dopamine D2 occupancy, clinical response, and side effects: A double-blind PET study of first-episode schizophrenia. Am J Psychiatry.

Kargl J, Brown AJ, Andersen L, Dorn G, Schicho R, Waldhoer M, Heinemann A (2013) A Selective Antagonist Reveals a Potential Role of G Protein–Coupled Receptor 55 in Platelet and Endothelial Cell Function. J Pharmacol Exp Ther 346:54–66.

Karwad MA, Couch DG, Wright KL, Tufarelli C, Larvin M, Lund J, O'Sullivan SE (2019) Endocannabinoids and endocannabinoid-like compounds modulate hypoxia-induced permeability in CaCo-2 cells via CB1, TRPV1, and PPARα. Biochem Pharmacol 168:465–472.

Kathuria S, Gaetani S, Fegley D, Valiño F, Duranti A, Tontini A, Mor M, Tarzia G, Rana G La, Calignano A, Giustino A, Tattoli M, Palmery M, Cuomo V, Piomelli D (2003) Modulation of anxiety through blockade of anandamide hydrolysis. Nat Med 9:76–81.

Katona I, Sperlágh B, Maglóczky Z, Sántha E, Köfalvi A, Czirják S, Mackie K, Vizi E., Freund T. (2000) GABAergic interneurons are the targets of cannabinoid actions in the human hippocampus. Neuroscience 100:797–804.

Kawamura Y (2006) The CB1 Cannabinoid Receptor Is the Major Cannabinoid Receptor at Excitatory Presynaptic Sites in the Hippocampus and Cerebellum. J Neurosci 26:2991–3001.

Kempton MJ, Stahl D, Williams SCR, DeLisi LE (2010) Progressive lateral ventricular enlargement in schizophrenia: A meta-analysis of longitudinal MRI studies. Schizophr Res 120:54–62.

Kenakin T (1988) Are receptors promiscuous? Intrinsic efficacy as a transduction phenomenon. Life Sci 43:1095–1101.

Kenakin T (2011) Functional Selectivity and Biased Receptor Signaling. J Pharmacol Exp Ther 336:296–302.

Kendler KS, Diehl SR (1993) The Genetics of Schizophrenia: A Current, Genetic-epidemiologic Perspective. Schizophr Bull 19:261–285.

Kennedy SG, Wagner AJ, Conzen SD, Jordan J, Bellacosa A, Tsichlis PN, Hay N (1997) The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. Genes Dev 11:701–713.

Keshavan M, Tandon R, Boutros N, Nasrallah H (2008) Schizophrenia, "just the facts": What we know in 2008Part 3: Neurobiology. Schizophr Res 106:89–107.

Kety SS (1987) The significance of genetic factors in the etiology of schizophrenia: Results from the national study of adoptees in Denmark. J Psychiatr Res 21:423–429.

Kety SS (1994) Mental Illness in the Biological and Adoptive Relatives of Schizophrenic Adoptees. Arch Gen Psychiatry 51:442.

Khashan AS, Abel KM, McNamee R, Pedersen MG, Webb RT, Baker PN, Kenny LC, Mortensen PB (2008) Higher Risk of Offspring Schizophrenia Following Antenatal Maternal Exposure to Severe Adverse Life Events. Arch Gen Psychiatry 65:146.

Kim JS, Kornhuber HH, Schmid-Burgk W, Holzmüller B (1980) Low cerebrospinal fluid glutamate in schizophrenic patients and a new hypothesis on schizophrenia. Neurosci Lett 20:379–382.

Kishimoto S, Gokoh M, Oka S, Muramatsu M, Kajiwara T, Waku K, Sugiura T (2003) 2-Arachidonoylglycerol Induces the Migration of HL-60 Cells Differentiated into Macrophage-like Cells and Human Peripheral Blood Monocytes through the Cannabinoid CB2 Receptor-dependent Mechanism. J Biol Chem 278:24469–24475.

Kitagishi Y, Kobayashi M, Kikuta K, Matsuda S (2012) Roles of PI3K/AKT/GSK3/mTOR Pathway in Cell Signaling of Mental Illnesses. Depress Res Treat 2012:1–8.

Knudsen P, Vilmar T (1984) Cannabis and neuroleptic agents in schizophrenia. Acta Psychiatr Scand 69:162–174.

Kochunov P et al. (2017) Association of White Matter With Core Cognitive Deficits in Patients With Schizophrenia. JAMA Psychiatry 74:958.

Koethe D, Gerth CW, Neatby MA, Haensel A, Thies M, Schneider U, Emrich HM, Klosterkötter J, Schultze-Lutter F, Leweke FM (2006) Disturbances of visual information processing in early states of psychosis and experimental delta-9-tetrahydrocannabinol altered states of consciousness. Schizophr Res 88:142–150.

Kokkinou M, Ashok AH, Howes OD (2018) The effects of ketamine on dopaminergic function: meta-analysis and review of the implications for neuropsychiatric disorders. Mol Psychiatry 23:59–69.

Kotlicka-Antczak M, Pawełczyk A, Pawełczyk T, Strzelecki D, Żurner N, Karbownik MS (2018) A history of obstetric complications is associated with the risk of progression from an at risk mental state to psychosis. Schizophr Res 197:498–503.

Kotov R (2011) New Dimensions in the Quantitative Classification of Mental Illness. Arch Gen Psychiatry 68:1003.

Kozak KR, Rowlinson SW, Marnett LJ (2000) Oxygenation of the Endocannabinoid, 2-Arachidonylglycerol, to Glyceryl Prostaglandins by Cyclooxygenase-2. J Biol Chem 275:33744–33749.

Kozlovsky N, Amar S, Belmaker RH, Agam G (2006) Psychotropic drugs affect Ser9-

phosphorylated GSK-3 beta protein levels in rodent frontal cortex. Int J Neuropsychopharmacol 9:337–342.

Krabbendam L (2005) Schizophrenia and Urbanicity: A Major Environmental Influence--Conditional on Genetic Risk. Schizophr Bull 31:795–799.

Krystal JH (1994) Subanesthetic Effects of the Noncompetitive NMDA Antagonist, Ketamine, in Humans. Arch Gen Psychiatry 51:199.

Kumar V, Rao NP, Narasimha V, Sathyanarayanan G, Muralidharan K, Varambally S, Venkatasubramanian G, Gangadhar BN (2016) Antipsychotic dose in maintenance treatment of schizophrenia: A retrospective study. Psychiatry Res.

Kumarasinghe N, Beveridge NJ, Gardiner E, Scott RJ, Yasawardene S, Perera A, Mendis J, Suriyakumara K, Schall U, Tooney PA (2013) Gene expression profiling in treatment-naive schizophrenia patients identifies abnormalities in biological pathways involving AKT1 that are corrected by antipsychotic medication. Int J Neuropsychopharmacol 16:1483–1503.

Kurrasch-Orbaugh DM, Parrish JC, Watts VJ, Nichols DE (2003) A complex signaling cascade links the serotonin2A receptor to phospholipase A2 activation: the involvement of MAP kinases. J Neurochem 86:980–991.

Lai W-S, Xu B, Westphal KGC, Paterlini M, Olivier B, Pavlidis P, Karayiorgou M, Gogos JA (2006) Akt1 deficiency affects neuronal morphology and predisposes to abnormalities in prefrontal cortex functioning. Proc Natl Acad Sci 103:16906–16911.

Laplante M, Sabatini DM (2012) mTOR Signaling in Growth Control and Disease. Cell 149:274–293.

Laprairie RB, Bagher AM, Kelly MEM, Denovan-Wright EM (2015) Cannabidiol is a negative allosteric modulator of the cannabinoid CB 1 receptor. Br J Pharmacol 172:4790–4805.

Laprairie RB, Bagher AM, Kelly MEM, Dupré DJ, Denovan-Wright EM (2014) Type 1 Cannabinoid Receptor Ligands Display Functional Selectivity in a Cell Culture Model of Striatal Medium Spiny Projection Neurons. J Biol Chem 289:24845–24862.

Large M, Sharma S, Compton MT, Slade T, Nielssen O (2011) Cannabis Use and Earlier Onset of Psychosis: a systematic meta-analysis. Arch Gen Psychiatry 68:555–561.

Larsen TK, Melle I, Auestad B, Friis S, Haahr U, Johannessen JO, Opjordsmoen S, Rund BR, Simonsen E, Vaglum P, McGlashan TH (2006) Substance abuse in first-episode non-affective psychosis. Schizophr Res 88:55–62.

Laruelle M (1993) Selective Abnormalities of Prefrontal Serotonergic Receptors in Schizophrenia. Arch Gen Psychiatry 50:810–818.

Lauckner JE, Hille B, Mackie K (2005) The cannabinoid agonist WIN55,212-2 increases intracellular calcium via CB1 receptor coupling to Gq/11 G proteins. Proc Natl Acad Sci 102:19144–19149.

Lauckner JE, Jensen JB, Chen H-Y, Lu H-C, Hille B, Mackie K (2008) GPR55 is a cannabinoid receptor that increases intracellular calcium and inhibits M current. Proc Natl Acad Sci 105:2699–2704.

Lee RS, House CM, Cristiano BE, Hannan RD, Pearson RB, Hannan KM (2011) Relative Expression Levels Rather Than Specific Activity Plays the Major Role in Determining In Vivo AKT Isoform Substrate Specificity. Enzyme Res 2011:1–18.

Lee T, Seeman P (1980) Elevation of brain neuroleptic/dopamine receptors in schizophrenia. Am J Psychiatry 137:191–197.

Leggett JD, Aspley S, Beckett SRG, D'Antona AM, Kendall DA, Kendall DA (2004) Oleamide is a selective endogenous agonist of rat and human CB 1 cannabinoid receptors. Br J Pharmacol 141:253–262.

Lehtonen M, Storvik M, Malinen H, Hyytiä P, Lakso M, Auriola S, Wong G, Callaway JC (2011) Determination of endocannabinoids in nematodes and human brain tissue by liquid chromatography electrospray ionization tandem mass spectrometry. J Chromatogr B 879:677–694.

Leucht S, Cipriani A, Spineli L, Mavridis D, Örey D, Richter F, Samara M, Barbui C, Engel RR, Geddes JR, Kissling W, Stapf MP, Lässig B, Salanti G, Davis JM (2013) Comparative efficacy and tolerability of 15 antipsychotic drugs in schizophrenia: a multiple-treatments meta-analysis. Lancet 382:951–962.

Leucht S, Corves C, Arbter D, Engel RR, Li C, Davis JM (2009) Second-generation versus first-generation antipsychotic drugs for schizophrenia: a meta-analysis. Lancet 373:31–41.

Leucht S, Davis JM (2017) Do antipsychotic drugs lose their efficacy for relapse prevention over time? Br J Psychiatry.

Leucht S, Tardy M, Komossa K, Heres S, Kissling W, Salanti G, Davis JM (2012) Antipsychotic drugs versus placebo for relapse prevention in schizophrenia: a systematic review and meta-analysis. Lancet 379:2063–2071.

Levin R, Peres FF, Almeida V, Calzavara MB, Zuardi AW, Hallak JEC, Crippa JAS, Abílio VC

(2014) Effects of cannabinoid drugs on the deficit of prepulse inhibition of startle in an animal model of schizophrenia: the SHR strain. Front Pharmacol 5:10.

Leweke FM, Mueller JK, Lange B, Rohleder C (2016) Therapeutic Potential of Cannabinoids in Psychosis. Biol Psychiatry 79:604–612.

Leweke FM, Schneider U, Radwan M, Schmidt E, Emrich HM (2000) Different Effects of Nabilone and Cannabidiol on Binocular Depth Inversion in Man. Pharmacol Biochem Behav 66:175–181.

Leysen JE, Gommeren W, De Clerck F (1983) Demonstration of S2-receptor binding sites on cat blood platelets using [3H]ketanserin. Eur J Pharmacol 88:125–130.

Li N, Lee B, Liu R-J, Banasr M, Dwyer JM, Iwata M, Li X-Y, Aghajanian G, Duman RS (2010) mTOR-Dependent Synapse Formation Underlies the Rapid Antidepressant Effects of NMDA Antagonists. Science (80-) 329:959–964.

Li Z et al. (2017) Genome-wide association analysis identifies 30 new susceptibility loci for schizophrenia. Nat Genet 49:1576–1583.

Lichtman AH, Varvel SA, Martin BR (2002) Endocannabinoids in cognition and dependence. Prostaglandins, Leukot Essent Fat Acids 66:269–285.

Lindahl JS, Kjellsen BR, Tigert J, Miskimins R (2008) In utero PCP exposure alters oligodendrocyte differentiation and myelination in developing rat frontal cortex. Brain Res 1234:137–147.

Little PJ, Compton DR, Johnson MR, Melvin LS, Martin BR (1988) Pharmacology and stereoselectivity of structurally novel cannabinoids in mice. J Pharmacol Exp Ther 247:1046–1051.

Liu B, Chen X, Wang Z-Q, Tong W-M (2014) Nbn gene inactivation in the CNS of mouse inhibits the myelinating ability of the mature cortical oligodendrocytes. Glia 62:133–144.

Liu J, Wang L, Harvey-White J, Osei-Hyiaman D, Razdan R, Gong Q, Chan AC, Zhou Z, Huang BX, Kim H-Y, Kunos G (2006) A biosynthetic pathway for anandamide. Proc Natl Acad Sci 103:13345–13350.

Liu L, Luo Y, Zhang G, Jin C, Zhou Z, Cheng Z, Yuan G (2016) The mRNA expression of DRD2, PI3KCB, and AKT1 in the blood of acute schizophrenia patients. Psychiatry Res 243:397–402.

Liu X, Wu Z, Lian J, Hu C-H, Huang X-F, Deng C (2017) Time-dependent changes and

potential mechanisms of glucose-lipid metabolic disorders associated with chronic clozapine or olanzapine treatment in rats. Sci Rep 7:2762.

Loewe S (1946) Studies on the pharmacology and acute toxicity of compounds with marihuana activity. J Pharmacol Exp Ther 88:154–161.

Loh HC, Chow TJ, Tang PY, Yong H Sen (2013) No association between AKT1 gene variants and schizophrenia: A Malaysian case-control study and meta-analysis. Psychiatry Res 209:732–733.

Long T, Wagner M, Demske D, Leipe C, Tarasov PE (2017) Cannabis in Eurasia: origin of human use and Bronze Age trans-continental connections. Veg Hist Archaeobot 26:245–258.

López-Giménez JF, González-Maeso J (2017) Hallucinogens and Serotonin 5-HT2A Receptor-Mediated Signaling Pathways. In: Current Topics in Behavioral Neurosciences, pp 45–73.

Luby ED (1959) Study of a New Schizophrenomimetic Drug—Sernyl. Arch Neurol Psychiatry 81:363.

Ludányi A, Hu SS-J, Yamazaki M, Tanimura A, Piomelli D, Watanabe M, Kano M, Sakimura K, Maglóczky Z, Mackie K, Freund TF, Katona I (2011) Complementary synaptic distribution of enzymes responsible for synthesis and inactivation of the endocannabinoid 2-arachidonoylglycerol in the human hippocampus. Neuroscience 174:50–63.

Luxenburger H (1928) Vorläufiger Bericht über psychiatrische Serienuntersuchungen an Zwillingen. Zeitschrift für die gesamte Neurol und Psychiatr 116:297–326.

Maas DA, Vallès A, Martens GJM (2017) Oxidative stress, prefrontal cortex hypomyelination and cognitive symptoms in schizophrenia. Transl Psychiatry 7:e1171.

Maccarrone M (2002) Low fatty acid amide hydrolase and high anandamide levels are associated with failure to achieve an ongoing pregnancy after IVF and embryo transfer. Mol Hum Reprod 8:188–195.

Maccarrone M, Bari M, Del Principe D, Finazzi-Agrò A (2003) Activation of human platelets by 2-arachidonoylglycerol is enhanced by serotonin. Thromb Haemost 89:340–347.

Maccarrone M, Bari M, Menichelli A, Del Principe D, Finazzi Agrò A (1999) Anandamide activates human platelets through a pathway independent of the arachidonate cascade. FEBS Lett 447:277–282.

Maccarrone M, Bari M, Menichelli A, Giuliani E, Del Principe D, Finazzi-Agrò A (2001) Human platelets bind and degrade 2-arachidonoylglycerol, which activates these cells

through a cannabinoid receptor. Eur J Biochem 268:819–825.

Maejima T, Hashimoto K, Yoshida T, Aiba A, Kano M (2001) Presynaptic Inhibition Caused by Retrograde Signal from Metabotropic Glutamate to Cannabinoid Receptors. Neuron 31:463–475.

Mah S, Nelson MR, DeLisi LE, Reneland RH, Markward N, James MR, Nyholt DR, Hayward N, Handoko H, Mowry B, Kammerer S, Braun A (2006) Identification of the semaphorin receptor PLXNA2 as a candidate for susceptibility to schizophrenia. Mol Psychiatry 11:471–478.

Maier RM, Visscher PM, Robinson MR, Wray NR (2018) Embracing polygenicity: a review of methods and tools for psychiatric genetics research. Psychol Med 48:1055–1067.

Makara JK, Mor M, Fegley D, Szabó SI, Kathuria S, Astarita G, Duranti A, Tontini A, Tarzia G, Rivara S, Freund TF, Piomelli D (2005) Selective inhibition of 2-AG hydrolysis enhances endocannabinoid signaling in hippocampus. Nat Neurosci 8:1139–1141.

Mamo D, Graff A, Mizrahi R, Shammi CM, Romeyer F, Kapur S (2007) Differential Effects of Aripiprazole on D 2 , 5-HT 2 , and 5-HT 1A Receptor Occupancy in Patients With Schizophrenia: A Triple Tracer PET Study. Am J Psychiatry 164:1411–1417.

Manning BD, Cantley LC (2007a) AKT/PKB Signaling: Navigating Downstream. Cell 129:1261–1274.

Manning BD, Cantley LC (2007b) AKT/PKB Signaling: Navigating Downstream. Cell 129:1261–1274.

Manrique-Garcia E, Zammit S, Dalman C, Hemmingsson T, Andreasson S, Allebeck P (2014) Prognosis of schizophrenia in persons with and without a history of cannabis use. Psychol Med 44:2513–2521.

Marazziti D, Giannaccini G, Giromella A, Betti L, Pesce D, Nardi I, Rossi A, Lucacchini A, Cassano GB (2003) [3H]-ketanserin binding sites in different psychiatric disorders. Neurochem Int 42:511–516.

March D, Hatch SL, Morgan C, Kirkbride JB, Bresnahan M, Fearon P, Susser E (2008) Psychosis and Place. Epidemiol Rev 30:84–100.

Marchetti D, Spagnolo A, De Matteis V, Filograna L, De Giovanni N (2016) Coronary thrombosis and marijuana smoking: a case report and narrative review of the literature. Drug Test Anal 8:56–62.

Marcolin MA, Davis JM (1992) Platelet monoamine oxidase in schizophrenia: a meta-analysis. Schizophr Res.

Marconi A, Di Forti M, Lewis CM, Murray RM, Vassos E (2016) Meta-analysis of the Association Between the Level of Cannabis Use and Risk of Psychosis. Schizophr Bull 42:1262–1269.

Marek GJ, Aghajanian GK (1996) LSD and the phenethylamine hallucinogen DOI are potent partial agonists at 5-HT2A receptors on interneurons in rat piriform cortex. J Pharmacol Exp Ther 278:1373–1382.

Markon KE (2010) Modeling psychopathology structure: a symptom-level analysis of Axis I and II disorders. Psychol Med 40:273–288.

Marshall CR (1898) A contribution to the pharmacology of Cannbis indica. J Am Med Assoc XXXI:882.

Marshall CR et al. (2017) Contribution of copy number variants to schizophrenia from a genome-wide study of 41,321 subjects. Nat Genet 49:27–35.

Marsicano G, Wotjak CT, Azad SC, Bisogno T, Rammes G, Cascio MG, Hermann H, Tang J, Hofmann C, Zieglgänsberger W, Di Marzo V, Lutz B (2002) The endogenous cannabinoid system controls extinction of aversive memories. Nature 418:530–534.

Martin BR, Compton DR, Thomas BF, Prescott WR, Little PJ, Razdan RK, Johnson MR, Melvin LS, Mechoulam R, Susan J. W (1991) Behavioral, biochemical, and molecular modeling evaluations of cannabinoid analogs. Pharmacol Biochem Behav 40:471–478.

Martínez-Martínez E, Martín-Ruíz A, Martín P, Calvo V, Provencio M, García JM (2016) CB2 cannabinoid receptor activation promotes colon cancer progression via AKT/GSK3β signaling pathway. Oncotarget 7:68781–68791.

Martínez-Pinilla E, Varani K, Reyes-Resina I, Angelats E, Vincenzi F, Ferreiro-Vera C, Oyarzabal J, Canela EI, Lanciego JL, Nadal X, Navarro G, Borea PA, Franco R (2017) Binding and Signaling Studies Disclose a Potential Allosteric Site for Cannabidiol in Cannabinoid CB2 Receptors. Front Pharmacol 8.

Masure S, Haefner B, Wesselink J-J, Hoefnagel E, Mortier E, Verhasselt P, Tuytelaars A, Gordon R, Richardson A (1999) Molecular cloning, expression and characterization of the human serine/threonine kinase Akt-3. Eur J Biochem 265:353–360.

Matheny RW, Geddis A V., Abdalla MN, Leandry LA, Ford M, McClung HL, Pasiakos SM (2018) AKT2 is the predominant AKT isoform expressed in human skeletal muscle. Physiol

Rep 6:e13652.

Mathur A, Law MH, Megson IL, Shaw DJ, Wei J (2010) Genetic association of the AKT1 gene with schizophrenia in a British population. Psychiatr Genet 20:118–122.

Mato S, Aso E, Castro E, Martín M, Valverde O, Maldonado R, Pazos Á (2007) CB 1 knockout mice display impaired functionality of 5-HT 1A and 5-HT 2A/C receptors. J Neurochem 103:2111–2120.

Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346:561–564.

Matsumoto I, Inoue Y, Iwazaki T, Pavey G, Dean B (2005) 5-HT2A and muscarinic receptors in schizophrenia: a postmortem study. Neurosci Lett 379:164–168.

Mazereeuw G, Herrmann N, Bennett SAL, Swardfager W, Xu H, Valenzuela N, Fai S, Lanctôt KL (2013) Platelet activating factors in depression and coronary artery disease: A potential biomarker related to inflammatory mechanisms and neurodegeneration. Neurosci Biobehav Rev 37:1611–1621.

McGlashan TH (2000) Schizophrenia as a Disorder of Developmentally Reduced Synaptic Connectivity. Arch Gen Psychiatry 57:637–648.

McGrath J, Saha S, Chant D, Welham J (2008) Schizophrenia: A Concise Overview of Incidence, Prevalence, and Mortality. Epidemiol Rev 30:67–76.

McGrath J, Saha S, Welham J, El Saadi O, MacCauley C, Chant D (2004) A systematic review of the incidence of schizophrenia: the distribution of rates and the influence of sex, urbanicity, migrant status and methodology. BMC Med 2:13.

McGrath J, Welham J, Scott J, Varghese D, Degenhardt L, Hayatbakhsh MR, Alati R, Williams GM, Bor W, Najman JM (2010) Association Between Cannabis Use and Psychosis-Related Outcomes Using Sibling Pair Analysis in a Cohort of Young Adults. Arch Gen Psychiatry 67:440.

