eman ta zabal zazu

Universidad del País Vasco
Euskal Herriko Unibertsitatea
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DOKTOREGO-TESIA

Ingurune aberastuaren eragina MK-801 eskizofrenia animalia-ereduan: hobekuntza kognitiboaren oinarri neurokimikoak.

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Universidad del País Vasco
Euskal Herriko Unibertsitatea
Department of Neuroscience

DOCTORAL THESIS

Neurochemical Basis of Cognitive Enhancement in MK-801 Animal Model of Schizophrenia: The Role of Enriched Environment

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(Albert Szent-Györyi)

"The function of education is to teach one to think intensively and to think critically. Intelligence plus character - that is the goal of true education"

(Martin Luther King, Jr.)

"A mind that has been stretched by a new experience can never go back to its old dimensions"

(Oliver Wendell Holmes, Jr.)

Aurkibidea

ABSTRACT

NMDAR hypofunction hypothesis has been notably successful in explaining the pathophysiological findings and symptomatology of schizophrenia. Thereby, NMDAR blockade in rodents may represent a useful tool to identify new therapeutic approaches for cognitive and neurochemical alterations associated with the disease. In this respect, enriched environment (EE) could play an essential role. Investigating the underlying neurochemical modifications would shed new light on the neurobiological mechanism used by EE to reverse the actions of MK-801.

Using a multilevel approach of behavior, cellular quantification and protein analysis, we tested whether adult exposure to EE could improve behavioral, cognitive, structural and cellular impairments resulting from early postnatal MK-801 treatment (0.5mg/kg, P10-P20). Here we demonstrate for the first time that adult-life short exposure to EE ameliorated postnatal MK-801-induced cognitive alterations in Morris Water Maze, Novel Object Recognition and Object-in-Place tasks, and counteracted the reductions in brain volume, microvascular supply and interneurons markers. Moreover, we found increased cell proliferation because of MK-801, but only EE-housed animals presented increased granule cell survival. We also examined how MK-801 and EE influenced on NMDAR subunit expression, BDNF-TrkB signaling pathway, and downstream signaling pathways to provide a molecular basis to cellular and cognitive improvements. In the present study, we report that cognitive and cellular changes were associated with upregulated BDNF-TrkB signaling and a set of modifications on the components of glutamatergic neurotransmission.

Taken together, our findings reveal new insights into the beneficial effects of experience in EE in adulthood of animals treated postnatally with MK-801, and renders EE a useful strategy to improve cognitive impairments, network disturbances and neurochemical alterations relevant to schizophrenia.

1. Sarrera

Eskizofrenia gizartearen %1ari eragiten dion buru nahasmendua da. Gaixo eskizofrenikoek erikortasun eta heriotza tasa altuak erakusten dituzte eta haien bizi-itxaropena %20an murrizten da biztanleri orokorrarekin alderatuta (Auquier et al., 2007). Eskizofreniaren ikuspegi klinikotik azpimarratzekoak dira sintoma kognitiboak, hauek eskizofrenia pairatzen duten gaixoen gizarteratzea eragozten baitute. Gaur egun, sintoma kognitiboak tratagaitzak dira eta luzeko ezintasun eta langabeziarekin erlazio zuzena dute. Hauen artean, aipatzekoak dira oroimen hutsegiteak, lan-memoriako defizitak eta arrazoiketa prozesuetan zein ahozko jariotasunean erakusten dituzten zailtasunak.

Azken bi hamarkadetan, eskizofreniaren disfuntzio kognitiboa ulertzeko ahalegin ugari egin dira. Eragin handiko patologia izan arren, bere jatorri etiopatogenikoa ez da guztiz ezaguna. Hala, gaixotasunaren erantzule, besteak beste faktore genetikoak, ingurumen faktoreak edo hauen arteko konbinazio konplexuak proposatzen dituzten hainbat hipotesi argitaratu dira *(Singh et al., 2014; Uher, 2014)*. Garuneko eskanerrek eta *post mortem* egindako ikerketek aldaketa esanguratsuak azaleratu dituzte eskizofrenia duten gaixoetan, batez ere kortex prefrontal eta lobulu tenporaleko eremuetan. Hauen artean, kortexeko bolumen murriztua *(Ordóñez et al., 2016)*, zirkuituen konektibitate arazoak *(Garey L, 2010)* eta neuronen aldaketak deskribatu dira *(Liu et al., 2014)*. Aldaketa guzti hauek eskizofreniaren ezaugarri den disfuntzio kognitiboarekin erlazionatuta daude. Horrez gain, hipokanpoko barne- eta kanpo-zirkuituak ere aldatuta aurkitzen dira *(Benes, 2000)*, zelula GABAergikoen zirkuituak barne. Interneurona GABAergikoek zelula kitzikatzaileekin duten tokiko elkarrekintza nolabait eraldatuta dago eskizofrenian eta honek gaitasun kognitiboaren galera sortzen du *(Rotaru et al., 2012)*. Era berean, hipokanpoa eta kortex prefrontalaren arteko konexio aferente eta eferenteak ere kaltetuak daudela deskribatu izan da *(Godsil et al., 2013)*. *Post mortem* egindako ikerlanak eskizofreniaren etiologia kortex prefrontaleko eta hipokanpoko zelula GABAergiekoetan aurkitzen denaren euskarri dira. Zehazki, ikerketa inmunohistokimikoek parbalbumina eta somatostatina adierazten duten interneuronen murrizketa egiaztatu dute *(Hashimoto et al., 2003)*.

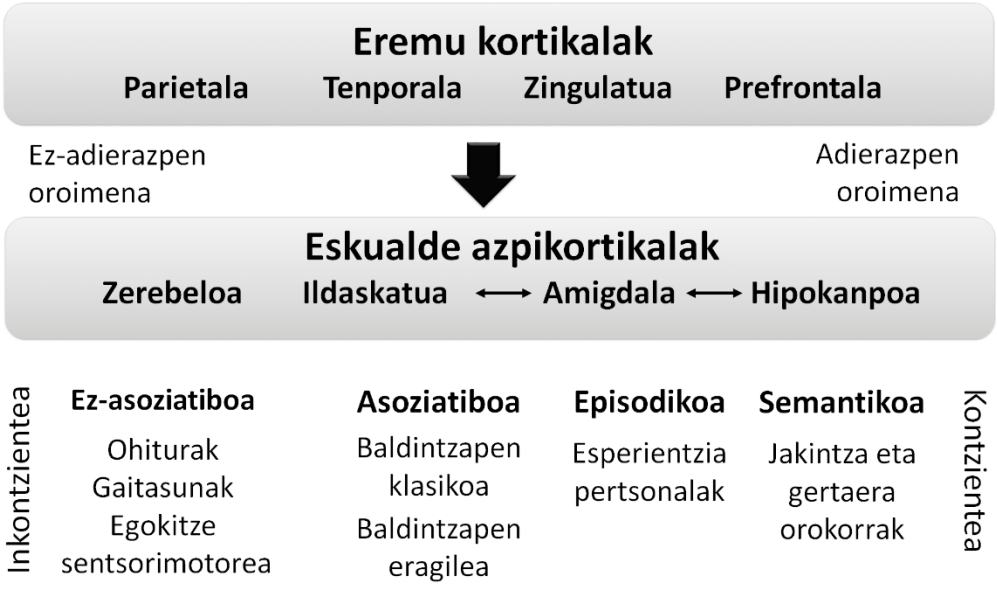
Eskizofreniaren oinarriak ikertu eta baliagarriak izan daitezkeen terapia eta estrategiak aztertzeko hainbat animalia-eredu erabili izan dira. Animalia-ereduak gaixotasun psikiatrikoak ikertzeko erabiltzearen eragozpenetako bat gaixotasun hauen oinarri neurobiologikoen ezagutza urria da. Hala ere, eskizofreniaren prebalentzia handia eta gaixotasun honi lotutako ondorio kaltegarriak kontuan izanik, animalia-ereduak