McGuire JL, Depasquale EA, Funk AJ, O'Donnovan SM, Hasselfeld K, Marwaha S, Hammond JH, Hartounian V, Meador-Woodruff JH, Meller J, McCullumsmith RE (2017) Abnormalities of signal transduction networks in chronic schizophrenia. npj Schizophr 3:30.

McGuire P, Robson P, Cubala WJ, Vasile D, Morrison PD, Barron R, Taylor A, Wright S (2018) Cannabidiol (CBD) as an adjunctive therapy in schizophrenia: A multicenter randomized controlled trial. Am J Psychiatry.

McGuire PK, Jones P, Harvey I, Williams M, McGuffin P, Murray RM (1995) Morbid risk of schizophrenia for relatives of patients with cannabis-associated psychosis. Schizophr Res 15:277–281.

McIntosh BT, Hudson B, Yegorova S, Jollimore CAB, Kelly MEM (2009) Agonist-dependent cannabinoid receptor signalling in human trabecular meshwork cells. Br J Pharmacol 152:1111–1120.

McKinney MK, Cravatt BF (2005) Structure and function of fatty acid amide hydrolase. Annu Rev Biochem 74:411–432.

Meador-Woodruff J (2000) Glutamate receptor expression in schizophrenic brain. Brain Res Rev 31:288–294.

Meana JJ, Callado LF, Morentin B (2014) Do post-mortem brain studies provide useful information for Psychiatry? Rev Psiquiatr Salud Ment 7:101–103.

Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, Gopher A, Almog S, Martin BR, Compton DR, Pertwee RG, Griffin G, Bayewitch M, Barg J, Vogel Z (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochem Pharmacol 50:83–90.

Mechoulam R, Feigenbaum JJ, Lander N, Segal M, Järbe TUC, Hiltunen AJ, Consroe P (1988) Enantiomeric cannabinoids: stereospecificity of psychotropic activity. Experientia 44:762–764.

Mechoulam R, Shvo Y (1963) Hashish—I. Tetrahedron 19:2073–2078.

Meltzer HY, Matsubara S, Lee JC (1989a) Classification of typical and atypical antipsychotic drugs on the basis of dopamine D-1, D-2 and serotonin2 pKi values. J Pharmacol Exp Ther 251:238–246.

Meltzer HY, Matsubara S, Lee JC (1989b) Classification of typical and atypical antipsychotic drugs on the basis of dopamine D-1, D-2 and serotonin2 pKi values. J Pharmacol Exp Ther 251:238–246.

Mercado CP, Kilic F (2010) Molecular mechanisms of sert in platelets: Regulation of plasma serotonin levels. Mol Interv.

Merlin MD (2003) Archaeological Evidence for the Tradition of Psychoactive Plant Use in the Old World. Econ Bot 57:295–323.

Merritt K, Egerton A, Kempton MJ, Taylor MJ, McGuire PK (2016) Nature of Glutamate Alterations in Schizophrenia. JAMA Psychiatry 73:665.

Meyer HC, Lee FS, Gee DG (2018) The Role of the Endocannabinoid System and Genetic Variation in Adolescent Brain Development. Neuropsychopharmacology 43:21–33.

Meyuhas O (2015) Ribosomal Protein S6 Phosphorylation. In: International Review of Cell and Molecular Biology, pp 41–73.

Miettunen J, Törmänen S, Murray GK, Jones PB, Mäki P, Ebeling H, Moilanen I, Taanila A, Heinimaa M, Joukamaa M, Veijola J (2008) Association of cannabis use with prodromal symptoms of psychosis in adolescence. Br J Psychiatry 192:470–471.

Miller S, Daily L, Leishman E, Bradshaw H, Straiker A (2018) Δ 9 -Tetrahydrocannabinol and Cannabidiol Differentially Regulate Intraocular Pressure. Investig Opthalmology Vis Sci 59:5904.

Mitchell R, McCulloch D, Lutz E, Johnson M, MacKenzie C, Fennell M, Fink G, Zhou W, Sealfon SC (1998) Rhodopsin-family receptors associate with small G proteins to activate phospholipase D. Nature 392:411–414.

Mittleman MA, Lewis RA, Maclure M, Sherwood JB, Muller JE (2001) Triggering Myocardial Infarction by Marijuana. Circulation 103:2805–2809.

Miyamoto S, Miyake N, Jarskog LF, Fleischhacker WW, Lieberman JA (2012) Pharmacological treatment of schizophrenia: A critical review of the pharmacology and clinical effects of current and future therapeutic agents. Mol Psychiatry 17:1206–1227.

Moghaddam B, Adams B, Verma A, Daly D (1997) Activation of glutamatergic neurotransmission by ketamine: a novel step in the pathway from NMDA receptor blockade to dopaminergic and cognitive disruptions associated with the prefrontal cortex. J Neurosci 17:2921–2927.

Moghaddam B, Javitt D (2012) From Revolution to Evolution: The Glutamate Hypothesis of Schizophrenia and its Implication for Treatment. Neuropsychopharmacology 37:4–15.

Monsma FJ, Mahan LC, McVittie LD, Gerfen CR, Sibley DR (1990) Molecular cloning and expression of a D1 dopamine receptor linked to adenylyl cyclase activation. Proc Natl Acad Sci 87:6723–6727.

Monsma FJ, McVittie LD, Gerfen CR, Mahan LC, Sibley DR (1989) Multiple D2 dopamine receptors produced by alternative RNA splicing. Nature 342:926–929.

Monteleone P, Matias I, Martiadis V, De Petrocellis L, Maj M, Di Marzo V (2005) Blood Levels of the Endocannabinoid Anandamide are Increased in Anorexia Nervosa and in Binge-Eating Disorder, but not in Bulimia Nervosa. Neuropsychopharmacology 30:1216–1221.

Moore H, Jentsch JD, Ghajarnia M, Geyer MA, Grace AA (2006) A Neurobehavioral Systems Analysis of Adult Rats Exposed to Methylazoxymethanol Acetate on E17: Implications for the Neuropathology of Schizophrenia. Biol Psychiatry 60:253–264.

Moore TH, Zammit S, Lingford-Hughes A, Barnes TR, Jones PB, Burke M, Lewis G (2007) Cannabis use and risk of psychotic or affective mental health outcomes: a systematic review. Lancet 370:319–328.

Morgan C, Charalambides M, Hutchinson G, Murray RM (2010) Migration, Ethnicity, and Psychosis: Toward a Sociodevelopmental Model. Schizophr Bull 36:655–664.

Morgan CJA, Freeman TP, Powell J, Curran H V (2016) AKT1 genotype moderates the acute psychotomimetic effects of naturalistically smoked cannabis in young cannabis smokers. Transl Psychiatry 6:e738.

Moyer CE, Shelton MA, Sweet RA (2015) Dendritic spine alterations in schizophrenia. Neurosci Lett 601:46–53.

Muccioli GG (2010) Endocannabinoid biosynthesis and inactivation, from simple to complex. Drug Discov Today 15:474–483.

Muguruza C, Lehtonen M, Aaltonen N, Morentin B, Meana JJ, Callado LF (2013a) Quantification of endocannabinoids in postmortem brain of schizophrenic subjects. Schizophr Res 148:145–150.

Muguruza C, Moreno JL, Umali A, Callado LF, Meana JJ, González-Maeso J (2013b) Dysregulated 5-HT2A receptor binding in postmortem frontal cortex of schizophrenic subjects. Eur Neuropsychopharmacol 23:852–864.

Muguruza C, Morentin B, Meana JJ, Alexander SPH, Callado LF (2019) Endocannabinoid system imbalance in the postmortem prefrontal cortex of subjects with schizophrenia. J Psychopharmacol 33:1132–1140.

Munro S, Thomas KL, Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. Nature 365:61–65.

Murga C, Laguinge L, Wetzker R, Cuadrado A, Gutkind JS (1998) Activation of Akt/Protein Kinase B by G Protein-coupled Receptors. J Biol Chem 273:19080–19085.

Murillo-Rodrıǵuez E, Sánchez-Alavez M, Navarro L, Martıńez-González D, Drucker-Colıń R, Prospéro-Garcıá O (1998) Anandamide modulates sleep and memory in rats. Brain Res 812:270–274.

Murphy DL, Wyatt RJ (1972) Reduced Monoamine Oxidase Activity in Blood Platelets from Schizophrenic Patients. Nature 238:225–226.

Mwansisya TE, Hu A, Li Y, Chen X, Wu G, Huang X, Lv D, Li Z, Liu C, Xue Z, Feng J, Liu Z (2017) Task and resting-state fMRI studies in first-episode schizophrenia: A systematic review. Schizophr Res 189:9–18.

Nassa G, Giurato G, Cimmino G, Rizzo F, Ravo M, Salvati A, Nyman TA, Zhu Y, Vesterlund M, Lehtiö J, Golino P, Weisz A, Tarallo R (2018) Splicing of platelet resident pre-mRNAs upon activation by physiological stimuli results in functionally relevant proteome modifications. Sci Rep 8:498.

Nestler EJ, Hyman SE (2010) Animal models of neuropsychiatric disorders. Nat Neurosci 13:1161–1169.

Netzeband JG, Conroy SM, Parsons KL, Gruol DL (1999) Cannabinoids Enhance NMDA-Elicited Ca 2+ Signals in Cerebellar Granule Neurons in Culture. J Neurosci 19:8765–8777.

Newcomer J (1999) Ketamine-Induced NMDA Receptor Hypofunction as a Model of Memory Impairment and Psychosis. Neuropsychopharmacology 20:106–118.

Newcomer JW (2007) Metabolic considerations in the use of antipsychotic medications: a review of recent evidence. J Clin Psychiatry 68 Suppl 1:20–27.

Nguyen JD, Aarde SM, Vandewater SA, Grant Y, Stouffer DG, Parsons LH, Cole M, Taffe MA (2016) Inhaled delivery of Δ9-tetrahydrocannabinol (THC) to rats by e-cigarette vapor technology. Neuropharmacology 109:112–120.

Nichols DE (2004) Hallucinogens. Pharmacol Ther 101:131–181.

Nielsen RE, Levander S, Kjaersdam Telléus G, Jensen SOW, Østergaard Christensen T, Leucht S (2015) Second-generation antipsychotic effect on cognition in patients with schizophrenia-a meta-analysis of randomized clinical trials. Acta Psychiatr Scand 131:185–196.

Norton N, Williams HJ, Dwyer S, Carroll L, Peirce T, Moskvina V, Segurado R, Nikolov I, Williams NM, Ikeda M, Iwata N, Owen MJ, O'Donovan MC (2007) Association analysis of AKT1 and schizophrenia in a UK case control sample. Schizophr Res 93:58–65.

Núñez C, Ochoa S, Huerta-Ramos E, Baños I, Barajas A, Dolz M, Sánchez B, del Cacho N, Usall J (2016) Cannabis use and cognitive function in first episode psychosis: differential effect of heavy use. Psychopharmacology (Berl) 233:809–821.

Núñez E, Benito C, Pazos MR, Barbachano A, Fajardo O, González S, Tolón RM, Romero J (2004) Cannabinoid CB 2 receptors are expressed by perivascular microglial cells in the human brain: An immunohistochemical study. Synapse 53:208–213.

O'Neill A, Wilson R, Blest-Hopley G, Annibale L, Colizzi M, Brammer M, Giampietro V, Bhattacharyya S (2020) Normalization of mediotemporal and prefrontal activity, and mediotemporal-striatal connectivity, may underlie antipsychotic effects of cannabidiol in psychosis. Psychol Med.

O'Reilly KE, Rojo F, She Q-B, Solit D, Mills GB, Smith D, Lane H, Hofmann F, Hicklin DJ, Ludwig DL, Baselga J, Rosen N (2006) mTOR Inhibition Induces Upstream Receptor Tyrosine Kinase Signaling and Activates Akt. Cancer Res 66:1500–1508.

O'Tuathaigh CMP, O'Connor A-M, O'Sullivan GJ, Lai D, Harvey R, Croke DT, Waddington JL (2008) Disruption to social dyadic interactions but not emotional/anxiety-related behaviour in mice with heterozygous 'knockout' of the schizophrenia risk gene neuregulin-1. Prog Neuro-Psychopharmacology Biol Psychiatry 32:462–466.

Ohara K (1997) Analysis of the 5′-Flanking Promoter Region of the 5-HT2A Receptor Gene in Schizophrenia. Neuropsychopharmacology 17:274–278.

Ohtsuki T, Inada T, Arinami T (2004) Failure to confirm association between AKT1 haplotype and schizophrenia in a Japanese case–control population. Mol Psychiatry 9:981–983.

Okamoto Y, Morishita J, Tsuboi K, Tonai T, Ueda N (2004) Molecular Characterization of a Phospholipase D Generating Anandamide and Its Congeners. J Biol Chem 279:5298–5305.

Okamoto Y, Morishita J, Wang J, Schmid PC, Krebsbach RJ, Schmid HHO, Ueda N (2005) Mammalian cells stably overexpressing N-acylphosphatidylethanolamine-hydrolysing phospholipase D exhibit significantly decreased levels of N-acylphosphatidylethanolamines. Biochem J 389:241–247.

Olabi B, Ellison-Wright I, McIntosh AM, Wood SJ, Bullmore E, Lawrie SM (2011) Are There Progressive Brain Changes in Schizophrenia? A Meta-Analysis of Structural Magnetic Resonance Imaging Studies. Biol Psychiatry 70:88–96.

Onstad S, Skre I, Torgersen S, Kringlen E (1991) Twin concordance for DSM-III-R schizophrenia. Acta Psychiatr Scand 83:395–401.

Osimo EF, Beck K, Reis Marques T, Howes OD (2019) Synaptic loss in schizophrenia: a meta-analysis and systematic review of synaptic protein and mRNA measures. Mol Psychiatry 24:549–561.

Owen F, Crow TJ, Poulter M, Cross AJ, Longden A, Riley GJ (1978) Increased dopamine-receptor sensitivity in schizphrenia. Lancet 312:223–226.

Ozaita A, Puighermanal E, Maldonado R (2007) Regulation of PI3K/Akt/GSK-3 pathway by cannabinoids in the brain. J Neurochem 102:1105–1114.

Pandey SC, Sharma RP, Janicak PG, Marks RC, Davis JM, Pandey GN (1993) Platelet serotonin-2 receptors in schizophrenia: Effects of illness and neuroleptic treatment. Psychiatry Res 48:57–68.

Pardiñas AF et al. (2018) Common schizophrenia alleles are enriched in mutation-intolerant genes and in regions under strong background selection. Nat Genet 50:381–389.

Park KK, Liu K, Hu Y, Smith PD, Wang C, Cai B, Xu B, Connolly L, Kramvis I, Sahin M, He Z (2008) Promoting Axon Regeneration in the Adult CNS by Modulation of the PTEN/mTOR Pathway. Science (80-) 322:963–966.

Parrish JC, Nichols DE (2006) Serotonin 5-HT 2A receptor activation induces 2-arachidonoylglycerol release through a phospholipase c-dependent mechanism. J Neurochem 99:1164–1175.

Pasman JA et al. (2018) GWAS of lifetime cannabis use reveals new risk loci, genetic overlap with psychiatric traits, and a causal effect of schizophrenia liability. Nat Neurosci 21:1161–1170.

Paton WDM (1975) Pharmacology of Marijuana. Annu Rev Pharmacol 15:191–220.

Pazos A, Probst A, Palacios JM (1987) Serotonin receptors in the human brain—IV. Autoradiographic mapping of serotonin-2 receptors. Neuroscience 21:123–139.

Pedersen CB (2001) Evidence of a Dose-Response Relationship Between Urbanicity During Upbringing and Schizophrenia Risk. Arch Gen Psychiatry 58:1039–1046.

Pedrazzi JFC, Issy AC, Gomes F V., Guimarães FS, Del-Bel EA (2015) Cannabidiol effects in the prepulse inhibition disruption induced by amphetamine. Psychopharmacology (Berl) 232:3057–3065.

Peitl V, Vidrih B, Karlović Z, Getaldić B, Peitl M, Karlović D (2016) Platelet serotonin concentration and depressive symptoms in patients with schizophrenia. Psychiatry Res 239:105–110.

Peralta L, Agirregoitia E, Mendoza R, Expósito A, Casis L, Matorras R, Agirregoitia N (2011) Expression and localization of cannabinoid receptors in human immature oocytes and

unfertilized metaphase-II oocytes. Reprod Biomed Online 23:372–379.

Perry T, Buchanan J, Kish S, Hansen S (1979) γ-aminobutyric-acid deficiency in brain of schizphrenic patients. Lancet 313:237–239.

Perry TL (1982) Normal cerebrospinal fluid and brain glutamate levels in schizophrenia do not support the hypothesis of glutamatergic neuronal dysfunction. Neurosci Lett 28:81–85.

Pertwee RG (1997) Pharmacology of cannabinoid CB1 and CB2 receptors. Pharmacol Ther 74:129–180.

Pertwee RG, Howlett AC, Abood ME, Alexander SPH, Di Marzo V, Elphick MR, Greasley PJ, Hansen HS, Kunos G, Mackie K, Mechoulam R, Ross RA (2010) International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid Receptors and Their Ligands: Beyond CB 1 and CB 2. Pharmacol Rev 62:588–631.

Petronis A (2000) The Genes for Major Psychosis Aberrant Sequence or Regulation? Neuropsychopharmacology 23:1–12.

Petrosino S, Verde R, Vaia M, Allarà M, Iuvone T, Di Marzo V (2018) Anti-inflammatory Properties of Cannabidiol, a Nonpsychotropic Cannabinoid, in Experimental Allergic Contact Dermatitis. J Pharmacol Exp Ther 365:652–663.

Pham X, Song G, Lao S, Goff L, Zhu H, Valle D, Avramopoulos D (2016) The DPYSL2 gene connects mTOR and schizophrenia. Transl Psychiatry 6:e933.

Pietiläinen OPH, Paunio T, Loukola A, Tuulio-Henriksson A, Kieseppä T, Thompson P, Toga AW, van Erp TGM, Silventoinen K, Soronen P, Hennah W, Turunen JA, Wedenoja J, Palo OM, Silander K, Lönnqvist J, Kaprio J, Cannon TD, Peltonen L (2009) Association of AKT1 with verbal learning, verbal memory, and regional cortical gray matter density in twins. Am J Med Genet Part B Neuropsychiatr Genet 150B:683–692.

Pignon B et al. (2019) Relationship between childhood trauma and level of insight in schizophrenia: A path-analysis in the national FACE-SZ dataset. Schizophr Res 208:90–96.

Piomelli D (2003) The molecular logic of endocannabinoid signalling. Nat Rev Neurosci 4:873–884.

Pirbhoy PS, Farris S, Steward O (2016) Synaptic activation of ribosomal protein S6 phosphorylation occurs locally in activated dendritic domains. Learn Mem 23:255–269.

Pirbhoy PS, Farris S, Steward O (2017) Synaptically driven phosphorylation of ribosomal protein S6 is differentially regulated at active synapses versus dendrites and cell bodies by

MAPK and PI3K/mTOR signaling pathways. Learn Mem 24:341–357.

Pistis M, Porcu G, Melis M, Diana M, Luigi Gessa G (2001) Effects of cannabinoids on prefrontal neuronal responses to ventral tegmental area stimulation. Eur J Neurosci 14:96–102.

Pithadia A (2009) 5-Hydroxytryptamine Receptor Subtypes and their Modulators with Therapeutic Potentials. J Clin Med Res 1:72–80.

Poutsma M (1965) Additions and Corrections - Chlorination Studies of Unsaturated Materials in Nonpolar Media. IV. The Ionic Pathway for Alkylated Ethylenes. Products and Relative Reactivities. J Am Chem Soc 87:5811–5811.

PPålsson Å, Thulin SO, Tunving K (1982) Cannabis psychoses in south Sweden. Acta Psychiatr Scand 66:311–321.

Pralong D, Tomaskovic-Crook E, Opeskin K, Copolov D, Dean B (2000) Serotonin2A receptors are reduced in the planum temporale from subjects with schizophrenia. Schizophr Res 44:35–45.

Prather PL, Martin NA, Breivogel CS, Childers SR (2000) Activation of cannabinoid receptors in rat brain by WIN 55212-2 produces coupling to multiple G protein alpha-subunits with different potencies. Mol Pharmacol 57:1000–1010.

Presley CS, Abidi AH, Moore BM (2016) Cannabinoid receptor 1 ligands revisited: Pharmacological assessment in the ACTOne system. Anal Biochem 498:8–28.

Procopio M (2008) Maternal exposure to death of a first degree relative during first trimester of pregnancy increases risk of schizophrenia in offspring. Evid Based Ment Health 11:127.

Puig MV, Gulledge AT (2011) Serotonin and Prefrontal Cortex Function: Neurons, Networks, and Circuits. Mol Neurobiol 44:449–464.

Puighermanal E, Biever A, Pascoli V, Melser S, Pratlong M, Cutando L, Rialle S, Severac D, Boubaker-Vitre J, Meyuhas O, Marsicano G, Lüscher C, Valjent E (2017) Ribosomal Protein S6 Phosphorylation Is Involved in Novelty-Induced Locomotion, Synaptic Plasticity and mRNA Translation. Front Mol Neurosci 10.

Puighermanal E, Busquets-Garcia A, Gomis-González M, Marsicano G, Maldonado R, Ozaita A (2013) Dissociation of the Pharmacological Effects of THC by mTOR Blockade. Neuropsychopharmacology 38:1334–1343.

Puighermanal E, Marsicano G, Busquets-Garcia A, Lutz B, Maldonado R, Ozaita A (2009)

Cannabinoid modulation of hippocampal long-term memory is mediated by mTOR signaling. Nat Neurosci 12:1152–1158.

Radhakrishnan R, Wilkinson ST, D'Souza DC (2014) Gone to Pot: A Review of the Association between Cannabis and Psychosis. Front Psychiatry 5:54.

Ranganathan M, Cortes-Briones J, Radhakrishnan R, Thurnauer H, Planeta B, Skosnik P, Gao H, Labaree D, Neumeister A, Pittman B, Surti T, Huang Y, Carson RE, D'Souza DC (2016) Reduced Brain Cannabinoid Receptor Availability in Schizophrenia. Biol Psychiatry 79:997–1005.

Ranganathan M, D'Souza DC (2006) The acute effects of cannabinoids on memory in humans: a review. Psychopharmacology (Berl) 188:425–444.

Rao GK, Kaminski NE (2006) Cannabinoid-Mediated Elevation of Intracellular Calcium: A Structure-Activity Relationship. J Pharmacol Exp Ther 317:820–829.

Rasmussen H, Erritzoe D, Andersen R, Ebdrup BH, Aggernaes B, Oranje B, Kalbitzer J, Madsen J, Pinborg LH, Baaré W, Svarer C, Lublin H, Knudsen GM, Glenthoj B (2010) Decreased Frontal Serotonin2A Receptor Binding in Antipsychotic-Naive Patients With First-Episode Schizophrenia. Arch Gen Psychiatry 67:9.

Rasmussen H, Frokjaer VG, Hilker RW, Madsen J, Anhøj S, Oranje B, Pinborg LH, Glenthøj B, Knudsen GM (2016) Low frontal serotonin 2A receptor binding is a state marker for schizophrenia? Eur Neuropsychopharmacol 26:1248–1250.