ezinbesteko tresna bilakatu dira. Karraskarietan eskizofrenia eragiteko hainbat eredu proposatu badira ere, jaiotza inguruan N-metil-D-aspartato hartzaileak (NMDAR) blokeatzeak eskizofrenia pairatzen duten gizakien antzeko sintomak eta aurkikuntza patologikoak azaleratzen ditu: kortex prefrontalaren bolumenaren murrizketa, hiperaktibitatea eta ahalmen kognitiboaren akatsak, besteak beste. Beraz, animalia-eredu hau bereziki interesgarria da gaixotasunaren aurkako terapia edo estrategia egokiak bilatzeko *(Bubeníková-Valesová et al., 2008; Nakazawa et al., 2017)*.

Gaixotasun neurologikoen sintomatologia imitatzen duten animalietan badago eritasun hauen sintomatologiaren murrizketan funtzio garrantzitsua betetzen duen eragile bat: ingurune aberastua. Ingurune aberastuak zentzumenen erabilera, ariketa fisikoa eta sozializazioa areagotzen ditu, eta hauen bidez, ikas-ahalmenaren eta oroimenaren areagotzea eragiten du, bai baldintza patologiko zein baldintza arruntetan. Ingurune aberastuko kaiolak ohiko laborategi-kaiolak baino handiagoak dira eta bertan tamaina, kolore eta testura desberdinetako objektuak kokatzen dira, hala nola, jostailuak, tunelak, aldapak eta ariketa fisikoa egiteko gurpilak. Halaber, aberastutako ingurunean hazitako animaliek neurogenesia, gliogenesia eta dendriten adarkatze handiagoa erakusten dute *(van Praag et al., 2000)* eta gertakari guzti hauek urrituta daude eskizofrenian. Nerbio sistema zentralaren gainean eragiten duten beste hainbat gaixotasunetan ingurune aberastuak efektu onuragarriak dituela egiaztatu da *(Nithianantharajah eta Hannan, 2006)*. Emaitza faboragarri hauek kontuan izanik, ingurune aberastuak eskizofrenian izan dezakeen eragina aztertzea da lan honen xedea. Orain arte egindako ikerketa guztietan, ingurune aberastuak animalien garunetan eragindako onurak nabarmenak dira, bai gaitasun kognitiboari dagokionez, bai aldaketa zelular eta molekularrei dagokienez ere. Hala ere, ingurune aberastua eta eskizofrenia uztartzen dituzten lan gutxi argitaratu dira. Eskizofrenia jaio ondorengo garapeneko gaixotasun bat dela susmatzen den arren, sintomak nerabezaro/helduaro goiztiarreraino agertzen ez direnez, erronka garrantzitsuenetako bat iraupen luzeko aberrazio sinaptikoak dituzten zirkuitoak eraldatzea da. Ildo honetatik jarraituz, NMDA hartzaileen hipofuntzioa eragiten zaion animalia-eredu batean ingurune aberastuak izan ditzakeen efektu onuragarriak ikertzeak, farmako espezifikoagoak garatzen lagunduko luke. Azpimarratzekoa da baita, ingurune aberastua albo-ondoriorik gabeko estrategia seguru bat dela, eskizofreniaren kasuan sintoma kongnitiboen tratamendurako interes translazionala duelarik.

1.1. Ikasketa eta Oroimena

Ikasketa eta oroimena ingurumenari buruzko informazio eguneratua barneratzea ahalbidetzen duten prozesu dinamikoak dira, azken xede bezala egokitzapenezko portaera gidatzea dutelarik *(Preston eta Eichenbaum, 2013)*. Ikasketa ingurunetik zentzumen-informazio ugari biltzen duen prozesu gisa definitzen bada, oroimenak informazio hori kodifikatzea, biltzea eta berreskuratzea ahalbidetzen du. Ikasketa eta oroimen-sistemak kategoria desberdinetan bana daitezke eta oroimen-sistema horietako bakoitza garuneko egitura eta zirkuitu neuronal desberdinetan oinarritzen da (1. irudia). Hala ere, objektu, leku eta gertaerei buruzko informazioa egitura azpikortikaletara konektatuta dauden garuneko integrazio multimodal edo asoziazio eremuetara helduko da, eta egitura azpikortikalek kortexeko adierazpena moldatuko dute. Elkarrekintza konplexu horien oinarriak oraindik argitzeko badaude ere, ikasketa eta oroimenak egunerokotasunean duen funtsezko rola dela eta, mekanismo zelular, molekular eta zirkuitu mekanismoak argitzeko ahalegin nabarmenak egiten ari dira.

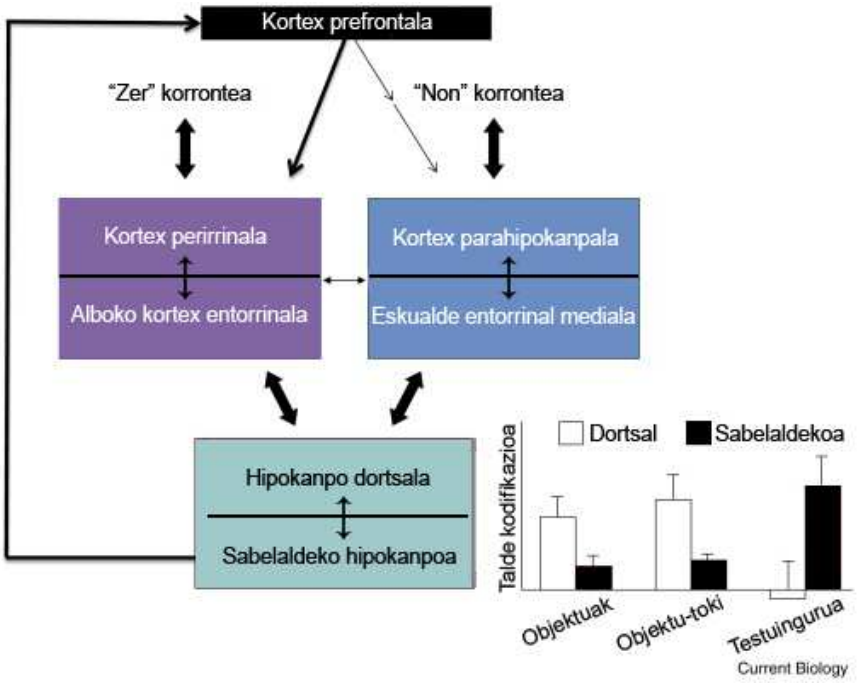


1. irudia. Oroimen-sistema ezberdinen irudikapen eskematikoa

1.1.1. Ikasketa eta oroimen prozesuetan parte hartzen duten garuneko eremuak

1.1.1.1. Hipokanpoa eta kortex prefrontalaren arteko elkarrekintza

Asko dira ikasketa eta oroimen prozesuetan parte hartzen duten eremu telentzefalikoak, baina hipokanpoak eta kortex prefrontal medialak (mPFC) jokatzen duten papera nabarmentzekoa da. Hipokanpoak objektu espezifikoak eta haien kokapenak kodifikatzen ditu testuinguru batean, karraskarien nabigazio espazialaren gaitasunerako bereziki garrantzitsua delarik *(Preston eta Eichenbaum, 2013)*. Gizakien kasuan aldiz, hipokanpoa adierazpen episodikoaren oroimena kodifikatzeko behar-beharrezko egitura da eta mPFC-ak funtzio exekutiboak gobernatzen ditu, arreta eta malgutasun kognitiboa barne. Horrez gain, mPFC-ak rol garrantzitsua betetzen du urruneko, azkenaldiko eta epe laburrerako oroitzapenetan ere *(Euston et al., 2012)*.