Read J, Os J, Morrison AP, Ross CA (2005) Childhood trauma, psychosis and schizophrenia: a literature review with theoretical and clinical implications. Acta Psychiatr Scand 112:330–350.

Reed GL, Fitzgerald ML, Polgar J (2000) Molecular mechanisms of platelet exocytosis: Insights into the "secrete" life of thrombocytes. Blood 96:3334–3342.

Regier DA (1990) Comorbidity of Mental Disorders With Alcohol and Other Drug Abuse. JAMA 264:2511.

Reibaud M, Obinu MC, Ledent C, Parmentier M, Böhme GA, Imperato A (1999) Enhancement of memory in cannabinoid CB1 receptor knock-out mice. Eur J Pharmacol 379:R1–R2.

Reséndiz JC, Kroll MH, Lassila R (2007) Protease-activated receptor-induced Akt activation - regulation and possible function. J Thromb Haemost 5:2484–2493.

Resstel LBM, Tavares RF, Lisboa SFS, Joca SRL, Corrêa FMA, Guimarães FS (2009) 5-HT 1A receptors are involved in the cannabidiol-induced attenuation of behavioural and cardiovascular responses to acute restraint stress in rats. Br J Pharmacol 156:181–188.

Reynolds GP, Rossor MN, Iversen LL (1983) Preliminary studies of human cortical 5-HT2 receptors and their involvement in schizophrenia and neuroleptic drug action. J Neural Transm Suppl 18:273–277.

Rhee M-H, Bayewitch M, Avidor-Reiss T, Levy R, Vogel Z (2002) Cannabinoid Receptor Activation Differentially Regulates the Various Adenylyl Cyclase Isozymes. J Neurochem 71:1525–1534.

Robbe D, Alonso G, Duchamp F, Bockaert J, Manzoni OJ (2001) Localization and Mechanisms of Action of Cannabinoid Receptors at the Glutamatergic Synapses of the Mouse Nucleus Accumbens. J Neurosci 21:109–116.

Robertson DN, Johnson MS, Moggach LO, Holland PJ, Lutz EM, Mitchell R (2003) Selective Interaction of ARF1 with the Carboxy-Terminal Tail Domain of the 5-HT 2A Receptor. Mol Pharmacol 64:1239–1250.

Roland AB, Ricobaraza A, Carrel D, Jordan BM, Rico F, Simon A, Humbert-Claude M, Ferrier J, McFadden MH, Scheuring S, Lenkei Z (2014) Cannabinoid-induced actomyosin contractility shapes neuronal morphology and growth. Elife 3:e03159.

Romigi A, Bari M, Placidi F, Marciani MG, Malaponti M, Torelli F, Izzi F, Prosperetti C, Zannino S, Corte F, Chiaramonte C, Maccarrone M (2010) Cerebrospinal fluid levels of the endocannabinoid anandamide are reduced in patients with untreated newly diagnosed temporal lobe epilepsy. Epilepsia 51:768–772.

Rosanoff AJ, Handy LM, Plesset IR, Brush S (1934) The etiology of so-called schizophrenic psychoses. Am J Psychiatry 91:247–286.

Ross RA (2009) The enigmatic pharmacology of GPR55. Trends Pharmacol Sci 30:156–163.

Rossato M, Ion Popa F, Ferigo M, Clari G, Foresta C (2005) Human Sperm Express Cannabinoid Receptor Cb 1 , the Activation of Which Inhibits Motility, Acrosome Reaction, and Mitochondrial Function. J Clin Endocrinol Metab 90:984–991.

Roth BL (2011) Irving Page Lecture: 5-HT2A serotonin receptor biology: Interacting proteins, kinases and paradoxical regulation. Neuropharmacology 61:348–354.

Rottanburg D (1982) Cannabis-associated psychosis with hypomanic features. Lancet 320:1364–1366.

Rowland LM, Bustillo JR, Mullins PG, Jung RE, Lenroot R, Landgraf E, Barrow R, Yeo R, Lauriello J, Brooks WM (2005) Effects of Ketamine on Anterior Cingulate Glutamate Metabolism in Healthy Humans: A 4-T Proton MRS Study. Am J Psychiatry 162:394–396.

Rozengurt E, Soares HP, Sinnet-Smith J (2014) Suppression of Feedback Loops Mediated by PI3K/mTOR Induces Multiple Overactivation of Compensatory Pathways: An Unintended Consequence Leading to Drug Resistance. Mol Cancer Ther 13:2477–2488.

Rubino T, Parolaro D (2016) The Impact of Exposure to Cannabinoids in Adolescence: Insights From Animal Models. Biol Psychiatry 79:578–585.

Rubino T, Viganò D, Massi P, Parolaro D (2001) The psychoactive ingredient of marijuana induces behavioural sensitization. Eur J Neurosci 14:884–886.

Rubino T, Viganò D, Massi P, Parolaro D (2003) Cellular mechanisms of Δ 9 - tetrahydrocannabinol behavioural sensitization. Eur J Neurosci 17:325–330.

Ruiz J, Gabilondo AM, Javier Meana J, García-Sevilla JA (1992) Increased [3H] raclopride binding sites in postmortem brains from schizophrenic violent suicide victims. Psychopharmacology (Berl) 109:410–414.

Russo EB, Burnett A, Hall B, Parker KK (2005) Agonistic Properties of Cannabidiol at 5-HT1a Receptors. Neurochem Res 30:1037–1043.

Ruvinsky I, Meyuhas O (2006) Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. Trends Biochem Sci 31:342–348.

Ryberg E, Larsson N, Sjögren S, Hjorth S, Hermansson N-O, Leonova J, Elebring T, Nilsson K, Drmota T, Greasley PJ (2009) The orphan receptor GPR55 is a novel cannabinoid receptor. Br J Pharmacol 152:1092–1101.

Sáiz PA, García-Portilla MP, Arango C, Morales B, Alvarez V, Coto E, Fernández JM, Bascarán MT, Bousoño M, Bobes J (2007) Association study of serotonin 2A receptor (5-HT2A) and serotonin transporter (5-HTT) gene polymorphisms with schizophrenia. Prog Neuro-Psychopharmacology Biol Psychiatry 31:741–745.

Saladrigas-Manjón S, Dučić T, Galindo L, Fernández-Avilés C, Pérez V, de la Torre R, Robledo P (2020) Effects of Cannabis Use on the Protein and Lipid Profile of Olfactory Neuroepithelium Cells from Schizophrenia Patients Studied by Synchrotron-Based FTIR Spectroscopy. Biomolecules 10:329.

Samara MT, Klupp E, Helfer B, Rothe PH, Schneider-Thoma J, Leucht S (2018) Increasing antipsychotic dose for non response in schizophrenia. Cochrane Database Syst Rev

5:CD011883.

Samara MT, Nikolakopoulou A, Salanti G, Leucht S (2019) How Many Patients With Schizophrenia Do Not Respond to Antipsychotic Drugs in the Short Term? An Analysis Based on Individual Patient Data From Randomized Controlled Trials. Schizophr Bull 45:639–646.

Samartzis L, Dima D, Fusar-Poli P, Kyriakopoulos M (2014) White Matter Alterations in Early Stages of Schizophrenia: A Systematic Review of Diffusion Tensor Imaging Studies. J Neuroimaging 24:101–110.

Sánchez-Fernández G, Cabezudo S, García-Hoz C, Benincá C, Aragay AM, Mayor F, Ribas C (2014) Gαq signalling: The new and the old. Cell Signal 26:833–848.

Sang N, Zhang J, Chen C (2007) COX-2 oxidative metabolite of endocannabinoid 2-AG enhances excitatory glutamatergic synaptic transmission and induces neurotoxicity. J Neurochem 102:1966–1977.

Sarbassov DD, Ali SM, Sengupta S, Sheen J-H, Hsu PP, Bagley AF, Markhard AL, Sabatini DM (2006) Prolonged Rapamycin Treatment Inhibits mTORC2 Assembly and Akt/PKB. Mol Cell 22:159–168.

Saunders C, Siuta M, Robertson SD, Davis AR, Sauer J, Matthies HJG, Gresch PJ, Airey DC, Lindsley CW, Schetz JA, Niswender KD, Veenstra-Vanderweele JM, Galli A (2014) Neuronal ablation of p-Akt at Ser473 leads to altered 5-HT1A/2A receptor function. Neurochem Int 73:113–121.

Scarr E, Pavey G, Copolov D, Dean B (2004) Hippocampal 5-hydroxytryptamine receptors: abnormalities in postmortem brain from schizophrenic subjects. Schizophr Res 71:383–392.

Schachter M, Geaney D, Grahame-Smith D, Cowen P, Elliott J (1985) Increased platelet membrane [3H]-LSD binding in patients on chronic neuroleptic treatment. Br J Clin Pharmacol 19:453–457.

Schizophrenia Working Group of the Psychiatric Genomics Consortium - (2014) Biological Insights From 108 Schizophrenia-Associated Genetic Loci. Nature 511:421–427.

Schmid CL, Bohn LM (2010) Serotonin, But Not N-Methyltryptamines, Activates the Serotonin 2A Receptor Via a -Arrestin2/Src/Akt Signaling Complex In Vivo. J Neurosci 30:13513–13524.

Schmid CL, Raehal KM, Bohn LM (2008) Agonist-directed signaling of the serotonin 2A receptor depends on -arrestin-2 interactions in vivo. Proc Natl Acad Sci 105:1079–1084.

Schoeler T, Petros N, Di Forti M, Pingault J-B, Klamerus E, Foglia E, Small A, Murray R, Bhattacharyya S (2016) Association Between Continued Cannabis Use and Risk of Relapse in First-Episode Psychosis. JAMA Psychiatry 73:1173–1179.

Schousboe A (1981) Transport and Metabolism of Glutamate and Gaba in Neurons and Glial Cells. Int Rev Neurobiol 22:1–45.

Schuel H, Burkman LJ, Lippes J, Crickard K, Forester E, Piomelli D, Giuffrida A (2002) N-Acylethanolamines in human reproductive fluids. Chem Phys Lipids 121:211–227.

Schwab SG, Hoefgen B, Hanses C, Hassenbach MB, Albus M, Lerer B, Trixler M, Maier W, Wildenauer DB (2005) Further Evidence for Association of Variants in the AKT1 Gene with Schizophrenia in a Sample of European Sib-Pair Families. Biol Psychiatry 58:446–450.

Seeman P (2011) All Roads to Schizophrenia Lead to Dopamine Supersensitivity and Elevated Dopamine D2High Receptors. CNS Neurosci Ther 17:118–132.

Seeman P, Guan H-C, Van Tol HHM (1993) Dopamine D4 receptors elevated in schizophrenia. Nature 365:441–445.

Seeman P, Lee T (1975) Antipsychotic drugs: direct correlation between clinical potency and presynaptic action on dopamine neurons. Science (80-) 188:1217–1219.

Seeman P, Lee T, Chau-Wong M, Wong K (1976) Antipsychotic drug doses and neuroleptic/dopamine receptors. Nature 261:717–719.

Selemon LD, Zecevic N (2015) Schizophrenia: a tale of two critical periods for prefrontal cortical development. Transl Psychiatry 5:e623.

Selten J-P, Cantor-Graae E (2005) Social defeat: Risk factor for schizophrenia? Br J Psychiatry 187:101–102.

Selvaraj S, Arnone D, Cappai A, Howes O (2014) Alterations in the serotonin system in schizophrenia: A systematic review and meta-analysis of postmortem and molecular imaging studies. Neurosci Biobehav Rev 45:233–245.

Setién-Suero E, Neergaard K, Ortiz-García de la Foz V, Suárez-Pinilla P, Martínez-García O, Crespo-Facorro B, Ayesa-Arriola R (2019) Stopping cannabis use benefits outcome in psychosis: findings from 10-year follow-up study in the PAFIP-cohort. Acta Psychiatr Scand 140:349–359.

Shan J, Khelashvili G, Mondal S, Mehler EL, Weinstein H (2012) Ligand-Dependent Conformations and Dynamics of the Serotonin 5-HT2A Receptor Determine Its Activation

and Membrane-Driven Oligomerization Properties Livesay DR, ed. PLoS Comput Biol 8:e1002473.

Sheffler DJ, Kroeze WK, Garcia BG, Deutch AY, Hufeisen SJ, Leahy P, Bruning JC, Roth BL (2006) p90 ribosomal S6 kinase 2 exerts a tonic brake on G protein-coupled receptor signaling. Proc Natl Acad Sci 103:4717–4722.

Sherman AD, Davidson AT, Baruah S, Hegwood TS, Waziri R (1991) Evidence of glutamatergic deficiency in schizophrenia. Neurosci Lett 121:77–80.

Shin M, Buckner A, Prince J, Bullock TNJ, Hsu K-L (2019) Diacylglycerol Lipase-β Is Required for TNF-α Response but Not CD8+ T Cell Priming Capacity of Dendritic Cells. Cell Chem Biol 26:1036-1041.e3.

Signorello MG, Leoncini G (2014) Effect of 2-arachidonoylglycerol on myosin light chain phosphorylation and platelet activation: The role of phosphatidylinositol 3 kinase/AKT pathway. Biochimie 105:182–191.

Signorello MG, Leoncini G (2016) Regulation of cAMP Intracellular Levels in Human Platelets Stimulated by 2-Arachidonoylglycerol. J Cell Biochem 117:1240–1249.

Sim-Selley LJ, Schechter NS, Rorrer WK, Dalton GD, Hernandez J, Martin BR, Selley DE (2006) Prolonged Recovery Rate of CB 1 Receptor Adaptation after Cessation of Long-Term Cannabinoid Administration. Mol Pharmacol 70:986–996.

Simpson MDC, Slater P, Deakin JFW, Royston MC, Skan WJ (1989) Reduced GABA uptake sites in the temporal lobe in schizophrenia. Neurosci Lett 107:211–215.

Simpson MDC, Slater P, Royston MC, Deakin JFW (1992) Regionally selective deficits in uptake sites for glutamate and gamma-aminobutyric acid in the basal ganglia in schizophrenia. Psychiatry Res 42:273–282.

Singh T et al. (2016) Rare loss-of-function variants in SETD1A are associated with schizophrenia and developmental disorders. Nat Neurosci 19:571–577.

Siris SG (2000) Depression in Schizophrenia: Perspective in the Era of "Atypical" Antipsychotic Agents. Am J Psychiatry 157:1379–1389.

Siu M, Chong CS, Lo WT (2018) Prevalence and clinicians' awareness of psychiatric comorbidities among first-episode schizophrenia. Early Interv Psychiatry 12:1128–1136.

Skene NG, Bryois J, Bakken TE, Breen G, Crowley JJ, Gaspar HA, Giusti-Rodriguez P, Hodge RD, Miller JA, Muñoz-Manchado AB, O'Donovan MC, Owen MJ, Pardiñas AF, Ryge J, Walters

JTR, Linnarsson S, Lein ES, Sullivan PF, Hjerling-Leffler J (2018) Genetic identification of brain cell types underlying schizophrenia. Nat Genet 50:825–833.

Skosnik PD, Hajós M, Cortes-Briones JA, Edwards CR, Pittman BP, Hoffmann WE, Sewell AR, D'Souza DC, Ranganathan M (2018) Cannabinoid receptor-mediated disruption of sensory gating and neural oscillations: A translational study in rats and humans. Neuropharmacology 135:412–423.

Smoller JW et al. (2013) Identification of risk loci with shared effects on five major psychiatric disorders: a genome-wide analysis. Lancet 381:1371–1379.

Sokoloff P, Giros B, Martres M-P, Bouthenet M-L, Schwartz J-C (1990) Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics. Nature 347:146–151.

Spies M, Knudsen GM, Lanzenberger R, Kasper S (2015) The serotonin transporter in psychiatric disorders: insights from PET imaging. The Lancet Psychiatry 2:743–755.

Spiller KJ, Bi G, He Y, Galaj E, Gardner EL, Xi Z (2019) Cannabinoid CB 1 and CB 2 receptor mechanisms underlie cannabis reward and aversion in rats. Br J Pharmacol 176:1268–1281.

Spokes EGS, Garrett NJ, Rossor MN, Iversen LL (1980) Distribution of GABA in post-mortem brain tissue from control, psychotic and Huntington's chorea subjects. J Neurol Sci.

Stahl SM (1977) The Human Platelet. Arch Gen Psychiatry 34:509–516.

Stahl SM, Woo DJ, Mefford IN, Berger PA, Ciaranello RD (1983) Hyperserotonemia and platelet serotonin uptake and release in schizophrenia and affective disorders. Am J Psychiatry 140:26–30.

Stam NJ, Van Huizen F, Van Alebeek C, Dijkema R, Tonnaer JADM, Olijve W (1992) Genomic organization, coding sequence and functional expression of human 5-HT2 and 5-HT1A receptor genes. Eur J Pharmacol Mol Pharmacol 227:153–162.

Steeds H, Carhart-Harris RL, Stone JM (2015) Drug models of schizophrenia. Ther Adv Psychopharmacol 5:43–58.

Stella N, Schweitzer P, Piomelli D (1997) A second endogenous cannabinoid that modulates long-term potentiation. Nature 388:773–778.

Stone JM, Dietrich C, Edden R, Mehta MA, De Simoni S, Reed LJ, Krystal JH, Nutt D, Barker GJ (2012) Ketamine effects on brain GABA and glutamate levels with 1H-MRS: relationship to ketamine-induced psychopathology. Mol Psychiatry 17:664–665.

Stone JM, Erlandsson K, Arstad E, Squassante L, Teneggi V, Bressan RA, Krystal JH, Ell PJ, Pilowsky LS (2008) Relationship between ketamine-induced psychotic symptoms and NMDA receptor occupancy—a [123I]CNS-1261 SPET study. Psychopharmacology (Berl) 197:401–408.

Storozhuk M V., Zholos A V. (2018) TRP Channels as Novel Targets for Endogenous Ligands: Focus on Endocannabinoids and Nociceptive Signalling. Curr Neuropharmacol 16:137–150.

Strachan RT, Sciaky N, Cronan MR, Kroeze WK, Roth BL (2010) Genetic Deletion of p90 Ribosomal S6 Kinase 2 Alters Patterns of 5-Hydroxytryptamine 2A Serotonin Receptor Functional Selectivity. Mol Pharmacol 77:327–338.

Strachan RT, Sheffler DJ, Willard B, Kinter M, Kiselar JG, Roth BL (2009) Ribosomal S6 Kinase 2 Directly Phosphorylates the 5-Hydroxytryptamine 2A (5-HT 2A) Serotonin Receptor, Thereby Modulating 5-HT 2A Signaling. J Biol Chem 284:5557–5573.

Straiker AJ, Borden CR, Sullivan JM (2002) G-Protein α Subunit Isoforms Couple Differentially to Receptors that Mediate Presynaptic Inhibition at Rat Hippocampal Synapses. J Neurosci 22:2460–2468.

Stroup TS, Gerhard T, Crystal S, Huang C, Tan Z, Wall MM, Mathai C, Olfson M (2019) Comparative Effectiveness of Adjunctive Psychotropic Medications in Patients With Schizophrenia. JAMA Psychiatry 76:508.

Subburaju S, Sromek AW, Seeman P, Neumeyer JL (2018) New Dopamine D2 Receptor Agonist, [3 H]MCL-536, for Detecting Dopamine D2high Receptors in Vivo. ACS Chem Neurosci 9:1283–1289.

Sullivan PF, Daly MJ, O'Donovan M (2012) Genetic architectures of psychiatric disorders: the emerging picture and its implications. Nat Rev Genet 13:537–551.

Sullivan PF, Kendler KS, Neale MC (2003) Schizophrenia as a Complex Trait. Arch Gen Psychiatry 60:1187.

Sundquist K, Frank G, Sundquist J (2004) Urbanisation and incidence of psychosis and depression. Br J Psychiatry 184:293–298.

Sykes DA, Moore H, Stott L, Holliday N, Javitch JA, Robert Lane J, Charlton SJ (2017) Extrapyramidal side effects of antipsychotics are linked to their association kinetics at dopamine D2 receptors. Nat Commun.

Takahashi N, Sakurai T, Davis KL, Buxbaum JD (2011) Linking oligodendrocyte and myelin dysfunction to neurocircuitry abnormalities in schizophrenia. Prog Neurobiol 93:13–24.

Takei N, Nawa H (2014) mTOR signaling and its roles in normal and abnormal brain development. Front Mol Neurosci 7:28.

Tan H-Y, Nicodemus KK, Chen Q, Li Z, Brooke JK, Honea R, Kolachana BS, Straub RE, Meyer-Lindenberg A, Sei Y, Mattay VS, Callicott JH, Weinberger DR (2008) Genetic variation in AKT1 is linked to dopamine-associated prefrontal cortical structure and function in humans. J Clin Invest 118:2200–2208.

Tang G, Gudsnuk K, Kuo S-H, Cotrina ML, Rosoklija G, Sosunov A, Sonders MS, Kanter E, Castagna C, Yamamoto A, Yue Z, Arancio O, Peterson BS, Champagne F, Dwork AJ, Goldman J, Sulzer D (2014) Loss of mTOR-Dependent Macroautophagy Causes Autistic-like Synaptic Pruning Deficits. Neuron 83:1131–1143.

Tao R, Davis KN, Li C, Shin JH, Gao Y, Jaffe AE, Gondré-Lewis MC, Weinberger DR, Kleinman JE, Hyde TM (2018) GAD1 alternative transcripts and DNA methylation in human prefrontal cortex and hippocampus in brain development, schizophrenia. Mol Psychiatry 23:1496–1505.

Teroganova N, Girshkin L, Suter CM, Green MJ (2016) DNA methylation in peripheral tissue of schizophrenia and bipolar disorder: a systematic review. BMC Genet 17:27.

Théberge J, Bartha R, Drost DJ, Menon RS, Malla A, Takhar J, Neufeld RW, Rogers J, Pavlosky W, Schaefer B, Densmore M, Al-Semaan Y, Williamson PC (2002) Glutamate and Glutamine Measured With 4.0 T Proton MRS in Never-Treated Patients With Schizophrenia and Healthy Volunteers. Am J Psychiatry 159:1944–1946.

Thieme U, Schelling G, Hauer D, Greif R, Dame T, Laubender RP, Bernhard W, Thieme D, Campolongo P, Theiler L (2014) Quantification of anandamide and 2-arachidonoylglycerol plasma levels to examine potential influences of tetrahydrocannabinol application on the endocannabinoid system in humans. Drug Test Anal 6:17–23.

Thiselton DL, Vladimirov VI, Kuo P-H, McClay J, Wormley B, Fanous A, O'Neill FA, Walsh D, Van den Oord EJCG, Kendler KS, Riley BP (2008) AKT1 Is Associated with Schizophrenia Across Multiple Symptom Dimensions in the Irish Study of High Density Schizophrenia Families. Biol Psychiatry 63:449–457.

Thomas EA, Carson MJ, Neal MJ, Sutcliffe JG (1997) Unique allosteric regulation of 5-hydroxytryptamine receptor-mediated signal transduction by oleamide. Proc Natl Acad Sci 94:14115–14119.

Thompson JL, Urban N, Slifstein M, Xu X, Kegeles LS, Girgis RR, Beckerman Y, Harkavy-Friedman JM, Gil R, Abi-Dargham A (2013) Striatal dopamine release in schizophrenia comorbid with substance dependence. Mol Psychiatry 18:909–915.