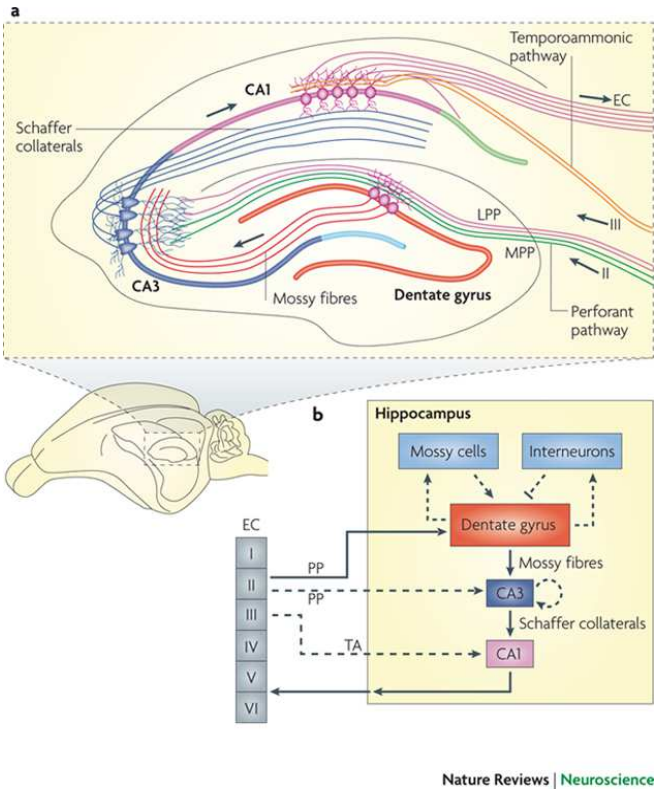
2. irudia. Hipokanpo eta kortex prefrontalaren arteko informazio-fluxu bideak. Preston eta Eichenbaum. Curr Biol. 2013-tik hartua eta moldatua.

Hipokanpoaren eta mPFC-aren zereginak oso desberdinak badirudite ere, bi eremu hauen eta beste eskualde kortikalen arteko elkarreragina funtsezkoa da portaera kognitibo egoki bat izateko *(Euston et al., 2012)*. Izan ere, hipokanpoa eta mPFC-a anatomikoki norantza bakarreko bide batez daude loturik *(Godsil et al., 2013)*. Sabelaldeko hipokanpotik eta subikulutik mPFC-era doazen proiekzioak bereziki indartsuak dira. Hipokanpo-prefrontal bideak testuinguruari buruzko informazioa bidaltzen du mPFC-era, testuinguruaren, gertaeren eta erantzunen arteko asoziazioen formazio eta sendotze azkarra ahalbidetzeko. Horrela, mPFC-ak egungo testuinguruaren eta gertaeren informazioa jasotzen du hipokanpotik eta iraganeko esperientzietan oinarrituz erantzun moldagarriena iragartzen du *(Euston et al., 2012)*. Kortex prefrontal medialak, atzeranzko proiekzioak bidaltzen ditu zeharkako bideetatik, kortex perirrinal eta alboko entorrinaletik, hipokanpoko jardueran eraginez hain zuzen (2. irudia). Halaber, talamoko nuleus reuniens deritzon nukleotik hipokanpora iristen den azpikortexeko bide bat ere badago. Beraz, kortex prefrontal mediala eta hipokanpoa elkarlanean ari dira ikasketa eta oroimen prozesuak sustatzeko.