Thomson AM, West DC, Lodge D (1985) An N-methylaspartate receptor-mediated synapse in rat cerebral cortex: a site of action of ketamine? Nature 313:479–481.

Tibbo P, Hanstock C, Valiakalayil A, Allen P (2004) 3-T Proton MRS Investigation of Glutamate and Glutamine in Adolescents at High Genetic Risk for Schizophrenia. Am J Psychiatry 161:1116–1118.

Tien AY, Anthony JC (1990) Epidemiological Analysis of Alcohol and Drug Use as Risk Factors for Psychotic Experiences. J Nerv Ment Dis 178:473–480.

Tienari P (1991) Interaction between genetic vulnerability and family environment: the Finnish adoptive family study of schizophrenia. Acta Psychiatr Scand 84:460–465.

Tiihonen J, Taipale H, Mehtälä J, Vattulainen P, Correll CU, Tanskanen A (2019) Association of Antipsychotic Polypharmacy vs Monotherapy With Psychiatric Rehospitalization Among Adults With Schizophrenia. JAMA Psychiatry 76:499–507.

Toda M, Abi-Dargham A (2007) Dopamine hypothesis of schizophrenia: Making sense of it all. Curr Psychiatry Rep 9:329–336.

Toto S, Grohmann R, Bleich S, Frieling H, Maier HB, Greil W, Cordes J, Schmidt-Kraepelin C, Kasper S, Stübner S, Degner D, Druschky K, Zindler T, Neyazi A (2019) Psychopharmacological Treatment of Schizophrenia Over Time in 30 908 Inpatients: Data From the AMSP Study. Int J Neuropsychopharmacol 22:560–573.

Tournier BB, Tsartsalis S, Dimiziani A, Millet P, Ginovart N (2016) Time-dependent effects of repeated THC treatment on dopamine D2/3 receptor-mediated signalling in midbrain and striatum. Behav Brain Res 311:322–329.

Trauelsen AM, Bendall S, Jansen JE, Nielsen H-GL, Pedersen MB, Trier CH, Haahr UH, Simonsen E (2015) Childhood adversity specificity and dose-response effect in non-affective first-episode psychosis. Schizophr Res 165:52–59.

Treffert DA (1978) Marijuana use in schizophrenia: a clear hazard. Am J Psychiatry 135:1213–1215.

Trezza V, Vanderschuren LJMJ (2008) Bidirectional cannabinoid modulation of social behavior in adolescent rats. Psychopharmacology (Berl) 197:217–227.

Üçok A, Gorwood P, Karadayi G (2012) Employment and its relationship with functionality and quality of life in patients with schizophrenia: EGOFORS Study. Eur Psychiatry.

Ujike H, Takaki M, Nakata K, Tanaka Y, Takeda T, Kodama M, Fujiwara Y, Sakai A, Kuroda S

(2002) CNR1, central cannabinoid receptor gene, associated with susceptibility to hebephrenic schizophrenia. Mol Psychiatry 7:515–518.

United Nations Office on Drugs and Crime (UNODC) (2017) Global overview of drug demand and supply. World Drug Rep 2018:1–66.

Upthegrove R, Marwaha S, Birchwood M (2017) Depression and Schizophrenia: Cause, Consequence or Trans-diagnostic Issue? Schizophr Bull 43:240–244.

Urigüen L, García-Fuster MJ, Callado LF, Morentin B, La Harpe R, Casadó V, Lluis C, Franco R, García-Sevilla JA, Meana JJ (2009) Immunodensity and mRNA expression of A2A adenosine, D2 dopamine, and CB1 cannabinoid receptors in postmortem frontal cortex of subjects with schizophrenia: effect of antipsychotic treatment. Psychopharmacology (Berl) 206:313–324.

Uzuneser TC, Speidel J, Kogias G, Wang A-L, de Souza Silva MA, Huston JP, Zoicas I, von Hörsten S, Kornhuber J, Korth C, Müller CP (2019) Disrupted-in-Schizophrenia 1 (DISC1) Overexpression and Juvenile Immune Activation Cause Sex-Specific Schizophrenia-Related Psychopathology in Rats. Front Psychiatry 10:222.

van der Ven E, Selten J-P (2018) Migrant and ethnic minority status as risk indicators for schizophrenia. Curr Opin Psychiatry 31:231–236.

van Haren NEM, Pol HEH, Schnack HG, Cahn W, Brans R, Carati I, Rais M, Kahn RS (2008) Progressive Brain Volume Loss in Schizophrenia Over the Course of the Illness: Evidence of Maturational Abnormalities in Early Adulthood. Biol Psychiatry 63:106–113.

van Kesteren CFMG, Gremmels H, de Witte LD, Hol EM, Van Gool AR, Falkai PG, Kahn RS, Sommer IEC (2017) Immune involvement in the pathogenesis of schizophrenia: a meta-analysis on postmortem brain studies. Transl Psychiatry 7:e1075.

Van Oekelen D, Luyten WHML, Leysen JE (2003) 5-HT2A and 5-HT2C receptors and their atypical regulation properties. Life Sci 72:2429–2449.

van Os J (2002) Cannabis Use and Psychosis: A Longitudinal Population-based Study. Am J Epidemiol 156:319–327.

van Os J, Kenis G, Rutten BPF (2010) The environment and schizophrenia. Nature 468:203–212.

van Os J, Pedersen CB, Mortensen PB (2004) Confirmation of Synergy Between Urbanicity and Familial Liability in the Causation of Psychosis. Am J Psychiatry 161:2312–2314.

Van Sickle MD (2005) Identification and Functional Characterization of Brainstem Cannabinoid CB2 Receptors. Science (80-) 310:329–332.

van Winkel R, (GROUP) GR and O of P (2011) Family-Based Analysis of Genetic Variation Underlying Psychosis-Inducing Effects of Cannabis: Sibling Analysis and Proband Follow-up. Arch Gen Psychiatry 68:148–157.

Varese F, Smeets F, Drukker M, Lieverse R, Lataster T, Viechtbauer W, Read J, van Os J, Bentall RP (2012) Childhood Adversities Increase the Risk of Psychosis: A Meta-analysis of Patient-Control, Prospective- and Cross-sectional Cohort Studies. Schizophr Bull 38:661–671.

Vaucher J, Keating BJ, Lasserre AM, Gan W, Lyall DM, Ward J, Smith DJ, Pell JP, Sattar N, Paré G, Holmes M V. (2018) Cannabis use and risk of schizophrenia: a Mendelian randomization study. Mol Psychiatry 23:1287–1292.

Veling W, Mackenbach JP, van Os J, Hoek HW (2008) Cannabis use and genetic predisposition for schizophrenia: a case-control study. Psychol Med 38:1251–1256.

Verdoux H, Gindre C, Sorbara F, Tournier M, Swendsen JD (2003) Effects of cannabis and psychosis vulnerability in daily life: an experience sampling test study. Psychol Med 33:23–32.

Verweij KJH, Abdellaoui A, Nivard MG, Sainz Cort A, Ligthart L, Draisma HHM, Minică CC, Gillespie NA, Willemsen G, Hottenga J-J, Boomsma DI, Vink JM (2017) Short communication: Genetic association between schizophrenia and cannabis use. Drug Alcohol Depend 171:117–121.

Viñals X, Moreno E, Lanfumey L, Cordomí A, Pastor A, de La Torre R, Gasperini P, Navarro G, Howell LA, Pardo L, Lluís C, Canela EI, McCormick PJ, Maldonado R, Robledo P (2015) Cognitive Impairment Induced by Delta9-tetrahydrocannabinol Occurs through Heteromers between Cannabinoid CB1 and Serotonin 5-HT2A Receptors Nestler EJ, ed. PLOS Biol 13:e1002194.

Vita A, De Peri L, Deste G, Barlati S, Sacchetti E (2015) The Effect of Antipsychotic Treatment on Cortical Gray Matter Changes in Schizophrenia: Does the Class Matter? A Meta-analysis and Meta-regression of Longitudinal Magnetic Resonance Imaging Studies. Biol Psychiatry 78:403–412.

Volk DW, Eggan SM, Horti AG, Wong DF, Lewis DA (2014) Reciprocal alterations in cortical cannabinoid receptor 1 binding relative to protein immunoreactivity and transcript levels in schizophrenia. Schizophr Res 159:124–129.

Volk DW, Lewis DA (2016) The Role of Endocannabinoid Signaling in Cortical Inhibitory

Neuron Dysfunction in Schizophrenia. Biol Psychiatry 79:595–603.

Vollenweider FX, Vollenweider-Scherpenhuyzen MFI, Bäbler A, Vogel H, Hell D (1998) Psilocybin induces schizophrenia-like psychosis in humans via a serotonin-2 agonist action. Neuroreport 9:3897–3902.

Voruganti LNP, Slomka P, Zabel P, Mattar A, Awad AG (2001) Cannabis induced dopamine release: An in-vivo SPECT study. Psychiatry Res - Neuroimaging 107:173–177.

Waldeck-Weiermair M, Zoratti C, Osibow K, Balenga N, Goessnitzer E, Waldhoer M, Malli R, Graier WF (2008) Integrin clustering enables anandamide-induced Ca2+ signaling in endothelial cells via GPR55 by protection against CB1-receptor-triggered repression. J Cell Sci 121:1704–1717.

Wang H-X, Gao W-J (2009) Cell Type-Specific Development of NMDA Receptors in the Interneurons of Rat Prefrontal Cortex. Neuropsychopharmacology 34:2028–2040.

Wang Q, Chen R, Cheng F, Wei Q, Ji Y, Yang H, Zhong X, Tao R, Wen Z, Sutcliffe JS, Liu C, Cook EH, Cox NJ, Li B (2019) A Bayesian framework that integrates multi-omics data and gene networks predicts risk genes from schizophrenia GWAS data. Nat Neurosci 22:691–699.

Wang Q, Liu L, Pei L, Ju W, Ahmadian G, Lu J, Wang Y, Liu F, Wang YT (2003) Control of Synaptic Strength, a Novel Function of Akt. Neuron 38:915–928.

Watts SW, Morrison SF, Davis RP, Barman SM (2012) Serotonin and Blood Pressure Regulation Daws LC, ed. Pharmacol Rev 64:359–388.

Wei BQ, Mikkelsen TS, McKinney MK, Lander ES, Cravatt BF (2006) A Second Fatty Acid Amide Hydrolase with Variable Distribution among Placental Mammals. J Biol Chem 281:36569–36578.

Whiteford HA, Degenhardt L, Rehm J, Baxter AJ, Ferrari AJ, Erskine HE, Charlson FJ, Norman RE, Flaxman AD, Johns N, Burstein R, Murray CJL, Vos T (2013) Global burden of disease attributable to mental and substance use disorders: findings from the Global Burden of Disease Study 2010. Lancet 382:1575–1586.

Wiles NJ, Zammit S, Bebbington P, Singleton N, Meltzer H, Lewis G (2006) Self-reported psychotic symptoms in the general population. Br J Psychiatry 188:519–526.

Wiley JL, Lowe JA, Balster RL, Martin BR (1995) Antagonism of the discriminative stimulus effects of delta 9-tetrahydrocannabinol in rats and rhesus monkeys. J Pharmacol Exp Ther 275:1–6.

Williams CM, Rogers PJ, Kirkham TC (1998) Hyperphagia in pre-fed rats following oral δ9-THC. Physiol Behav 65:343–346.

Williams E-J, Walsh FS, Doherty P (2003) The FGF receptor uses the endocannabinoid signaling system to couple to an axonal growth response. J Cell Biol 160:481–486.

Williams G V., Rao SG, Goldman-Rakic PS (2002) The Physiological Role of 5-HT 2A Receptors in Working Memory. J Neurosci 22:2843–2854.

Willins DL, Meltzer HY (1997) Direct injection of 5-HT2A receptor agonists into the medial prefrontal cortex produces a head-twitch response in rats. J Pharmacol Exp Ther 282:699–706.

Wilson RI, Nicoll RA (2001) Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. Nature 410:588–592.

Wilson RI, Nicoll RA (2002) Endocannabinoid signaling in the brain. Science (80-) 296:678–682.

Witt SH et al. (2017) Genome-wide association study of borderline personality disorder reveals genetic overlap with bipolar disorder, major depression and schizophrenia. Transl Psychiatry 7:e1155.

Witthaus H, Brune M, Kaufmann C, Bohner G, Ozgurdal S, Gudlowski Y, Heinz A, Klingebiel R, Juckel G (2008) White matter abnormalities in subjects at ultra high-risk for schizophrenia and first-episode schizophrenic patients. Schizophr Res 102:141–149.

Woolley DW, Shaw E (1954) A Biochemical and Pharmacological Suggestion about Certain Mental Disorders. Proc Natl Acad Sci U S A 40:228–231.

World Health Organization (1993) The ICD-10 classification of mental and behavioural disorders: Diagnostic criteria for research.

Woulfe DS (2010) Akt signaling in platelets and thrombosis. Expert Rev Hematol 3:81–91.

Yang Z-Z, Tschopp O, Di-Poi N, Bruder E, Baudry A, Dummler B, Wahli W, Hemmings BA (2005) Dosage-Dependent Effects of Akt1/Protein Kinase B (PKB) and Akt3/PKB on Thymus, Skin, and Cardiovascular and Nervous System Development in Mice. Mol Cell Biol 25:10407–10418.

Yi W, Li Q, Shen J, Ren L, Liu X, Wang Q, He S, Wu Q, Hu H, Mao X, Zhu L (2014) Modulation of Platelet Activation and Thrombus Formation Using a Pan-PI3K Inhibitor S14161 Li Z, ed. PLoS One 9:e102394.

Yilmaz Z, Zai CC, Hwang R, Mann S, Arenovich T, Remington G, Daskalakis ZJ (2012) Antipsychotics, dopamine D2 receptor occupancy and clinical improvement in schizophrenia: A meta-analysis. Schizophr Res 140:214–220.

Yoshino H, Miyamae T, Hansen G, Zambrowicz B, Flynn M, Pedicord D, Blat Y, Westphal RS, Zaczek R, Lewis DA, Gonzalez-Burgos G (2011) Postsynaptic diacylglycerol lipase α mediates retrograde endocannabinoid suppression of inhibition in mouse prefrontal cortex. J Physiol 589:4857–4884.

Zammit S (2002) Self reported cannabis use as a risk factor for schizophrenia in Swedish conscripts of 1969: historical cohort study. BMJ 325:1199–1199.

Zanda MT, Fattore L (2018) Old and new synthetic cannabinoids: lessons from animal models. Drug Metab Rev 50:54–64.

Zanelati T, Biojone C, Moreira F, Guimarães F, Joca S (2010) Antidepressant-like effects of cannabidiol in mice: possible involvement of 5-HT1A receptors. Br J Pharmacol 159:122–128.

Zhao Z, Ksiezak-Reding H, Riggio S, Haroutunian V, Pasinetti GM (2006) Insulin receptor deficits in schizophrenia and in cellular and animal models of insulin receptor dysfunction. Schizophr Res 84:1–14.

Zhu Y, Krause M, Huhn M, Rothe P, Schneider-Thoma J, Chaimani A, Li C, Davis JM, Leucht S (2017) Antipsychotic drugs for the acute treatment of patients with a first episode of schizophrenia: a systematic review with pairwise and network meta-analyses. The Lancet Psychiatry 4:694–705.

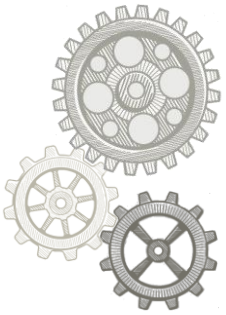
Zou S, Kumar U (2018) Cannabinoid Receptors and the Endocannabinoid System: Signaling and Function in the Central Nervous System. Int J Mol Sci 19:E833.

Zou Y, Jiang W, Wang J, Li Z, Zhang J, Bu J, Zou J, Zhou L, Yu S, Cui Y, Yang W, Luo L, Lu QR, Liu Y, Chen M, Worley PF, Xiao B (2014) Oligodendrocyte Precursor Cell-Intrinsic Effect of Rheb1 Controls Differentiation and Mediates mTORC1-Dependent Myelination in Brain. J Neurosci 34:15764–15778.

Zuckerman L, Rehavi M, Nachman R, Weiner I (2003) Immune Activation During Pregnancy in Rats Leads to a PostPubertal Emergence of Disrupted Latent Inhibition, Dopaminergic Hyperfunction, and Altered Limbic Morphology in the Offspring: A Novel Neurodevelopmental Model of Schizophrenia. Neuropsychopharmacology 28:1778–1789.

Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sørgård M, Di Marzo V, Julius D, Högestätt ED (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of

anandamide. Nature 400:452–457.



ANNEX

ARTICLE 5

The endocannabinoid system in mental disorders: Evidence from human brain studies

Inés Ibarra-Lecue, Fuencisla Pilar-Cuéllar, Carolina Muguruza, Eva Florensa-Zanuy, Álvaro Díaz, Leyre Urigüen, Elena Castro, Ángel Pazos, Luis F Callado

Biochemical Pharmacology (2018), **157**: 97-107

DOI: https://doi.org/10.1016/j.bcp.2018.07.009

Impact factor 2017: 4.2

Relative position: 36/261, Q1 Pharmacology and Pharmacy

Biochemical Pharmacology 157 (2018) 97–107

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Review

The endocannabinoid system in mental disorders: Evidence from human brain studies

Inés Ibarra-Lecue[a,b,1], Fuencisla Pilar-Cuéllar[b,c,d,1], Carolina Muguruza[a,b], Eva Florensa-Zanuy[b,c,d], Álvaro Díaz[b,c,d], Leyre Urigüen[a,b,e], Elena Castro[b,c,d], Angel Pazos[b,c,d], Luis F. Callado[a,b,e,*]

[a] Department of Pharmacology, University of the Basque Country UPV/EHU, Leioa, Spain
[b] Centro de Investigación Biomédica en Red de Salud Mental CIBERSAM, Spain
[c] Instituto de Biomedicina y Biotecnología de Cantabria (IBBTEC); Universidad de Cantabria-CSIC, Santander, Spain
[d] Departamento de Fisiología y Farmacología, Facultad de Medicina, Universidad de Cantabria, Santander, Spain
[e] Biocruces Health Research Institute, Bizkaia, Spain

ARTICLE INFO

Keywords:
Human brain
Depression
Anxiety
Schizophrenia
Endocannabinoids
Cannabinoid receptors

ABSTRACT

Mental disorders have a high prevalence compared with many other health conditions and are the leading cause of disability worldwide. Several studies performed in the last years support the involvement of the endocannabinoid system in the etiopathogenesis of different mental disorders. The present review will summarize the latest information on the role of the endocannabinoid system in psychiatric disorders, specifically depression, anxiety, and schizophrenia. We will focus on the findings from human brain studies regarding alterations in endocannabinoid levels, cannabinoid receptors and endocannabinoid metabolizing enzymes in patients suffering mental disorders.

Studies carried out in humans have consistently demonstrated that the endocannabinoid system is fundamental for emotional homeostasis and cognitive function. Thus, deregulation of the different elements that are part of the endocannabinoid system may contribute to the pathophysiology of several mental disorders. However, the results reported are controversial. In this sense, different alterations in gene and/or protein expression of CB1 receptors have been shown depending on the technical approach used or the brain region studied. Despite the current discrepancies regarding cannabinoid receptors changes in depression and schizophrenia, present findings point to the endocannabinoid system as a pivotal neuromodulatory pathway relevant in the pathophysiology of mental disorders.

1. Introduction

Mental disorders are responsible for the largest proportion of the global burden of disease worldwide. It has been suggested that by 2030 depression will be the leading cause of disease burden globally. In this way, mood-related disorders contribute most of the non-fatal burden of mental illness followed by anxiety-related disorders, substance abuse and schizophrenia [1]. They present a major medical, societal and economic burden that has a large impact on individuals, families and communities.

Actual knowledge about the etiology and pathophysiology of mental disorders is mainly a result of an interaction between the development of new technology and the direct study of the brain tissue of patients. Thus, the description of morphological differences, functional deficits and molecular alterations is widely accepted today as existing in the brain of psychiatric patients due to the advance of *in vivo* neuroimaging techniques, genetic and genomic development, and the use of post-mortem brain tissue as a key substrate of the disease [2]. Nevertheless, despite the huge economic and scientific effort developed in the last decades, the pathophysiology of mental disorders remains elusive. In this context, many studies have focused in the possible involvement of alterations of the endocannabinoid system (ECS) in the pathophysiology of mental disorders such as depression or schizophrenia. The ECS participates, in part, in the control of emotional behavior and mood

* Corresponding author at: Department of Pharmacology, Medical School, University of the Basque Country (UPV/EHU), Barrio Sarriena s/n, 48940 Leioa, Bizkaia, Spain.
E-mail address: LF.callado@ehu.eus (L.F. Callado).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.bcp.2018.07.009
Received 29 May 2018; Accepted 12 July 2018
Available online 17 July 2018
0006-2952/

through a functional coupling with monoaminergic systems in the brain [3]. These functional interactions have suggested a potential role for ECS signaling in the neurobiology of various psychiatric disorders [4–7]. The ECS is composed of two inhibitory G-protein coupled receptors (GPCRs), cannabinoid receptor 1 and 2 (CB1 and CB2, respectively), and two major endogenous ligands, N-arachidonoylethanolamine (anandamide/AEA) and 2-arachidonoylglycerol (2-AG). The ECS also includes two main metabolic enzymes, the fatty acid amide hydrolase (FAAH) and the monoacylglycerol lipase (MAGL) which hydrolyze AEA and 2-AG, respectively; and two main synthetizing enzymes, N-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD) and the diacylglycerol lipase (DAGL) which synthesize AEA and 2-AG, respectively. The correct interplay between all these ECS elements plays an important role in central nervous system (CNS) development, synaptic plasticity, and the homeostatic maintenance of cognitive, behavioral, emotional, developmental, and physiological processes [8,9]. In the brain, CB1 receptors are present in GABAergic and glutamatergic neurons, exerting a presynaptic inhibitory function when they are activated by the released endocannabinoids [10,11]. They are the most abundant G-protein coupled receptors and are widely expressed all throughout the brain, being located in cortical, subcortical, cerebellar and brainstem structures [8]. The CB2 receptors are less numerous and were initially thought to be located mainly in the immune system; however, currently they seem to be widely distributed in the CNS, taking part in immune-mediated responses and supporting a neuroprotective role against inflammation [12]. The two main endogenous ligands, AEA and 2-AG, are eicosanoid neuromodulatory lipids derived from membrane phospholipids, synthesized when and where they are required, and acting presynaptically on both type of cannabinoid receptors [8].

In the present review, we will summarize data obtained from human studies providing evidence about the role of the different ECS components (endocannabinoids, metabolizing/synthetizing enzymes and cannabinoid receptors) in the pathophysiology and treatment of several psychiatric disorders, with a focus on results from postmortem and living human brain studies. We will review findings from patients suffering a mood-related disorder (depression, anxiety, posttraumatic stress disorder (PTSD)) or schizophrenia compared to healthy subjects.

2. The endocannabinoid system and the emotional homeostasis

The ECS influences the activity of multiple brain areas involved in the regulation of the hypothalamic-pituitary-adrenal system (HPA), mood, anxiety and other related behaviors (i.e. extinction of fear learning, reward…). Indeed, the ECS enables the efficient interaction within and between brain regions that modulate cognitive and behavioral functioning.

A considerable number of studies suggest the relationship between changes in one or more components of the ECS and some of the symptoms that are present in depression and anxiety-related disorders. The ECS modulates fear and anxiety-related behaviors in both humans and rodents [13–15]. Augmented ECS signaling is usually followed by reduced conditioned fear and anxiety, whereas the opposite effect is observed when it is inhibited [16–19]. This is not surprising since the ECS is present in key structures within the brain such as prefrontal cortex (PFC), amygdala, and hippocampus [20–25].

There are also animal studies showing the correlation between CB1 receptor-deficiency and depressive/comorbid symptoms (anhedonia, anxiety, and heightened stress-response) [26–28]. In line with this relationship, chronic stress, as a pathogenic factor for depressive-behavior, has been associated with a dysfunctional endocannabinoid signaling in the brain [29]. Thus, strategies that are directed to the augmentation of the endocannabinoid signaling are reported to mitigate many of the adverse effects of chronic stress, such as anhedonia and anxiety [30–32]. The readers are directed to comprehensive reviews on this topic that is beyond the scope of this review [33–38].

3. The endocannabinoid system and depression

Depression is one of the most prevalent major neuropsychiatric diseases, affecting 20% of the population, being almost twice as common in females than males [39]. There are two main challenges to fight against depression. First, we still poorly understand its neurobiological and pathological bases. Second, there needs to be more effective antidepressant drugs overcoming the therapeutic lag between drug administration and the onset of clinical improvement, the lack of response in some patients and safety/tolerability issues [40,41].

The implication of the ECS in depression comes from observational findings regarding the mood-related effects of cannabis in humans, though contradictory results are reported. On one hand, the heavy use of cannabis is associated to a higher incidence of depressive disorders [42]. A recent meta-analysis showed a positive correlation between cannabis use and depression, being more evident among heavy cannabis users [43]. Moreover, the abuse of cannabis has been linked to a higher risk of suicide in patients with mood disorders [44]. On the other hand, other authors indicate that the use of marijuana, or its main psychoactive component Δ^9-tetrahidrocannabinol (THC), reduces the depressive behavior [45–48]. In addition, other authors describe that the administration of THC to patients with moderate to severe depression shows a lack of effect on mood [49], or on the suicidal ideation [50], but an increased anxiety [49].

Curiously, the pharmacological blockade of CB1 receptors using antagonists or inverse agonists was initially suggested as a potential novel target for antidepressant treatment, according to different evidences in preclinical studies [51]. However, further studies revealed that drugs as the CB1 receptor antagonist rimonabant (SR 141716A) that was marketed to treat obesity, induced depressed mood [52,53]. In animal studies, the pharmacological activation or blockade of CB1 receptors also give rise to contradictory results, since both approaches lead to an antidepressant-like effect [5,51,54–58].

Although these studies draw conflicting findings, they point to the involvement of the brain ECS in the modulation of mood and, especially, the contribution of CB1 receptors to major depression have received particular attention [59]. The activation of cannabinoid receptors produces the release of stress hormones as ACTH and cortisol in the HPA axis [60], which has been observed following acute marijuana administration [61]. However, this presents tolerance after chronic administration [62], as the THC-induced cortisol release is blunted in frequent marijuana users [63].

Neuroimaging studies in non-cannabis users show that the administration of THC produces a reduced activation of some brain areas in response to a negative content [64,65]. Conversely, there is an increased activation in response to a positive content, mediated by the activation of areas such as prefrontal and occipital cortices, amygdala, hippocampus and orbitofrontal gyrus [65], which is associated to a reduction in the negative attentional bias, and the potentiation of positive attentional bias [65]. This type of studies have also shown that the activation of cannabinoid receptors leads to morphological changes, such as the reduction in white matter (WM) observed in marijuana users that negatively correlates with the severity of the depressive disorder [66,67]. This decrease in WM volume has been associated with the presence of cannabinoid receptors in oligodendrocytes, the myelin-forming cells [68].

3.1. Cannabinoid receptors in depression

Human studies have corroborated the existence of an altered ECS activity associated to major depression [34,69]. The CB1 receptor density [70] and mRNA [71] is increased in the dorsolateral prefrontal cortex of patients with major depression, in parallel to CB1 receptor functionality (evaluated by [^{35}S]GTPγS binding studies) [70] (Mato et al., in this issue). However, no changes in CB1 immunoreactivity in the dorsolateral prefrontal cortex [72], or a reduction in CB1

Table 1
Studies about alterations of the different components of the ECS in the brain of patients with depression or anxiety-related disorders.

ECS element	Finding (% change)	Brain region	Cohort (n: disease-Ct)	Method/sample	References
CB1 (mRNA)	↑ (60%)	DLPC (BA46)	MDD:Ct 26:46	Gene expression microarray/PMBT	[71]
CB1 (protein)	≈	DLPC (BA46)	MDD:Ct 14:14	IHC/PMBT	[72]
	↑ (38%)	DLPC (BA9)	MDD with suicide:Ct 10:10	WB/PMBT	[70]
	↓ (22.1%)	Glial$^+$ cells in ACC GM	MDD:Ct 15:15	IHC/PMBT	[73]
	≈	Neurons$^+$ cells in ACC	MDD:Ct 15:15	IHC/PMBT	[73]
	↓ (~8.5–9.5%)	Neurons$^+$ cells in ACC	MDD + SSRI:MDD no-SSRI	IHC/PMBT	[73]
CB1 (density)	↑ (31%)	DLPC (BA9)	MDD with suicide:Ct 10:10	[^{3}H]CP-55,940 binding/PMBT	[70]
CB1 (functionality)	↑ (45%)	DLPC (BA9)	MDD with suicide:Ct 10:10	[^{3}H]CP-55,940 and [^{35}S]GTPγS binding/PMBT	[70]
CB1 (availability)	↑ (19.5%)	Brain-wide	PTSD:Ct 25:23	*In vivo* brain PET scan [^{11}C]OMAR	[100]
	↑ (14.5%)	Brain-wide	PTSD:TC 25:12	*In vivo* brain PET scan [^{11}C]OMAR	[100]
	↑	Amygdala	PTSD:TC:Ct 12:4:4	In vivo brain PET scan [^{11}C]OMAR	[101]
CNR1 gene rs1049353 (A)	↑ activity associated to emotional processing	Bilateral amygdala, putamen and pallidum	MDD(AG) MDD(GG) 13:20	Genetic association study with fMRI/peripheral cells	[80]
CB1-HINT1 (protein)	≈	PFC (BA9)	MDD:Ct 24:24	Co-IP/PMBT	[86]
CB1-NR1 C1 (protein)	≈	PFC (BA9)	MDD:Ct 24:24	Co-IP/PMBT	[86]
CB2 (mRNA)	≈	DLPC (BA46)	MDD:Ct 26:46	Gene expression microarray/PMBT	[71]

↑ Increase; ↓ decrease; ≈ no significant change. ACC: anterior cingulate cortex; BA: Brodmann's area; CB1, CNR1: cannabinoid receptor 1; CB2: cannabinoid receptor 2; Co-IP: co-immunoprecipitation; Ct: control; DLPC: dorsolateral prefrontal cortex; FAAH: fatty acid amide hydrolase; fMRI: functional magnetic resonance image; GM: grey matter; HINT-1: histidine triad nucleotide binding protein 1; IHC: immunohistochemistry; MDD: major depression disorder; PET: positron emission tomography; PFC: prefrontal cortex; PMBT: post-mortem brain tissue; PTSD: posttraumatic stress disorder; SSRI: serotonin selective reuptake inhibitor; TC: trauma controls; WB: western blot.

immunoreactivity in glial cells in the anterior cingulate cortex [73] have been reported (Table 1). Regarding CB2 receptor, no changes have been detected in its mRNA levels in prefrontal cortex of depressed patients [71] (Table 1).

The role of the ECS in the effect of antidepressant drugs has also been evidenced in studies reporting an increased CB1 receptor expression [74], and a lack of changes in CB1-mediated activation of Gi/o proteins in prefrontal cortex (Mato et al., in this issue) in the antidepressant-treated group. Other areas such as the hippocampus only elicited increased CB1 receptor density after chronic monoamine oxidase inhibitors (MAOI) treatment [74]. On contrast, studies in human brain samples have shown a reduction in CB1 receptor immunoreactivity in the anterior cingulate cortex of patients treated with serotonin selective reuptake inhibitors (SSRIs) [73].

The presence of different single nucleotide polymorphisms in the cannabinoid receptor 1 (*CNR1*) gene appears to modulate either the depressive phenotype, and/or the response to antidepressant treatment. The carriers of *CNR1* gene variants influences the vulnerability to suffer mental disorders, including major depression [75]. The frequency of the G allele of the *CNR1* gene polymorphism rs806371 is higher in patients with major depression showing comorbid psychotic symptoms, while the haplotype C-G-T (rs806368, rs1049353, rs806371) is associated with an increased risk for melancholic and psychotic symptoms of major depression [76]. This is consistent with the melancholic depressive-like symptoms observed in animal models with pharmacological or genetic blockade of the ECS [4]. Other studies report a lower incidence of depression in Parkinsons' disease patients carrying two long alleles of the *CNR1* gene polymorphism (AAT)n [77]. The C allele carriers of the *CNR1* gene polymorphism rs2023239 present a lower incidence of major depression within a group of methadone-responder patients [78].

In patients with the *CNR1* gene polymorphism rs1049353, carriers of one or more copies of the minor allele (AA/AG) exhibit a buffering effect to anhedonia and depression after early childhood trauma [79]. Moreover, the G allele of the rs1049353 polymorphism is associated to the resistance to the antidepressant treatment in females diagnosed with major depression that present comorbid anxiety [80]. Patients with the G allele of the *CNR1* rs1049353 also present a subcortical hypo-responsiveness in the bilateral amygdala, putamen, and pallidum activity and left lateralized caudate and thalamus activity, to specific cues, which might be linked to a deficient effect on the processing of emotional and social behavior [80]. On contrast, other authors report a better response to treatment of male presenting the GG genotype [76]. In patients treated with citalopram, TT homozygous carriers for the rs806368 and rs806371 polymorphisms, show a higher incidence of no remission, compared to the G carriers [76]. Moreover, the response in the rs806368 G carriers was different depending on the gender, presenting a better antidepressant outcome in men than in women [76].

Although the CB2 receptor subtype is less abundant than CB1 receptor subtype in the brain, some studies also describe an association with mental disorders. In this sense, the RR genotype of the Q63R polymorphism in the *CNR2* gene presents a higher association with depression in the Japanese population [81]. This Q63R polymorphism presents also a high incidence in patients with eating disorders (anorexia nervosa and bulimia nervosa) [82] and schizophrenia [83]. Studies in cells expressing this mutated form of the *CNR2* gene showed that the functional relevance of this polymorphism is due to changes in CB2 ligand affinity, constitutive activity and a reduced 2-AG-induced adenylyl cyclase inhibition [84]. Regarding the *CNR2* gene polymorphism rs2501431, the AA carriers present a higher severity of the disease, compared to the G carriers [85].

The activity of cannabinoid receptors is associated to the cross-regulation that the CB1 receptors exert over the NMDA receptors mediated by their interaction via the histidine triad nucleotide binding protein 1 (HINT-1), in which a reduction in the number of CB1 receptors may be associated to the NMDA hyperfunction observed in depression [86]. In this sense, molecular studies have shown an increase in the HINT-1 protein and the NR1 subunit of the NMDA receptors in the prefrontal cortex of depressed patients, in parallel to results obtained in CB1 receptor knockout mice [86].

3.2. Endocannabinoid metabolizing enzymes in depression

One of the most frequent polymorphism of FAAH in humans is a functional non-synonymous single-nucleotide polymorphism (C385A; rs324420) associated with a reduced cellular expression of this enzyme. The C385A polymorphism of the *FAAH* gene, presents a greater association in A allele carriers with pathologies such as depression and bipolar disorder [87]. Moreover, the presence of this polymorphism constitutes a susceptibility factor to develop depressive and anxious phenotypes in adult individuals that have been exposed to childhood trauma [88]. The high AEA levels because the reduced FAAH activity in

the A allele carriers, induce the desensitization of CB1 receptors. This reduction in CB1 receptors promotes a glutamatergic hyperactivity that, together with high cortisol levels due to childhood trauma in critic neurodevelopmental periods, results in anxious and/or depressive disorders [88]. The CC carriers of the rs324420 polymorphism in the *FAAH* gene present a reduction in the WM integrity of fibers that connect with the anterior cingulate cortex and the orbital cortex, and greater incidence of self-reported depressive symptoms [67].

3.3. Endocannabinoids levels in depression

The serum content of endocannabinoids is also altered in major depression. Some authors report lower levels of the circulating endocannabinoids AEA and 2-AG in patients with depression [89,90]. Moreover, the 2-AG levels are lower in patients with a longer duration of the depressive episode, while patients with minor depression present higher levels of AEA [89]. In contrast, other authors report no changes in AEA and 2-AG levels in depressed women [91].

The levels of the endocannabinoids AEA and 2-AG were not modified in response to SSRIs such as fluoxetine, while the chronic administration of MAOIs induced a reduction in areas such as prefrontal cortex, hippocampus and hypothalamus [74]. These data are consistent with a study that associates the beneficial effect of exercise in depression with an increase in the plasma levels of AEA, BDNF and cortisol [92].

4. The endocannabinoid system and anxiety-related disorders

Few neurochemical, molecular genetics and neuroimaging studies suggest a potential link between dysregulation of the endocannabinoid signaling and anxiety-related behavior in both healthy and patients with mental disorders in which anxiety is a core symptom (PTSD, social phobia, agoraphobia, etc…).

4.1. Cannabinoid receptors in anxiety

Two single nucleotide polymorphism (SNP) variants (C-A and C-G) of the haplotype formed by the polymorphisms rs806368 and rs1049353 at the *CNR1* gene showed a significant association with PTSD [93]. Moreover, a higher risk to suffer anxiety is observed when homozygous 'SS' of the polymorphism of serotonin transporter (5-HTTLPR) in the SLC6A4 promoter is combined with the homozygous 'GG' rs2180619 of *CNR1* gene [94]. This highlights the strong interaction between the serotonergic system and the ECS on anxiety disorders as extensively described in many preclinical and clinical studies, using pharmacological and genetic approaches [37,95,96].

Moreover, Heitland et al. [97] published the first evidence, in healthy medication-free human subjects, of the implication of ECS in the fear extinction phenomenon, a relevant mechanism underlying the pathophysiology of human anxiety disorders. These authors describe the effect of the *CNR1* gene polymorphism rs2180619 in the response to fear conditioning and extinction. They found that both homozygote (G/G) and heterozygote (A/G) G-allele carriers of this polymorphism showed a clear extinction of fear, whereas this response was absent in homozygotes (A/A).

Recent findings suggest that during childhood and adolescence, the ECS is critical to mediate a correct balance between excitatory and inhibitory neurotransmission, especially within the prefrontal cortex [98]. This makes the endocannabinoid signaling quite sensitive for developmental fluctuations due to environmental causes, which may increase the risk of anxiety and other stress-related disorders. Interestingly, a recent study shows the impact of the variation in the *CNR1*, *CNR2*, and *FAAH* genes in a sample of children with a primary anxiety disorder diagnosis [99]. These authors nicely reported an association between two SNPs (rs12133557 and rs6454676) in the *CNR1* gene and the change in symptom severity in both the entire sample and a subset of patients with fear based diagnoses [99]. Moreover, a favorable and a poorer response during the active treatment period were associated with minor allele of rs12133557 and rs6454676, respectively. Regarding the *CNR2* gene, unlike to previous findings in depression [81], the rs2501431 genotype was not associated with anxiety symptoms or treatment response [99].

In vivo neuroimaging studies also support the existence of an abnormal CB1 receptor-mediated signaling especially in PTSD. Using positron emission tomography (PET) with [^{11}C]OMAR, a CB1-selective tracer, Neumeister and colleagues [100] reported a higher CB1 receptor availability in untreated individuals with PTSD, relative to control subjects (with or without lifetime histories of trauma), which was most pronounced in women. This up-regulation of CB1 receptors was present in anxiety-related brain areas, especially the amygdala-hippocampal-cortico-striatal neural circuit. In a later report, the same group [101] assessed the attentional bias to threat, which is considered one of the main endophenotypic characteristics of trauma-related mental disorders. In line with their previous findings, they reported a positive correlation between an increased CB1 receptor availability ([^{11}C]OMAR binding) in the amygdala and increased in both attentional bias to threat and the severity of threat. Interestingly, this greater CB1 receptor availability in the amygdala was associated with lower plasma levels of AEA.

4.2. Endocannabinoid metabolizing enzymes in anxiety

Pharmacological strategies that reduce the activity of either FAAH or MAGL have been reported to reduce anxiety-like behaviors in rodents and humans [37]; however, dual FAAH/MAGL inhibitors did not reduce stress-related affective dysfunction regardless of treatment timing [102]. There are several studies linking the activity of FAAH, especially in the amygdala, with stress-reactivity and risk to suffer anxiety-disorders.

In healthy volunteers, there are some studies examining the impact of the *FAAH* gene polymorphism C385A (rs324420) on threat- and reward-related human brain function. Using imaging genetics, a decreased threat-related amygdala reactivity but increased reward-related ventral striatal reactivity was detected in carriers of the A allele of the *FAAH* enzyme gene [103]. In a later study, a quicker habituation of amygdala reactivity to threat and lower scores on the personality trait of stress-reactivity was found to be associated with carriers of a low-expressing FAAH variant (385A allele; rs324420) [104]. More recently, an enhanced fear extinction was demonstrated in both mouse and human A-allele carriers of this *FAAH* gene C385A polymorphism, highlighting again the association of the increased fronto-amygdala connectivity with enhanced stress-reactivity [105]. This genetic alteration appears to have functional consequences since a markedly reduced FAAH protein expression was detected in many cortico-limbic areas using the first available PET radiotracer ([^{11}C]CURB) in human brain [106]. Regarding the role of FAAH on PTSD, Pardini et al. [107] reported an association of the rs2295633 SNP of *FAAH* gene with PTSD diagnosis in male Vietnam War veterans without lesions in the ventromedial prefrontal cortex. Even more, the C allele was present in subjects that had a more negative reported experience of trauma.

4.3. Endocannabinoid levels in anxiety

Preclinical findings suggest that the pharmacological manipulation of endogenous either AEA or 2-AG levels under stressful conditions could represent a good strategy for treatment of anxiety-related disorders [108]. Thus, it is plausible to hypothesize the existence of altered endocannabinoid levels in the brain of patients diagnosed of psychiatric diseases in which anxiety is either core or a comorbid symptom.

Decreased plasma AEA levels are found in PTSD patients relative to healthy control subjects without trauma history [109], though elevations are also reported in chronic PTSD [110]. The AEA deficiency

seems to be specific of PTSD patients since healthy subjects with lifetime histories of trauma, but without PTSD, exhibit normal AEA plasma levels [100]. As mentioned above, a positive correlation between lower plasma levels of AEA and increased CB1 receptor availability in the amygdala has been reported; in addition, AEA levels were negatively associated with attentional bias to threat [101]. Intriguingly, this inverse relationship between AEA levels and anxiety appears to be disease-specific since acute stress increases the circulating levels of AEA and other endocannabinoids, in parallel with cortisol [111]. Regarding the 2-AG, reduced [112] and increased [110] levels among individuals meeting diagnostic criteria for PTSD were described. Healthy humans that were subjected to prolonged stress (520-day isolation period and simulating a flight to Mars) showed reduced blood levels of 2-AG, but not AEA [113].

All these findings suggest the interaction between the ECS and HPA axis activity in response to stress, especially at the level of the amygdala [19]. In line with this relationship, a recent imaging genetics study [114] revealed a molecular interaction between genetic polymorphisms associated with differential AEA levels (FAAH rs324420) and corticotrophin releasing hormone (*corticotropin-releasing hormone receptor 1*, *CRHR1* rs110402) signaling. and amygdala function. However, in a very recent study, the levels of circulating endocannabinoids were measured in subjects with and without history of psychiatric disorder and the relationships with categorically DSM-5 defined disorders or state dimensional measures of depression or anxiety were studied. Surprisingly, neither AEA nor 2-AG levels differed as a function of any syndromal/personality disorder and neither correlated significantly with state depression or state anxiety scores [115]. Therefore, we must be cautious since peripheral endocannabinoid levels may be not well correlated with brain concentrations.

5. The endocannabinoid system and schizophrenia

Schizophrenia is one of the main psychiatric syndromes together with Major Depression. It is a chronic and devastating disorder affecting 1% of the population worldwide. Individuals diagnosed with schizophrenia have impaired social and occupational functioning. Thus, the combined economic and social costs of schizophrenia place it as the world's 15th cause of disease-related disability [116]. The clinical features of schizophrenia are clustered in positive symptoms (i.e. hallucinations and delusions); negative symptoms (i.e. social withdrawal and blunted affect) and cognitive deficits (i.e. impaired working memory and cognitive flexibility). Current antipsychotic drugs, which are the main treatment for schizophrenia, are not effective in all patients and their benefits are restricted to the amelioration of the positive symptoms, having none or limited impact in negative symptoms and cognitive impairment.

Despite the efforts of the scientific community in the last decades to elucidate the etiological basis of schizophrenia, the etiopathogenesis of the disease remains unknown. Since many years, the predominant focus of studies concerning the biological substrates of schizophrenia has been primarily centered on unique neurotransmitters including dopamine, serotonin, glutamate and γ-aminobutyric acid (GABA). Nonetheless, the limited efficacy of current antipsychotic drugs to treat some of the symptoms of schizophrenia has lead up researchers to investigate other potential neurotransmitter systems that may be altered in this disease.

In this sense, the ECS has become a hot-topic in schizophrenia research in the last years. Several studies starting from the 40 s up to nowadays agree that the ECS represents a major neuromodulatory system participating in tones of physiological processes [117,118]. Thereby, deregulation of the ECS has been speculated to be a proximal pathology in some forms of schizophrenia. In this sense, two 'cannabinoid hypotheses' of schizophrenia have been proposed [119]. The endogenous hypothesis refers to the fact that deregulation of the ECS may contribute to the pathophysiology of schizophrenia, whereas the exogenous theory refers to the risk associated with cannabis abuse that could facilitate the onset of the disease in vulnerable individuals or aggravate the symptoms in schizophrenic patients. The psychotomimetic effects of cannabis plant are known since thousands of years, but the first systematic work concerning psychotic-like experiences after acute cannabis use came in the 19th century, by Jacques-Joseph Moreau [120]. In his book he described a plethora of symptoms resembling those of schizophrenia, including delusions, disorganized speech, and other psychotic symptoms. The high expression of CB1 receptors in the central nervous system, as well as the discovery that the psychoactive compound of cannabis THC actually binds to this receptor in the brain, seem sufficient reasons to consider this system as an interesting field of study in the context of psychiatric diseases, such as schizophrenia.

In this sense, different publications have reported alterations of the ECS components in the brain of patients with schizophrenia. The evidence for the implication of different elements of the central ECS in the pathophysiology and treatment of this psychiatric disorder are presented below.

5.1. Cannabinoid receptors in schizophrenia

Several studies have investigated the status of CB1 receptors in the brain of patients with schizophrenia. Both, imaging and postmortem brain studies, have reported alterations on CB1 receptor availability, density and/or mRNA expression but with different outcomes. Thus, the integration of these results seem to be complex and certain potential confounding factors might be underlying these discrepant findings.

Three neuroimaging PET studies have evaluated the CB1 receptor availability in schizophrenic patients compared to controls [121–123]. The first two studies, by Wong et al. [121] and Ceccarini et al. [122] reported a generalized increase in CB1 receptor density in most brain regions of schizophrenic patients compared to controls, being statistically significant only in certain areas (Table 2). Interestingly, these studies also reported that CB1 receptor binding in certain areas correlated with the severity of positive symptoms and inversely correlated with the severity of negative symptoms [121,122]. Opposite to these first reported imaging findings, a recent PET study by Ranganathan et al. [123], showed a significant decrease in CB1 receptor availability in patients with schizophrenia compared to healthy controls (Table 2). Moreover, this study showed a positive global association between both positive and negative symptoms and the availability of CB1 receptors in schizophrenia [104]. Given these discordant findings related to CB1 receptor availability in schizophrenia and its association with different symptoms of the disease, the necessity of further *in vivo* assessments in this regard becomes clear. Explanations for the contradictory results reported in imaging studies published so far, have been discussed in detail [124]. Thus, confounding factors such as sex and age of patients and controls included in the studies, the radiotracers and the procedures used for the analysis, the influence of cannabis and/or tobacco consumption and the impact of antipsychotic medication have been proposed as variables that can account for the discrepancies of these results [124].

Regarding postmortem studies in the brain of patients with schizophrenia, also different outcomes for gene and/or protein expression of CB1 receptors have been shown depending on the technical approach used. Thus, different reports have linked schizophrenia with increased, decreased or unaltered expression/density of CB1 receptors in the postmortem human brain. Postmortem brain radioligand binding studies consistently reported increased density of CB1 receptors in schizophrenia [125–131]. Six out of the eight radioligand binding studies published to date have reported an increase of CB1 receptor density in the brain of schizophrenic subjects compared to controls in areas known to be involved in schizophrenia, including the cingulate cortex and dorsolateral prefrontal cortex (Table 2). The only study that has evaluated CB1 receptor binding density in the superior temporal gyrus,

Table 2
Studies about alterations of the different components of the ECS in the brain of patients with schizophrenia.

ECS element	Finding in Sch (% change)	Brain region	Cohort (n: Sch-Ct)	Method/sample	Reference
CB1 (availability)	↓ (12%)	AM, CD, Insula, PCC, HC, HT	25–18	*In vivo* brain PET scan [^{11}C]OMAR	123
	↑ (10%-5%)	NAcc, Insula, CC, IFC	67–12	*In vivo* brain PET scan [^{18}F]MK-9470	122
	↑ (23%)	BS/pons	9–10	*In vivo* brain PET scan [^{11}C]OMAR	121
CB1	↑ (8%)	DLPC (BA9)	21–21	[^{3}H]-OMAR/PMBT	131
(density)	↑ (20%)	DLPC (BA9,46)	47–43	[^{3}H]MePPEP/PMBT	130
	↑ (22%)	DLPC (BA46)	37–37	[^{3}H]CP-55940/PMBT	129
	≈	STG	8–8	[^{3}H]SR141716A and [^{3}H]CP-55940/PMBT	128
	↑ (25%)	PCC	8–8	[^{3}H]CP-55940/PMBT	127
	↑ (64%)	ACC	10–9	[^{3}H]SR141716A/PMBT	126
	↑ (23%)	DLPC (BA9)	14–14	[^{3}H]CP-55940/PMBT	125
CB1 (protein)	↓ (19–20%)	DLPC (BA46)	26–26	Immunohistochemistry/PMBT	72
	≈ (AP-F)/↓ (29%)(AP-T)	DLPC (BA9)	25–25	Immunoblot/PMBT	137
	↓ (12–14%)	DLPC (BA9)	23–23	Immunohistochemistry/PMBT	136
	≈	ACC	15–15	Immunohistochemistry/PMBT	73
CB1	≈	DLPC (BA46)	37–37	RT-qPCR/PMBT	129
(mRNA)	≈	DLPC (BA9)	20–20	RT-qPCR/PMBT	137
	↓ (15%)	DLPC (BA9)	23–23	In situ hybridization/PMBT	136
CB2 (mRNA)	≈	DLPC (BA9)	23–24	RT-qPCR/PMBT	82
EC enzymes (mRNA)	↑ (18%) ABHD6 in < 40 years (n = 13–13)	DLPC (BA9)	42–42	RT-qPCR/PMBT	156
	≈ FAAH, MAGL, DAGLα, DAGLβ	DLPC (BA9)	42–42	RT-qPCR/PMBT	155

↑ Increase; ↓ decrease; ≈ no significant change. ABHD6: α-β-hydrolase domain 6; AP-F: antipsychotic free; AP-T: antipsychotic treated; BA: Brodmann's area; Ct: controls; DAGL: diacylglycerol lipase; EC: endocannabinoid; FAAH: fatty acid amide hydrolase; MAGL: monoacylglycerol lipase; RT-qPCR: real-time quantitative polymerase chain reaction; Sch: patients with schizophrenia; PET: positron emission tomography; PMBT: post-mortem brain tissue.
Brain regions: ACC: anterior cingulate cortex; AM: amygdala; BS; brain stem; CC: cingulate cortex; CD: caudate; DLPC: dorsolateral prefrontal cortex; HC: hippocampus; HT: hypothalamus; IFC: inferior frontal cortex; NAcc: nucleus acumens; PC: parietal cortex; PCC: posterior cingulate cortex; STG: superior temporal gyrus.

a brain area particularly involved in auditory hallucinations [132], found no differences between schizophrenic subjects and controls [128]. This fact, could suggest that the alterations found in CB1 receptor density in schizophrenia might be more associated to negative and/or cognitive symptoms of the disorder, which are linked with altered cortical functions integrated by the cingulate cortex (emotional processing and selective attention responses) and the dorsolateral prefrontal cortex (DLPC) (motivational responses and executive functions) [133]. Interestingly, the study by Dalton et al. [129] only found increased CB1 receptor binding in the DLPC of schizophrenic patients with a diagnosis of paranoid schizophrenia, characterized by the presence of prominent delusions or hallucinations, while no changes were reported in non-paranoid schizophrenic patients, indicating that also the type of diagnoses can influence the outcomes obtained. This fact highlights the relevance of the source of the diagnoses and the potential influence of changes in the clustering of schizophrenia spectrum disorders in the diagnosis manuals along time. One example of these changes, is the modification in schizophrenia definition in the Diagnostic and Statistical Manual of Mental Disorders (DSM), 5th Edition [134], respect to the previous one, that eliminates the clustering of schizophrenia classic subtypes—disorganized (hebephrenic), catatonic, paranoid, and undifferentiated—adding psychopathological dimensions instead [135]. This decision was made because the classic DSM-IV subtypes of schizophrenia provide poor description of the heterogeneity of schizophrenia, low diagnostic stability, do not exhibit distinctive patterns of treatment response or longitudinal course, and are not heritable [135]. Thus, the comparisons of the outcomes between different schizophrenia subtypes should be interpreted with caution.

The potential influence of antipsychotic medication in CB1 receptor radioligand binding studies has been also taken into account. These reports state that the increases in CB1 receptor density were not related to the antipsychotic treatment given to the patients [125–127,129–131]. However, it must be noticed that in all the studies more than 80% of schizophrenic subjects included were under antipsychotic medication at the time of death. The evaluation of the impact of antipsychotic drugs on CB1 receptors in schizophrenia postmortem studies is difficult to overcome due to the lack of brain tissue from drug naïve patients. Nevertheless, the inclusion of a greater number of schizophrenic patients with a negative toxicology for antipsychotics at the time of death could provide new information in this regard when compared with antipsychotic-treated patients.

Opposite to radioligand binding studies, CB1 receptor immunoreactivity has been found to be decreased in the postmortem DLPC of schizophrenic subjects compared to controls, with or without changes in *CB1* receptor mRNA [72,136,137] (Table 2). Urigüen et al. [137] reported a significant decrease in CB1 receptor immunoreactivity only in the DLPC of schizophrenic subjects that were antipsychotic-treated at the time of death without changes in those with a negative toxicology for antipsychotics. In the two reports by Eggan et al. [72,136] decreased CB1 receptor immunoreactivity was shown in the DLPC of subjects with schizophrenia compared to controls. Authors argued that this decrease was not a consequence of the antipsychotic treatment based on the lack of statistical correlation between CB1 receptor immunoreactivity and the presence of antipsychotic medication, and on the absence of alterations in CB1 expression in the brain of antipsychotic treated monkeys [72,136]. However, more than 75% of the schizophrenic subjects included in these studies were positive for antipsychotic drugs at the time of death, making it difficult to find statistical significance when assessing the potential impact of antipsychotics. CB1 mRNA expression has also been shown decreased in the DLPC of schizophrenic subjects [136], although absence of changes have also been reported [137]. In the cingulate cortex, no alterations in CB1 receptor immunoreactivity nor *CB1* receptor mRNA have been found [73] (Table 2). The reported changes in CB1 receptor immunoreactivity in the DLPC of medicated schizophrenic subjects point to a role of the antipsychotic treatment in the regulation of the ECS in this brain area. However, it is unknown whether this antipsychotic modulation of CB1 receptors could contribute or not to the therapeutic effects of these drugs. Taking into account all the results from postmortem studies regarding CB1 receptors in schizophrenia, showing lower or unchanged levels of mRNA, reduced immunoreactivity and higher receptor binding, two potential hypotheses have been proposed: 1) an altered trafficking of the receptor resulting in higher levels of membrane-bound CB1 receptor, and 2) a higher CB1 receptor affinity [131]. Both situations would entail a greater CB1 receptor availability, something that is not supported in all the neuroimaging studies reported to date [123].

Overall, the imaging and postmortem outcomes regarding CB1

receptor availability, density and expression in the brain of schizophrenic patients, although inconclusive, point towards a role of CB1 receptors in this pathology. Further research including functional assessment of the status of this receptor might help in understanding the potential pathophysiological consequences of the altered CB1 receptor availability, density and/or expression.

Little is yet known about the status of CB2 receptors in schizophrenia. To date, the only study reporting data related to CB2 receptor in the brain of schizophrenic patients found no significant correlation between the diagnosis of schizophrenia and total CB2 mRNA expression in the postmortem DLPC (BA9) [82] (Table 2). The main goal of this study was to test the association between tag SNPs in the *CNR2* gene and schizophrenia. In this regard, authors showed two SNPs associated with schizophrenia that were also related to reduced function of CB2 receptors, thus concluding that people with genetically predetermined lower functioning of CB2 receptors has an increased susceptibility to suffer schizophrenia when combined with other risk factors [82]. Previously, in 2003, De Marchi and co-workers [138] reported that clinical remission in schizophrenic patients was accompanied by a significant decrease in CB2 receptor mRNA in peripheral blood mononuclear cells. This finding does not agree with the observations of potential reduced CB2 receptor function associated with increased risk for schizophrenia [82], but authors also suggested that these peripheral changes might be related to several immunological alterations described in this pathology [138]. Nevertheless, further research is needed to support the potential role of CB2 receptors in schizophrenia.

Several genetic studies investigating different components of the ECS in patients with schizophrenia have been carried out, most of them focusing on different polymorphisms of the *CNR1* gene. Nevertheless, it must be noted that all genetic studies have been carried out in peripheral blood samples, and not in the brain. As these studies go beyond the main scope of this review, only a brief summary of the studies will be provided.

Nineteen studies have addressed different *CNR1* polymorphisms in relation with schizophrenia. Four of them studied the triplet AAT in *CNR1* repeat, finding no linkage when comparing with the general population of schizophrenic patients from different genetic backgrounds [139–142]. Another study from Seifert et al. studied two other SNPs apart from the triplet AAT (rs6454674 and rs1049353), not finding any association with any of them [143]. Nevertheless, a nine-time repetition of the triplet AAT in *CNR1* has been associated with the hebephrenic subtype of schizophrenia in two different studies, carried out in Caucasian and Japanese population [144,145]. Ujike et al. [144] also addressed the association of rs1049353 in their cohort, but, as in the study of Seifert, they did not find any linkage. These data reflect the heterogeneity of the schizophrenia and suggest that variations in the *CNR1* gene may contribute to the pathogenesis of specific subtypes of this disorder.

Some other studies have analyzed the SNP rs1049353 in schizophrenic patients [87,146], none of them showing any significant linkage. Several other studies have failed in trying to find associations between different SNPs of *CNR1* (rs806366, rs806368, rs806376, rs806379, rs806380, rs6454674, sr1535255 among other) and schizophrenia [147–150]. However, some SNPs such as rs6454674 [151], rs2023239 [152] and interactions with rs1049353, rs1535255, and rs2023239 [152] have been associated with positive and negative symptoms. At the same time, a study from Tiwari et al. have found an association between *CNR1* SNP rs806378 and the weight gain as a consequence of antipsychotic treatment [153].

An interesting study evaluated interactions between *CNR1* gene polymorphisms, cannabis use, cerebral volume and cognitive function [154]. They compared patients with schizophrenia or schizoaffective disorder with cannabis abuse/dependency and patients without cannabis use and observed smaller frontotemporal white matter (WM) volumes in those that smoked cannabis. They also observed associations between SNPs rs12720071, rs7766029, rs9450898 and WM volumes, as well as between SNP rs12720071 and processing speed/attention and problem-solving tests. Those results suggest that the use of cannabis in association with specific *CNR1* genotypes can contribute to alterations in WM and cognitive deficits in a subgroup of schizophrenic patients, which supports the hypothesis that both genetic and environmental factors could work together to determine the phenotypic expression in patients with schizophrenia.

Regarding genetic studies involving *CNR2*, data are critically scarce. One study has reported a close relationship between a polymorphism of the *CNR2* and increased susceptibility to schizophrenia in a large Japanese population [83]. This association was also confirmed in postmortem PFC from schizophrenic and control subjects with other ethnicities, being the risk allele also associated with low *CB2* receptor mRNA levels [83]. Furthermore, culture cell experiments showed that this *CNR2* gene polymorphism was linked to a lower functionality of the CB2 receptor, suggesting an increased risk of schizophrenia for people with low CB2 receptor function [83].

5.2. Endocannabinoid synthesizing and metabolizing enzymes in schizophrenia

Only a few studies have evaluated the endocannabinoid enzymes in the brain of patients with schizophrenia (Table 2). In the first study [155], quantitative polymerase chain reaction (PCR) was used to measure mRNA levels of DAGL (DAGLα and DAGLβ), MAGL, and FAAH. The mRNA level quantification of these enzymes was carried out in the prefrontal cortex Brodmann's area 9 of 42 schizophrenia subjects and matched control comparison subjects. No differences between subject groups were found in mRNA levels for endocannabinoid synthesizing and metabolizing enzymes.

In a more recent study, the same authors studied the transcript levels for the recently discovered 2-AG metabolizing enzyme, α-β-hydrolase domain 6 (ABHD6), in the prefrontal cortex of schizophrenia and healthy subjects (n = 84), using quantitative PCR [156]. This study showed that ABHD6 mRNA levels were elevated in schizophrenia subjects who were younger and had a shorter illness duration relative to age-matched comparison subjects. Furthermore, age and illness duration were strongly correlated in schizophrenia subjects, which made it difficult to differentiate between their effects on ABHD6 mRNA levels.

On the other hand, Morita et al. investigated a possible relationship between the non-synonymous polymorphism in Pro129Thr (rs324420) of the *FAAH* gene and schizophrenia. No differences were found in a group of 260 patients with schizophrenia (127 paranoids, 127 hebephrenics and 6 not classified) as compared to 63 controls in a Japanese population, regardless of the disorder subtype [157].

5.3. Endocannabinoid levels in schizophrenia

To date, only one study has evaluated endocannabinoid levels directly in the brain of patients with schizophrenia [158]. In this postmortem study, contents of the two main endocannabinoids, 2-AG and AEA, as well as other endocannabinoid and cannabimimetic compounds were quantified in three brain regions of subjects with schizophrenia and matched controls. The study revealed an opposite pattern for the regulation of endocannabinoids in schizophrenia. Authors found increased levels of 2-AG in cerebellum, hippocampus, and DLPC, whereas decreased levels of AEA and other *N*-acylethanolamines—dihomo-γ-linolenoylethanolamine (LEA), oleoylethanolamide (OEA), palmytoylethanolamide (PEA), and docosahexaenoylethanolamine (DHEA)—were reported [158]. In this way, antipsychotic medications reduced the content of endocannabinoids in the prefrontal cortex and hippocampus, but not in cerebellum, of antipsychotic-treated patients compared to antipsychotic-free subjects [158].

Before this study, several works focused their attention in the link between endocannabinoid levels, in both cerebrospinal fluid (CSF) and blood, and schizophrenia [138,159–162]. Thus, four studies of the same

research group have reported elevated AEA levels in CSF of schizophrenic patients, with no significant differences in serum AEA levels between schizophrenic patients and controls [159–162]. Moreover, in non-medicated acute schizophrenics, a negative correlation was found between CSF AEA levels and psychotic symptoms [160]. The increases of AEA levels reported in CSF from patients with schizophrenia contrast with the reduced AEA found in postmortem human brain of schizophrenics, but the neuronal origin of CSF endocannabinoids remains conjectural and it might reflect peripheral alterations of these signaling messengers [138]. In this sense, an increase in AEA levels in the blood of patients with acute schizophrenia respect to healthy volunteers has been reported [138]. Furthermore, in schizophrenic patients, pharmacologically-induced remission of the symptoms was accompanied by a significant decrease of blood AEA levels and of the mRNA transcripts for the degrading enzyme FAAH [138]. Thus, it has been proposed that the increased blood AEA levels observed in patients with acute schizophrenia might be due to the modified immune response observed during the course of the disease [138]. In fact, patients in initial prodromal states of psychosis with lower levels of AEA in CSF showed a higher risk for transiting to psychosis earlier [162]. Regarding the effect of antipsychotic medication on CSF endocannabinoid levels, AEA concentrations remained increased in patients that were treated with atypical antipsychotics, but not in those treated with typical ones [160].

In this line, a clinical trial in acute schizophrenia has evaluated the antipsychotic effects of the non-psychoactive phytocannabinoid cannabidiol (CBD) versus the atypical antipsychotic amisulpride, assessing in turn endocannabinoid serum levels along treatments [163]. Either treatment was safe and led to significant clinical improvement, but CBD displayed a markedly superior side effects profile. Results also showed that treatment with CBD, but not with amisulpride, was accompanied by a significant increase in serum AEA levels that were also associated with clinical improvement [163]. These authors suggest that inhibition of AEA deactivation may contribute to the antipsychotic effects of CBD potentially representing a completely new mechanism in the treatment of schizophrenia [163].

Besides AEA, other endocannabinoids have also been evaluated in CSF or serum of patients with schizophrenia. There is only one study which has attempted to determine 2-AG levels in CSF of schizophrenic patients [159]. However, despite being the most abundant endocannabinoid in the brain [164], significant levels of 2-AG could not be detected in any of the samples analyzed, suggesting that these endocannabinoids concentrations are exceedingly low in CSF of both controls and schizophrenic patients. The levels of the endogenous analogues of AEA, OEA and PEA, have also been explored out of the brain in schizophrenia. In observational studies, no differences were found in CSF or serum OEA levels between controls and schizophrenic patients [159–162]. By contrast, a 2-fold increase in PEA CSF levels was found in schizophrenic patients compared to controls [159]. However, this finding was not replicated in subsequent studies [160,161]. In previously mentioned clinical trial with CBD performed in patients with acute schizophrenia, both OEA and PEA serum levels were significantly elevated in schizophrenic patients treated with CBD, compared to those treated with amisulpride [163].

It is also noteworthy that CSF endocannabinoid levels have been shown to be affected depending on the history of cannabis use. Thus, markedly altered AEA concentrations (> 10-fold higher) were reported in CSF of a subgroup of schizophrenic patients who had low frequency cannabis use compared to controls (with high and low frequency use), as well as compared to schizophrenic high-frequency users [161]. However, this impact of cannabis use was not observed in other studies [160,162].

Compiling the information available from these studies is evident that the relationship between levels of endocannabinoids measured in the CSF, peripheral blood and concentrations of endocannabinoids in brain tissue is not clear yet. Thus, the understanding of functional implications of altered levels of endocannabinoids in each type of sample of schizophrenic patients remains to be elucidated.

6. Conclusions

Several evidences suggest the relationship between changes in one or more components of the ECS and some of the symptoms that are present in depression, anxiety-related disorders and schizophrenia. Indeed, recent human postmortem and *in vivo* neuroimaging studies are providing more knowledge about the implication of the ECS in these mental disorders. Most of the findings in depression and anxiety are related to the expression and/or functionality of CB1 receptors and FAAH in brain areas belonging to the amygdala-hippocampal-cortico-striatal neural circuit, especially the frontal cortex in depression and the amygdala in anxiety disorders. Regarding schizophrenia, the findings in postmortem and living human brains highlight a deregulation of CB1 receptor in specific brain areas that are highly affected in this disease. The findings on peripheral endocannabinoid levels are in good consonance with these adaptive changes. However, we must be cautious since peripheral endocannabinoid levels may not be well correlated with brain concentrations.

The pharmacological manipulation of the ECS is envisaged as an attractive alternative treatment for these mental disorders. For instance, drugs as the phytocannabinoid compound CBD have been reported to be effective to treat schizophrenia.

The advance in this field, together with the translational preclinical research is opening an attractive research scenario for the development of promising new pharmacological strategies based on drugs targeting the ECS to treat mental disorders.

Acknowledgements

This study was supported by the Spanish Ministry of Economy and Competitiveness (SAF2015-67457-R, MINECO/FEDER), the Plan Estatal de I+D+i 2013-2016, the Instituto de Salud Carlos III-Subdirección General de Evaluación y Fomento de la Investigación, Spanish Ministry of Economy, FEDER (PI13/01529) and the Basque Government (IT616/13). I I-L is a recipient of a Predoctoral Fellowship from the Basque Government. E F-Z is a recipient of a Predoctoral Fellowship from the University of Cantabria. CM is a recipient of a Postdoctoral Marie Skłodowska-Curie Individual Fellowship (H2020-MSCA-IF-2016, ID 747487).

Conflict of interests

There is no conflict of interest to declare.

References

[1] H.A. Whiteford, L. Degenhardt, J. Rehm, A.J. Baxter, A.J. Ferrari, H.E. Erskine, et al., Global burden of disease attributable to mental and substance use disorders: findings from the Global Burden of Disease Study 2010, Lancet 382 (9904) (2013) 1575–1586.
[2] J.J. Meana, L.F. Callado, B. Morentin, Do post-mortem brain studies provide useful information for Psychiatry? Rev. Psiquiatr. Salud Ment (Barc.) 7 (2014) 3.
[3] C.H. Ashton, P.B. Moore, Endocannabinoid system dysfunction in mood and related disorders, Acta Psychiatr. Scand. 124 (4) (2011) 250–261.
[4] M.N. Hill, B.B. Gorzalka, Is there a role for the endocannabinoid system in the etiology and treatment of melancholic depression? Behav. Pharmacol. 16 (5–6) (2005) 333–352.
[5] M.N. Hill, B.B. Gorzalka, Pharmacological enhancement of cannabinoid CB1 receptor activity elicits an antidepressant-like response in the rat forced swim test, Eur. Neuropsychopharmacol. 15 (6) (2005) 593–599.
[6] D. Parolaro, N. Realini, D. Vigano, C. Guidali, T. Rubino, The endocannabinoid system and psychiatric disorders, Exp. Neurol. 224 (1) (2010) 3–14.
[7] A.F. Carvalho, E.J. Van Bockstaele, Cannabinoid modulation of noradrenergic circuits: implications for psychiatric disorders, Prog. Neuro-Psychopharmacol. Biol. Psychiatry 38 (1) (2012) 59–67.
[8] R. Mechoulam, L.A. Parker, The endocannabinoid system and the brain, Annu. Rev. Psychol. 64 (2013) 21–47.
[9] H.C. Lu, K. Mackie, An introduction to the endogenous cannabinoid system, Biol. Psychiatry 79 (7) (2016) 516–525.

[10] S. Zou, U. Kumar, Cannabinoid receptors and the endocannabinoid system: signaling and function in the central nervous system, Int. J. Mol. Sci. 19 (3) (2018).
[11] A.C. Howlett, F. Barth, T.I. Bonner, G. Cabral, P. Casellas, W.A. Devane, et al., International union of pharmacology. XXVII. Classification of cannabinoid receptors, Pharmacol. Rev. 54 (2) (2002) 161–202.
[12] J.C. Ashton, M. Glass, The cannabinoid CB2 receptor as a target for inflammation-dependent neurodegeneration, Curr. Neuropharmacol. 5 (2) (2007) 73–80.
[13] M.P. Viveros, E.M. Marco, S.E. File, Endocannabinoid system and stress and anxiety responses, Pharmacol. Biochem. Behav. 81 (2) (2005) 331–342.
[14] C.J. Riebe, F.A. Pamplona, K. Kamprath, C.T. Wotjak, Fear relief-toward a new conceptual frame work and what endocannabinoids gotta do with it, Neuroscience 204 (2012) 159–185.
[15] S. Ruehle, A.A. Rey, F. Remmers, B. Lutz, The endocannabinoid system in anxiety, fear memory and habituation, J. Psychopharmacol. 26 (1) (2012) 23–39.
[16] I. Akirav, The role of cannabinoids in modulating emotional and non-emotional memory processes in the hippocampus, Front. Behav. Neurosci. 5 (2011) 34.
[17] O. Gunduz-Cinar, K.P. MacPherson, R. Cinar, J. Gamble-George, K. Sugden, B. Williams, et al., Convergent translational evidence of a role for anandamide in amygdala-mediated fear extinction, threat processing and stress-reactivity, Mol. Psychiatry 18 (7) (2013) 813–823.
[18] R.J. Bluett, J.C. Gamble-George, D.J. Hermanson, N.D. Hartley, L.J. Marnett, S. Patel, Central anandamide deficiency predicts stress-induced anxiety: behavioral reversal through endocannabinoid augmentation, Transl. Psychiatry 4 (2014) e408.
[19] J.M. Gray, H.A. Vecchiarelli, M. Morena, T.T. Lee, D.J. Hermanson, A.B. Kim, et al., Corticotropin-releasing hormone drives anandamide hydrolysis in the amygdala to promote anxiety, J. Neurosci. 35 (9) (2015) 3879–3892.
[20] M. Herkenham, A.B. Lynn, M.R. Johnson, L.S. Melvin, B.R. de Costa, K.C. Rice, Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study, J. Neurosci. 11 (2) (1991) 563–583.
[21] T. Rubino, C. Guidali, D. Vigano, N. Realini, M. Valenti, P. Massi, et al., CB1 receptor stimulation in specific brain areas differently modulate anxiety-related behaviour, Neuropharmacology 54 (1) (2008) 151–160.
[22] M.N. Hill, S. Patel, P. Campolongo, J.G. Tasker, C.T. Wotjak, J.S. Bains, Functional interactions between stress and the endocannabinoid system: from synaptic signaling to behavioral output, J. Neurosci. 30 (45) (2010) 14980–14986.
[23] M.N. Hill, J.G. Tasker, Endocannabinoid signaling, glucocorticoid-mediated negative feedback, and regulation of the hypothalamic-pituitary-adrenal axis, Neuroscience 204 (2012) 5–16.
[24] T.T. Lee, B.B. Gorzalka, Timing is everything: evidence for a role of corticolimbic endocannabinoids in modulating hypothalamic-pituitary-adrenal axis activity across developmental periods, Neuroscience 204 (2012) 17–30.
[25] M. Morena, S. Patel, J.S. Bains, M.N. Hill, Neurobiological interactions between stress and the endocannabinoid system, Neuropsychopharmacology 41 (1) (2016) 80–102.
[26] E. Aso, A. Ozaita, M.A. Serra, R. Maldonado, Genes differentially expressed in CB1 knockout mice: involvement in the depressive-like phenotype, Eur. Neuropsychopharmacol. 21 (1) (2011) 11–22.
[27] L. Uriguen, S. Perez-Rial, C. Ledent, T. Palomo, J. Manzanares, Impaired action of anxiolytic drugs in mice deficient in cannabinoid CB1 receptors, Neuropharmacology 46 (7) (2004) 966–973.
[28] O. Valverde, M. Torrens, CB1 receptor-deficient mice as a model for depression, Neuroscience 204 (2012) 193–206.
[29] M.N. Hill, S. Patel, E.J. Carrier, D.J. Rademacher, B.K. Ormerod, C.J. Hillard, et al., Downregulation of endocannabinoid signaling in the hippocampus following chronic unpredictable stress, Neuropsychopharmacology 30 (3) (2005) 508–515.
[30] M. Bortolato, R.A. Mangieri, J. Fu, J.H. Kim, O. Arguello, A. Duranti, et al., Antidepressant-like activity of the fatty acid amide hydrolase inhibitor URB597 in a rat model of chronic mild stress, Biol. Psychiatry 62 (10) (2007) 1103–1110.
[31] M.N. Hill, S.A. Kumar, S.B. Filipski, M. Iverson, K.L. Stuhr, J.M. Keith, et al., Disruption of fatty acid amide hydrolase activity prevents the effects of chronic stress on anxiety and amygdalar microstructure, Mol. Psychiatry 18 (10) (2013) 1125–1135.
[32] E. Lomazzo, L. Bindila, F. Remmers, R. Lerner, C. Schwitter, U. Hoheisel, et al., Therapeutic potential of inhibitors of endocannabinoid degradation for the treatment of stress-related hyperalgesia in an animal model of chronic pain, Neuropsychopharmacology 40 (2) (2015) 488–501.
[33] S. Gaetani, P. Dipasquale, A. Romano, L. Righetti, T. Cassano, D. Piomelli, et al., The endocannabinoid system as a target for novel anxiolytic and antidepressant drugs, Int. Rev. Neurobiol. 85 (2009) 57–72.
[34] M.N. Hill, C.J. Hillard, F.R. Bambico, S. Patel, B.B. Gorzalka, G. Gobbi, The therapeutic potential of the endocannabinoid system for the development of a novel class of antidepressants, Trends Pharmacol. Sci. 30 (9) (2009) 484–493.
[35] M.A. Katzman, M. Furtado, L. Anand, Targeting the endocannabinoid system in psychiatric illness, J. Clin. Psychopharmacol. 36 (6) (2016) 691–703.
[36] E.M. Marco, G. Laviola, The endocannabinoid system in the regulation of emotions throughout lifespan: a discussion on therapeutic perspectives, J. Psychopharmacol. 26 (1) (2012) 150–163.
[37] S. Patel, M.N. Hill, J.F. Cheer, C.T. Wotjak, A. Holmes, The endocannabinoid system as a target for novel anxiolytic drugs, Neurosci. Biobehav. Rev. 76 (Pt A) (2017) 56–66.
[38] T. Rubino, E. Zamberletti, D. Parolaro, Endocannabinoids and mental disorders, Handb. Exp. Pharmacol. 231 (2015) 261–283.
[39] R.M. Hirschfeld, The epidemiology of depression and the evolution of treatment, J. Clin. Psychiatry 73 (Suppl. 1) (2012) 5–9.
[40] A. Adell, E. Castro, P. Celada, A. Bortolozzi, A. Pazos, F. Artigas, Strategies for producing faster acting antidepressants, Drug Discov. Today 10 (8) (2005) 578–585.
[41] R. Vidal, E. Castro, F. Pilar-Cuellar, J. Pascual-Brazo, A. Diaz, M.L. Rojo, et al., Serotonin 5-HT4 receptors: a new strategy for developing fast acting antidepressants? Curr. Pharm. Des. 20 (23) (2014) 3751–3762.
[42] L. Degenhardt, W. Hall, M. Lynskey, Exploring the association between cannabis use and depression, Addiction 98 (11) (2003) 1493–1504.
[43] S. Lev-Ran, M. Roerecke, B. Le Foll, T.P. George, K. McKenzie, J. Rehm, The association between cannabis use and depression: a systematic review and meta-analysis of longitudinal studies, Psychol. Med. 44 (4) (2014) 797–810.
[44] A.L. Beautrais, P.R. Joyce, R.T. Mulder, Personality traits and cognitive styles as risk factors for serious suicide attempts among young people, Suicide Life Threat. Behav. 29 (1) (1999) 37–47.
[45] H. Ashton, J. Golding, V.R. Marsh, J.E. Millman, J.W. Thompson, The seed and the soil: effect of dosage, personality and starting state on the response to delta 9 tetrahydrocannabinol in man, Br. J. Clin. Pharmacol. 12 (5) (1981) 705–720.
[46] T.F. Denson, M. Earleywine, Decreased depression in marijuana users, Addict. Behav. 31 (4) (2006) 738–742.
[47] A.J. Gruber, H.G. Pope Jr., M.E. Brown, Do patients use marijuana as an antidepressant? Depression 4 (2) (1996) 77–80.
[48] D. Prentiss, R. Power, G. Balmas, G. Tzuang, D.M. Israelski, Patterns of marijuana use among patients with HIV/AIDS followed in a public health care setting, J. Acquir. Immune Defic. Syndr. 35 (1) (2004) 38–45.
[49] J. Kotin, R.M. Post, F.K. Goodwin, 9 -Tetrahydrocannabinol in depressed patients, Arch. Gen. Psychiatry 28 (3) (1973) 345–348.
[50] C. Price, T. Hemmingsson, G. Lewis, S. Zammit, P. Allebeck, Cannabis and suicide: longitudinal study, Br. J. Psychiatry 195 (6) (2009) 492–497.
[51] J.M. Witkin, E.T. Tzavara, G.G. Nomikos, A role for cannabinoid CB1 receptors in mood and anxiety disorders, Behav. Pharmacol. 16 (5–6) (2005) 315–331.
[52] R. Christensen, P.K. Kristensen, E.M. Bartels, H. Bliddal, A. Astrup, Efficacy and safety of the weight-loss drug rimonabant: a meta-analysis of randomised trials, Lancet 370 (9600) (2007) 1706–1713.
[53] S.E. Nissen, S.J. Nicholls, K. Wolski, J. Rodes-Cabau, C.P. Cannon, J.E. Deanfield, et al., Effect of rimonabant on progression of atherosclerosis in patients with abdominal obesity and coronary artery disease: the STRADIVARIUS randomized controlled trial, JAMA 299 (13) (2008) 1547–1560.
[54] F.R. Bambico, N. Katz, G. Debonnel, G. Gobbi, Cannabinoids elicit antidepressant-like behavior and activate serotonergic neurons through the medial prefrontal cortex, J. Neurosci. 27 (43) (2007) 11700–11711.
[55] G. Griebel, J. Stemmelin, B. Scatton, Effects of the cannabinoid CB1 receptor antagonist rimonabant in models of emotional reactivity in rodents, Biol. Psychiatry 57 (3) (2005) 261–267.
[56] R.J. McLaughlin, M.N. Hill, A.C. Morrish, B.B. Gorzalka, Local enhancement of cannabinoid CB1 receptor signalling in the dorsal hippocampus elicits an antidepressant-like effect, Behav. Pharmacol. 18 (5–6) (2007) 431–438.
[57] A.C. Morrish, M.N. Hill, C.J. Riebe, B.B. Gorzalka, Protracted cannabinoid administration elicits antidepressant behavioral responses in rats: role of gender and noradrenergic transmission, Physiol. Behav. 98 (1–2) (2009) 118–124.
[58] M.A. Steiner, G. Marsicano, E.J. Nestler, F. Holsboer, B. Lutz, C.T. Wotjak, Antidepressant-like behavioral effects of impaired cannabinoid receptor type 1 signaling coincide with exaggerated corticosterone secretion in mice, Psychoneuroendocrinology 33 (1) (2008) 54–67.
[59] K.Y. Vinod, B.L. Hungund, Cannabinoid-1 receptor: a novel target for the treatment of neuropsychiatric disorders, Expert Opin. Ther. Targets 10 (2) (2006) 203–210.
[60] L.L. Murphy, R.M. Munoz, B.A. Adrian, M.A. Villanua, Function of cannabinoid receptors in the neuroendocrine regulation of hormone secretion, Neurobiol. Dis. 5 (6 Pt B) (1998) 432–446.
[61] E.J. Cone, R.E. Johnson, J.D. Moore, J.D. Roache, Acute effects of smoking marijuana on hormones, subjective effects and performance in male human subjects, Pharmacol. Biochem. Behav. 24 (6) (1986) 1749–1754.
[62] E.M. Dax, N.S. Pilotte, W.H. Adler, J.E. Nagel, W.R. Lange, The effects of 9-ene-tetrahydrocannabinol on hormone release and immune function, J. Steroid Biochem. 34 (1–6) (1989) 263–270.
[63] M. Ranganathan, G. Braley, B. Pittman, T. Cooper, E. Perry, J. Krystal, et al., The effects of cannabinoids on serum cortisol and prolactin in humans, Psychopharmacology 203 (4) (2009) 737–744.
[64] M.E. Ballard, G. Bedi, H. de Wit, Effects of delta-9-tetrahydrocannabinol on evaluation of emotional images, J. Psychopharmacol. 26 (10) (2012) 1289–1298.
[65] M.G. Bossong, H.H. van Hell, G. Jager, R.S. Kahn, N.F. Ramsey, J.M. Jansma, The endocannabinoid system and emotional processing: a pharmacological fMRI study with 9-tetrahydrocannabinol, Eur. Neuropsychopharmacol. 23 (12) (2013) 1687–1697.
[66] K.L. Medina, B.J. Nagel, A. Park, T. McQueeny, S.F. Tapert, Depressive symptoms in adolescents: associations with white matter volume and marijuana use, J. Child Psychol. Psychiatry 48 (6) (2007) 592–600.
[67] S.G. Shollenbarger, J. Price, J. Wieser, K. Lisdahl, Poorer frontolimbic white matter integrity is associated with chronic cannabis use, FAAH genotype, and increased depressive and apathy symptoms in adolescents and young adults, Neuroimage Clin. 8 (2015) 117–125.
[68] E. Molina-Holgado, J.M. Vela, A. Arevalo-Martin, G. Almazan, F. Molina-Holgado, J. Borrell, et al., Cannabinoids promote oligodendrocyte progenitor survival: involvement of cannabinoid receptors and phosphatidylinositol-3 kinase/Akt signaling, J. Neurosci. 22 (22) (2002) 9742–9753.
[69] B.B. Gorzalka, M.N. Hill, Putative role of endocannabinoid signaling in the

etiology of depression and actions of antidepressants, Prog. Neuro-Psychopharmacol. Biol. Psychiatry 35 (7) (2011) 1575–1585.
[70] B.L. Hungund, K.Y. Vinod, S.A. Kassir, B.S. Basavarajappa, R. Yalamanchili, T.B. Cooper, et al., Upregulation of CB1 receptors and agonist-stimulated [35S]GTPgammaS binding in the prefrontal cortex of depressed suicide victims, Mol. Psychiatry 9 (2) (2004) 184–190.
[71] K. Choi, T. Le, J. McGuire, G. Xing, L. Zhang, H. Li, et al., Expression pattern of the cannabinoid receptor genes in the frontal cortex of mood disorder patients and mice selectively bred for high and low fear, J. Psychiatr. Res. 46 (7) (2012) 882–889.
[72] S.M. Eggan, S.R. Stoyak, C.D. Verrico, D.A. Lewis, Cannabinoid CB1 receptor immunoreactivity in the prefrontal cortex: comparison of schizophrenia and major depressive disorder, Neuropsychopharmacology 35 (10) (2010) 2060–2071.
[73] D. Koethe, I.C. Llenos, J.R. Dulay, C. Hoyer, E.F. Torrey, F.M. Leweke, et al., Expression of CB1 cannabinoid receptor in the anterior cingulate cortex in schizophrenia, bipolar disorder, and major depression, J. Neural Transm. (Vienna) 114 (8) (2007) 1055–1063.
[74] M.N. Hill, W.S. Ho, C.J. Hillard, B.B. Gorzalka, Differential effects of the antidepressants tranylcypromine and fluoxetine on limbic cannabinoid receptor binding and endocannabinoid contents, J. Neural Transm. (Vienna) 115 (12) (2008) 1673–1679.
[75] G. Juhasz, D. Chase, E. Pegg, D. Downey, Z.G. Toth, K. Stones, et al., CNR1 gene is associated with high neuroticism and low agreeableness and interacts with recent negative life events to predict current depressive symptoms, Neuropsychopharmacology 34 (8) (2009) 2019–2027.
[76] M. Mitjans, A. Serretti, C. Fabbri, C. Gasto, R. Catalan, L. Fananas, et al., Screening genetic variability at the CNR1 gene in both major depression etiology and clinical response to citalopram treatment, Psychopharmacology 227 (3) (2013) 509–519.
[77] F.J. Barrero, I. Ampuero, B. Morales, F. Vives, J. de Dios Luna Del Castillo, J. Hoenicka, et al., Depression in Parkinson's disease is related to a genetic polymorphism of the cannabinoid receptor gene (CNR1), Pharmacogenomics J. 5 (2) (2005) 135–141.
[78] R. Icick, K. Peoc'h, E. Karsinti, K. Ksouda, A. Hajj, V. Bloch, et al., A cannabinoid receptor 1 polymorphism is protective against major depressive disorder in methadone-maintained outpatients, Am. J. Addict. 24 (7) (2015) 613–620.
[79] A. Agrawal, E.C. Nelson, A.K. Littlefield, K.K. Bucholz, L. Degenhardt, A.K. Henders, et al., Cannabinoid receptor genotype moderation of the effects of childhood physical abuse on anhedonia and depression, Arch. Gen. Psychiatry 69 (7) (2012) 732–740.
[80] K. Domschke, U. Dannlowski, P. Ohrmann, B. Lawford, J. Bauer, H. Kugel, et al., Cannabinoid receptor 1 (CNR1) gene: impact on antidepressant treatment response and emotion processing in major depression, Eur. Neuropsychopharmacol. 18 (10) (2008) 751–759.
[81] E.S. Onaivi, H. Ishiguro, J.P. Gong, S. Patel, P.A. Meozzi, L. Myers, et al., Brain neuronal CB2 cannabinoid receptors in drug abuse and depression: from mice to human subjects, PLoS One 3 (2) (2008) e1640.
[82] H. Ishiguro, O. Carpio, Y. Horiuchi, A. Shu, S. Higuchi, N. Schanz, et al., A nonsynonymous polymorphism in cannabinoid CB2 receptor gene is associated with eating disorders in humans and food intake is modified in mice by its ligands, Synapse 64 (1) (2010) 92–96.
[83] H. Ishiguro, Y. Horiuchi, M. Ishikawa, M. Koga, K. Imai, Y. Suzuki, et al., Brain cannabinoid CB2 receptor in schizophrenia, Biol. Psychiatry 67 (10) (2010) 974–982.
[84] A. Carrasquer, N.M. Nebane, W.M. Williams, Z.H. Song, Functional consequences of nonsynonymous single nucleotide polymorphisms in the CB2 cannabinoid receptor, Pharmacogenet. Genomics 20 (3) (2010) 157–166.
[85] M. Mitjans, C. Gasto, R. Catalan, L. Fananas, B. Arias, Genetic variability in the endocannabinoid system and 12-week clinical response to citalopram treatment: the role of the CNR1, CNR2 and FAAH genes, J. Psychopharmacol. 26 (10) (2012) 1391–1398.
[86] M. Rodriguez-Munoz, P. Sanchez-Blazquez, L.F. Callado, J.J. Meana, J. Garzon-Nino, Schizophrenia and depression, two poles of endocannabinoid system deregulation, Transl. Psychiatry 7 (12) (2017) 1291.
[87] P. Monteleone, M. Bifulco, G. Maina, A. Tortorella, P. Gazzerro, M.C. Proto, et al., Investigation of CNR1 and FAAH endocannabinoid gene polymorphisms in bipolar disorder and major depression, Pharmacol. Res. 61 (5) (2010) 400–404.
[88] J. Lazary, N. Eszlari, G. Juhasz, G. Bagdy, Genetically reduced FAAH activity may be a risk for the development of anxiety and depression in persons with repetitive childhood trauma, Eur. Neuropsychopharmacol. 26 (6) (2016) 1020–1028.
[89] M.N. Hill, G.E. Miller, W.S. Ho, B.B. Gorzalka, C.J. Hillard, Serum endocannabinoid content is altered in females with depressive disorders: a preliminary report, Pharmacopsychiatry 41 (2) (2008) 48–53.
[90] M.N. Hill, G.E. Miller, E.J. Carrier, B.B. Gorzalka, C.J. Hillard, Circulating endocannabinoids and N-acyl ethanolamines are differentially regulated in major depression and following exposure to social stress, Psychoneuroendocrinology 34 (8) (2009) 1257–1262.
[91] W.S. Ho, M.N. Hill, G.E. Miller, B.B. Gorzalka, C.J. Hillard, Serum contents of endocannabinoids are correlated with blood pressure in depressed women, Lipids Health Dis. 11 (2012) 32.
[92] E. Heyman, F.X. Gamelin, M. Goekint, F. Piscitelli, B. Roelands, E. Leclair, et al., Intense exercise increases circulating endocannabinoid and BDNF levels in humans–possible implications for reward and depression, Psychoneuroendocrinology 37 (6) (2012) 844–851.
[93] A.T. Lu, M.N. Ogdie, M.R. Jarvelin, I.K. Moilanen, S.K. Loo, J.T. McCracken, et al., Association of the cannabinoid receptor gene (CNR1) with ADHD and post-traumatic stress disorder, Am. J. Med. Genet. B Neuropsychiatr. Genet. 147B (8) (2008) 1488–1494.
[94] J. Lazary, A. Lazary, X. Gonda, A. Benko, E. Molnar, L. Hunyady, et al., Promoter variants of the cannabinoid receptor 1 gene (CNR1) in interaction with 5-HTTLPR affect the anxious phenotype, Am. J. Med. Genet. B Neuropsychiatr. Genet. 150B (8) (2009) 1118–1127.
[95] M. Haring, V. Enk, A. Aparisi Rey, S. Loch, I. Ruiz de Azua, T. Weber, et al., Cannabinoid type-1 receptor signaling in central serotonergic neurons regulates anxiety-like behavior and sociability, Front. Behav. Neurosci. 9 (2015) 235.
[96] A. Mendiguren, E. Aostri, J. Pineda, Regulation of noradrenergic and serotonergic systems by cannabinoids: relevance to cannabinoid-induced effects, Life Sci. 192 (2018) 115–127.
[97] I. Heitland, F. Klumpers, R.S. Oosting, D.J. Evers, J. Leon Kenemans, J.M. Baas, Failure to extinguish fear and genetic variability in the human cannabinoid receptor 1, Transl. Psychiatry 2 (2012) e162.
[98] H.C. Meyer, F.S. Lee, D.G. Gee, The role of the endocannabinoid system and genetic variation in adolescent brain development, Neuropsychopharmacology 43 (1) (2018) 21–33.
[99] K.J. Lester, J.R. Coleman, S. Roberts, R. Keers, G. Breen, S. Bogels, et al., Genetic variation in the endocannabinoid system and response to Cognitive Behavior Therapy for child anxiety disorders, Am. J. Med. Genet. B Neuropsychiatr. Genet. 174 (2) (2017) 144–155.
[100] A. Neumeister, M.D. Normandin, R.H. Pietrzak, D. Piomelli, M.Q. Zheng, A. Gujarro-Anton, et al., Elevated brain cannabinoid CB1 receptor availability in post-traumatic stress disorder: a positron emission tomography study, Mol. Psychiatry 18 (9) (2013) 1034–1040.
[101] R.H. Pietrzak, Y. Huang, S. Corsi-Travali, M.Q. Zheng, S.F. Lin, S. Henry, et al., Cannabinoid type 1 receptor availability in the amygdala mediates threat processing in trauma survivors, Neuropsychopharmacology 39 (11) (2014) 2519–2528.
[102] G. Bedse, R.J. Bluett, T.A. Patrick, N.K. Romness, A.D. Gaulden, P.J. Kingsley, et al., Therapeutic endocannabinoid augmentation for mood and anxiety disorders: comparative profiling of FAAH, MAGL and dual inhibitors, Transl. Psychiatry 8 (1) (2018) 92.
[103] A.R. Hariri, A. Gorka, L.W. Hyde, M. Kimak, I. Halder, F. Ducci, et al., Divergent effects of genetic variation in endocannabinoid signaling on human threat- and reward-related brain function, Biol. Psychiatry 66 (1) (2009) 9–16.
[104] O. Gunduz-Cinar, M.N. Hill, B.S. McEwen, A. Holmes, Amygdala FAAH and anandamide: mediating protection and recovery from stress, Trends Pharmacol. Sci. 34 (11) (2013) 637–644.
[105] I. Dincheva, A.T. Drysdale, C.A. Hartley, D.C. Johnson, D. Jing, E.C. King, et al., FAAH genetic variation enhances fronto-amygdala function in mouse and human, Nat. Commun. 6 (2015) 6395.
[106] I. Boileau, R.F. Tyndale, B. Williams, E. Mansouri, D.J. Westwood, B. Le Foll, et al., The fatty acid amide hydrolase C385A variant affects brain binding of the positron emission tomography tracer [11C]CURB, J. Cereb. Blood Flow Metab. 35 (8) (2015) 1237–1240.
[107] M. Pardini, F. Krueger, M. Koenigs, V. Raymont, C. Hodgkinson, S. Zoubak, et al., Fatty-acid amide hydrolase polymorphisms and post-traumatic stress disorder after penetrating brain injury, Transl. Psychiatry 2 (2012) e75.
[108] R. Wyrofsky, P. McGonigle, E.J. Van Bockstaele, Drug discovery strategies that focus on the endocannabinoid signaling system in psychiatric disease, Expert Opin. Drug Discov. 10 (1) (2015) 17–36.
[109] C.R. Bailey, E. Cordell, S.M. Sobin, A. Neumeister, Recent progress in understanding the pathophysiology of post-traumatic stress disorder: implications for targeted pharmacological treatment, CNS Drugs 27 (3) (2013) 221–232.
[110] D. Hauer, G. Schelling, H. Gola, P. Campolongo, J. Morath, B. Roozendaal, et al., Plasma concentrations of endocannabinoids and related primary fatty acid amides in patients with post-traumatic stress disorder, PLoS One 8 (5) (2013) e62741.
[111] A. Dlugos, E. Childs, K.L. Stuhr, C.J. Hillard, H. de Wit, Acute stress increases circulating anandamide and other N-acylethanolamines in healthy humans, Neuropsychopharmacology 37 (11) (2012) 2416–2427.
[112] M.N. Hill, L.M. Bierer, I. Makotkine, J.A. Golier, S. Galea, B.S. McEwen, et al., Reductions in circulating endocannabinoid levels in individuals with post-traumatic stress disorder following exposure to the World Trade Center attacks, Psychoneuroendocrinology 38 (12) (2013) 2952–2961.
[113] B. Yi, I. Nichiporuk, M. Nicolas, S. Schneider, M. Feuerecker, G. Vassilieva, et al., Reductions in circulating endocannabinoid 2-arachidonoylglycerol levels in healthy human subjects exposed to chronic stressors, Prog. Neuro-Psychopharmacol. Biol. Psychiatry 67 (2016) 92–97.
[114] C.H. Demers, E. Drabant Conley, R. Bogdan, A.R. Hariri, Interactions between anandamide and corticotropin-releasing factor signaling modulate human amygdala function and risk for anxiety disorders: an imaging genetics strategy for modeling molecular interactions, Biol. Psychiatry 80 (5) (2016) 356–362.
[115] E.F. Coccaro, M.N. Hill, L. Robinson, R.J. Lee, Circulating endocannabinoids and affect regulation in human subjects, Psychoneuroendocrinology 92 (2018) 66–71.
[116] A.A. Abajobir, T. Vos, K.H. Abate, C. Abbafati, K.M. Abbas, et al., Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016, Lancet 390 (10100) (2017) 1211–1259.
[117] V. Di Marzo, D. Melck, T. Bisogno, L. De Petrocellis, Endocannabinoids: endogenous cannabinoid receptor ligands with neuromodulatory action, Trends Neurosci. 21 (12) (1998) 521–528.
[118] I. Katona, T.F. Freund, Multiple functions of endocannabinoid signaling in the brain, Annu. Rev. Neurosci. 35 (2012) 529–558.
[119] K.R. Muller-Vahl, H.M. Emrich, Cannabis and schizophrenia: towards a cannabinoid hypothesis of schizophrenia, Expert Rev. Neurother. 8 (7) (2008) 1037–1048.

[120] J.J. Moreau, Du hachisch et de l'aliénation mentale: études psycholo-giques, Fortin Masson, Paris, 1845.
[121] D.F. Wong, H. Kuwabara, A.G. Horti, V. Raymont, J. Brasic, M. Guevara, et al., Quantification of cerebral cannabinoid receptors subtype 1 (CB1) in healthy subjects and schizophrenia by the novel PET radioligand [11C]OMAR, Neuroimage 52 (4) (2010) 1505–1513.
[122] J. Ceccarini, M. De Hert, R. Van Winkel, J. Peuskens, G. Bormans, L. Kranaster, et al., Increased ventral striatal CB1 receptor binding is related to negative symptoms in drug-free patients with schizophrenia, Neuroimage 79 (2013) 304–312.
[123] M. Ranganathan, P.D. Skosnik, D.C. D'Souza, Marijuana and madness: associations between cannabinoids and psychosis, Biol. Psychiatry 79 (7) (2016) 511–513.
[124] Y. Mihov, Positron emission tomography studies on cannabinoid receptor Type 1 in schizophrenia, Biol. Psychiatry 79 (12) (2016) e97–e99.
[125] B. Dean, S. Sundram, R. Bradbury, E. Scarr, D. Copolov, Studies on [3H]CP-55940 binding in the human central nervous system: regional specific changes in density of cannabinoid-1 receptors associated with schizophrenia and cannabis use, Neuroscience 103 (1) (2001) 9–15.
[126] K. Zavitsanou, T. Garrick, X.F. Huang, Selective antagonist [3H]SR141716A binding to cannabinoid CB1 receptors is increased in the anterior cingulate cortex in schizophrenia, Prog. Neuro-Psychopharmacol. Biol. Psychiatry 28 (2) (2004) 355–360.
[127] K.A. Newell, C. Deng, X.F. Huang, Increased cannabinoid receptor density in the posterior cingulate cortex in schizophrenia, Exp. Brain Res. 172 (4) (2006) 556–560.
[128] C. Deng, M. Han, X.F. Huang, No changes in densities of cannabinoid receptors in the superior temporal gyrus in schizophrenia, Neurosci. Bull. 23 (6) (2007) 341–347.
[129] V.S. Dalton, L.E. Long, C.S. Weickert, K. Zavitsanou, Paranoid schizophrenia is characterized by increased CB1 receptor binding in the dorsolateral prefrontal cortex, Neuropsychopharmacology 36 (8) (2011) 1620–1630.
[130] K.J. Jenko, J. Hirvonen, I.D. Henter, K.B. Anderson, S.S. Zoghbi, T.M. Hyde, et al., Binding of a tritiated inverse agonist to cannabinoid CB1 receptors is increased in patients with schizophrenia, Schizophr. Res. 141 (2–3) (2012) 185–188.
[131] D.W. Volk, S.M. Eggan, A.G. Horti, D.F. Wong, D.A. Lewis, Reciprocal alterations in cortical cannabinoid receptor 1 binding relative to protein immunoreactivity and transcript levels in schizophrenia, Schizophr. Res. 159 (1) (2014) 124–129.
[132] J.J. Kim, B. Crespo-Facorro, N.C. Andreasen, D.S. O'Leary, V. Magnotta, P. Nopoulos, Morphology of the lateral superior temporal gyrus in neuroleptic nai;ve patients with schizophrenia: relationship to symptoms, Schizophr. Res. 60 (2–3) (2003) 173–181.
[133] F.M. Benes, Amygdalocortical circuitry in schizophrenia: from circuits to molecules, Neuropsychopharmacology 35 (1) (2010) 239–257.
[134] **American, Psychiatric, Association, Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-5), in: Washington, DC, 2013.**
[135] R. Tandon, W. Gaebel, D.M. Barch, J. Bustillo, R.E. Gur, S. Heckers, et al., Definition and description of schizophrenia in the DSM-5, Schizophr. Res. 150 (1) (2013) 3–10.
[136] S.M. Eggan, T. Hashimoto, D.A. Lewis, Reduced cortical cannabinoid 1 receptor messenger RNA and protein expression in schizophrenia, Arch. Gen. Psychiatry 65 (7) (2008) 772–784.
[137] L. Uriguen, M.J. Garcia-Fuster, L.F. Callado, B. Morentin, R. La Harpe, V. Casado, et al., Immunodensity and mRNA expression of A2A adenosine, D2 dopamine, and CB1 cannabinoid receptors in postmortem frontal cortex of subjects with schizophrenia: effect of antipsychotic treatment, Psychopharmacology 206 (2) (2009) 313–324.
[138] N. De Marchi, L. De Petrocellis, P. Orlando, F. Daniele, F. Fezza, V. Di Marzo, Endocannabinoid signalling in the blood of patients with schizophrenia, Lipids Health Dis. 2 (2003) 5.
[139] S.J. Tsai, Y.C. Wang, C.J. Hong, Association study of a cannabinoid receptor gene (CNR1) polymorphism and schizophrenia, Psychiatr. Genet. 10 (3) (2000) 149–151.
[140] E. Dawson, Identification of a highly polymorphic triplet repeat marker for the brain cannabinoid receptor gene: use in linkage and association studies of schizophrenia, Schizophr. Res. 15 (1995) 1.
[141] N. Ballon, S. Leroy, C. Roy, M.C. Bourdel, A. Charles-Nicolas, M.O. Krebs, et al., (AAT)n repeat in the cannabinoid receptor gene (CNR1): association with cocaine addiction in an African-Caribbean population, Pharmacogenomics J. 6 (2) (2006) 126–130.
[142] I. Martinez-Gras, J. Hoenicka, G. Ponce, R. Rodriguez-Jimenez, M.A. Jimenez-Arriero, E. Perez-Hernandez, et al., (AAT)n repeat in the cannabinoid receptor gene, CNR1: association with schizophrenia in a Spanish population, Eur. Arch. Psychiatry Clin. Neurosci. 256 (7) (2006) 437–441.
[143] J. Seifert, S. Ossege, H.M. Emrich, U. Schneider, M. Stuhrmann, No association of CNR1 gene variations with susceptibility to schizophrenia, Neurosci. Lett. 426 (1) (2007) 29–33.
[144] H. Ujike, M. Takaki, K. Nakata, Y. Tanaka, T. Takeda, M. Kodama, et al., CNR1, central cannabinoid receptor gene, associated with susceptibility to hebephrenic schizophrenia, Mol. Psychiatry 7 (5) (2002) 515–518.
[145] I. Chavarria-Siles, J. Contreras-Rojas, E. Hare, C. Walss-Bass, P. Quezada, A. Dassori, et al., Cannabinoid receptor 1 gene (CNR1) and susceptibility to a quantitative phenotype for hebephrenic schizophrenia, Am. J. Med. Genet. B Neuropsychiatr. Genet. 147 (3) (2008) 279–284.
[146] S. Zammit, G. Spurlock, H. Williams, N. Norton, N. Williams, M.C. O'Donovan, et al., Genotype effects of CHRNA7, CNR1 and COMT in schizophrenia: interactions with tobacco and cannabis use, Br. J. Psychiatry 191 (2007) 402–407.
[147] N. Hamdani, J.P. Tabeze, N. Ramoz, J. Ades, M. Hamon, Y. Sarfati, et al., The CNR1 gene as a pharmacogenetic factor for antipsychotics rather than a susceptibility gene for schizophrenia, Eur. Neuropsychopharmacol. 18 (1) (2008) 34–40.
[148] R. van Winkel, R. Genetic, I. Outcome of Psychosis, Family-based analysis of genetic variation underlying psychosis-inducing effects of cannabis: sibling analysis and proband follow-up, Arch. Gen. Psychiatry 68 (2) (2011) 148–157.
[149] M. Costa, A. Squassina, D. Congiu, C. Chillotti, P. Niola, S. Galderisi, et al., Investigation of endocannabinoid system genes suggests association between peroxisome proliferator activator receptor-alpha gene (PPARA) and schizophrenia, Eur. Neuropsychopharmacol. 23 (7) (2013) 749–759.
[150] J.S. Bae, J.Y. Kim, B.L. Park, J.H. Kim, B. Kim, C.S. Park, et al., Genetic association analysis of CNR1 and CNR2 polymorphisms with schizophrenia in a Korean population, Psychiatr. Genet. 24 (5) (2014) 225–229.
[151] U.S. Copoglu, M. Igci, E. Bozgeyik, M.H. Kokacya, Y.Z. Igci, A. Ozden, et al., Cannabinoid receptor 1 (CNR1) gene polymorphisms in schizophrenia patients: Rs6454674 polymorphism is associated with disease severity, Klinik Psikofarmakoloji Bülteni-Bull. Clin. Psychopharmacol. 25 (4) (2015) 341–347.
[152] P. Suarez-Pinilla, R. Roiz-Santianez, V. Ortiz-Garcia de la Foz, P.C. Guest, R. Ayesa-Arriola, A. Cordova-Palomera, et al., Brain structural and clinical changes after first episode psychosis: Focus on cannabinoid receptor 1 polymorphisms, Psychiatry Res. 233 (2) (2015) 112–119.
[153] A.K. Tiwari, C.C. Zai, O. Likhodi, A. Lisker, D. Singh, R.P. Souza, et al., A common polymorphism in the cannabinoid receptor 1 (CNR1) gene is associated with antipsychotic-induced weight gain in Schizophrenia, Neuropsychopharmacology 35 (6) (2010) 1315–1324.
[154] B.C. Ho, T.H. Wassink, S. Ziebell, N.C. Andreasen, Cannabinoid receptor 1 gene polymorphisms and marijuana misuse interactions on white matter and cognitive deficits in schizophrenia, Schizophr. Res. 128 (1–3) (2011) 66–75.
[155] D.W. Volk, S.M. Eggan, D.A. Lewis, Alterations in metabotropic glutamate receptor 1α and regulator of G protein signaling 4 in the prefrontal cortex in Schizophrenia, Am. J. Psychiatry 167 (12) (2010) 1489–1498.
[156] D.W. Volk, B.I. Siegel, C.D. Verrico, D.A. Lewis, Endocannabinoid metabolism in the prefrontal cortex in schizophrenia, Schizophr. Res. 147 (1) (2013) 53–57.
[157] Y. Morita, H. Ujike, Y. Tanaka, N. Uchida, A. Nomura, K. Ohtani, et al., A non-synonymous polymorphism in the human fatty acid amide hydrolase gene did not associate with either methamphetamine dependence or schizophrenia, Neurosci. Lett. 376 (3) (2005) 182–187.
[158] C. Muguruza, M. Lehtonen, N. Aaltonen, B. Morentin, J.J. Meana, L.F. Callado, Quantification of endocannabinoids in postmortem brain of schizophrenic subjects, Schizophr. Res. 148 (1–3) (2013) 145–150.
[159] F.M. Leweke, A. Giuffrida, U. Wurster, H.M. Emrich, D. Piomelli, Elevated endogenous cannabinoids in schizophrenia, Neuroreport 10 (8) (1999) 1665–1669.
[160] A. Giuffrida, F.M. Leweke, C.W. Gerth, D. Schreiber, D. Koethe, J. Faulhaber, et al., Cerebrospinal anandamide levels are elevated in acute schizophrenia and are inversely correlated with psychotic symptoms, Neuropsychopharmacology 29 (11) (2004) 2108–2114.
[161] F.M. Leweke, A. Giuffrida, D. Koethe, D. Schreiber, B.M. Nolden, L. Kranaster, et al., Anandamide levels in cerebrospinal fluid of first-episode schizophrenic patients: impact of cannabis use, Schizophr. Res. 94 (1–3) (2007) 29–36.
[162] D. Koethe, A. Giuffrida, D. Schreiber, M. Hellmich, F. Schultze-Lutter, S. Ruhrmann, et al., Anandamide elevation in cerebrospinal fluid in initial prodromal states of psychosis, Br. J. Psychiatry 194 (4) (2009) 371–372.
[163] F.M. Leweke, D. Piomelli, F. Pahlisch, D. Muhl, C.W. Gerth, C. Hoyer, et al., Cannabidiol enhances anandamide signaling and alleviates psychotic symptoms of schizophrenia, Transl. Psychiatry 2 (2012) e94.
[164] N. Stella, P. Schweitzer, D. Piomelli, A second endogenous cannabinoid that modulates long-term potentiation, Nature 388 (6644) (1997) 773–778.

INFORMED CONSENT - *Document for patients*

FORMULARIO DE CONSENTIMIENTO INFORMADO

Estudio del receptor 5HT2A y de la vía Akt/GSK3 como mecanismos moleculares de la psicosis inducida por el consumo crónico de cannabis

Centro: Centro de Salud Mental Uribe/Osakidetza

Yo (nombre y apellidos)

.....

He leído la hoja de información que se me ha entregado.
He podido hacer preguntas sobre el estudio.
He recibido suficiente información sobre el estudio.

He hablado con: .....
(nombre del investigador)

Comprendo que la participación es voluntaria.
La información clínica recolectada descrita en el Proyecto se introducirá en una base de datos generando un código disociado que impida su identificación personal fuera del ámbito del Centro Asistencial. Los investigadores clínicos implicados en el presente Proyecto serán los responsables de custodiar la base de datos que permite la vinculación de los datos personales y clínicos con el código asignado a la información y a la muestra. Cada muestra tendrá asignado un código y sólo los facultativos del Centro de Salud tienen acceso a los datos personales de los pacientes. Las muestras que llegarán a la UPV/EHU serán muestras disociadas. La información asociada a cada código será introducida en una base de datos por la Investigadora Principal. La base de datos se guardará en un fichero de nivel de seguridad alto denominado INA-SCHIZO CANNABIS y que ya ha sido registrado en la Agencia Vasca de Protección de Datos y cuyo responsable interno es la IP Leyre Urigüen Echeverría.
Los resultados obtenidos serán presentados en publicaciones científicas de manera agrupada entre todos los participantes guardándose un estricto anonimato en lo referente a los datos personales que pudieran identificarle. En cualquier momento usted puede solicitar el acceso, rectificación, cancelación y oposición a sus datos conservados en el Centro de Salud para el presente estudio. También en cualquier momento, usted podrá solicitar la destrucción de su muestra sanguínea o los resultados de la misma, si ya ha sido procesada Para ello deberá contactar con los responsables clínicos.
Todo el proceso se desarrollará conforme a lo establecido en la Ley 14/2007 de Investigación Biomédica, la Ley 15/1999 de Protección de Datos de Carácter personal y la Normativa interna de Osakidetza y la UPV/EHU, en sus respectivos ámbitos de actuación.

Comprendo que puedo retirar mi consentimiento:
1º Cuando quiera
2º Sin tener que dar explicaciones.
3º Sin que esto repercuta en mis cuidados médicos.

Presto libremente mi conformidad para participar en el estudio.

FECHA: FIRMA DEL PACIENTE

FECHA: FIRMA DEL INVESTIGADOR

INFORMED CONSENT - *Document for controls*

FORMULARIO DE CONSENTIMIENTO INFORMADO

Estudio del receptor 5HT2A y de la vía Akt/GSK3 como mecanismos moleculares de la psicosis inducida por el consumo crónico de cannabis

Centro: Departamento de Farmacología, Facultad de Medicina y Odontología, Universidad del País Vasco UPV-EHU

Yo (nombre y apellidos)

.....

He leído la hoja de información que se me ha entregado.
He podido hacer preguntas sobre el estudio.
He recibido suficiente información sobre el estudio.

He hablado con: .....
(nombre del investigador)

Comprendo que la participación es voluntaria.
La información recolectada descrita en el Proyecto se introducirá en una base de datos generando un código disociado que impida su identificación personal. La información asociada a cada código será introducida en una base de datos por la Investigadora Principal. La base de datos se guardará en un fichero de nivel de seguridad alto denominado INA-SCHIZO CANNABIS y que ya ha sido registrado en la Agencia Vasca de Protección de Datos y cuyo responsable interno es la IP Leyre Urigüen Echeverría.
Los resultados obtenidos serán presentados en publicaciones científicas de manera agrupada entre todos los participantes guardándose un estricto anonimato en lo referente a los datos personales que pudieran identificarle. En cualquier momento usted puede solicitar el acceso, rectificación, cancelación y oposición a sus datos conservados en la Universidad del País Vasco UPV-EHU. También en cualquier momento, usted podrá solicitar la destrucción de su muestra sanguínea o los resultados de la misma, si ya ha sido procesada. Para ello deberá contactar con los responsables del Estudio.
Todo el proceso se desarrollará conforme a lo establecido en la Ley 14/2007 de Investigación Biomédica, la Ley 15/1999 de Protección de Datos de Carácter personal y la Normativa interna de la UPV/EHU.

Comprendo que puedo retirar mi consentimiento:

1º Cuando quiera
2º Sin tener que dar explicaciones.
3º Sin que esto repercuta en mis cuidados médicos.

Presto libremente mi conformidad para participar en el estudio.

FECHA: FIRMA DEL VOLUNTARIO

FECHA: FIRMA DEL INVESTIGADOR