1.1.1.2. Hipokanpo eta kortex prefrontalaren anatomia

Hipokanpoa sistema linbikoaren atala da eta eskualde ezberdinek osatzen dute: hortz-jiroa (HJ), cornus ammonis 3 (CA3) eta cornus ammonis 1 (CA1) (3a. irudia). Hortz-jiroan hiru geruza bereiz daitezke: geruza molekularra, granularra eta polimorfikoa. Geruza granularrean, zelula granularren dentsitate handia dago eta hauen dendritak geruza molekularrera zabaltzen dira bertan bide zulatzaileko axoi terminalekin sinapsia egiteko. Bide zulatzaileko zuntz gutxi batzuk interneuronekin ere burutzen dute sinapsia. Granaila-zuntzak (*mossy fibers*) CA3ra proiektatzen duten zelula granularren axoiak dira. Axoi hauek zuntz kolateral batzuk ematen dituzte geruza polimorfikoan granaila zelulekin (*mossy cells*) eta somatostatina adierazten duten zelula inhibitzaileekin kontaktatzen dutelarik. Hipokanpoko CA1 eta CA3 eskualdeak ere geruza desberdinetan banatzen dira. ***Stratum oriens***-a da kanporengo geruza eta bertan, zelula piramidalen hurbileko dendritak aurkitzeaz gain, saski-zelulen eta zelula horizontalen somak ere aurkitzen dira. Zelula kitzikatzaileen somak geruza piramidalean daude. Horrez gain, geruza honetan kokatzen dira interneurona ugariren somak ere, zelula axo-axoniko edo biestratifikatuenak kasu. ***Stratum lucidum***-a CA3 eskualdean aurkitzen den geruza fin

bat da. Granaila-zuntzak CA3ko geruza honetatik igarotzen dira CA2ko mugara heldu arte. Cornus ammonis 3-ko zelulen axoiak Schafferren bide kolateralaren bidez iristen dira CA1eko zelula piramidaletaraino eta komisura zuntzen bidez kontrako hipokanpoko CA1eraino, bidean zuntz hauek ***stratum radiatum***-etik pasatzen direlarik. Zelula piramidalen urruneko dendrita apikalak bide zulatzaileko zuntzekin eta *stratum oriens*-eko zelulen axoiekin egiten duten sinapsia ***stratum lacunosum-moleculare*** geruzan. CA3ko zelula piramidalen antzera, CA1eko neuronak bide zulatzailetik informazio zuzena jaso eta subikulura bidaltzen dute. Ondoren, neurona hauek hipokanpoko seinaleak berriro kortex entorrinalera bidaltzen dituzte, begizta bat sortuz (3b. irudia).

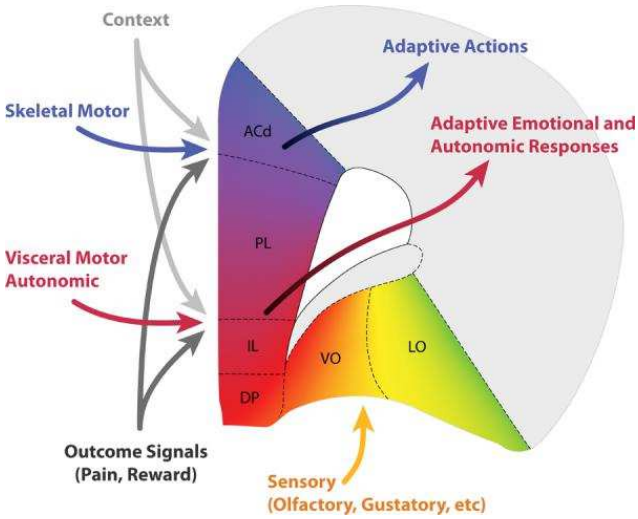


3. irudia. Karraskarien hipokanpoko zirkuitu neuralak. Deng et al. Nature Rev Neurosci. 2010-tik hartuta.

Funtzioaren ikuspuntutik, hipokanpoko eskualde nagusietan (HJ, CA3, CA1) bi atal bereiz daitezke: eremu dortsala eta sabelaldeko eremua. Eremu dortsalak (atzeko hipokanpoa gizakietan) funtzio kognitiboak bideratzen ditu eta sabelaldeko eremua (aurreko hipokanpoa gizakietan) hipokanpo afektibotzat jotzen da *(Fanselow eta Dong, 2010)*. Ikerketa anatomikoen arabera, hipokanpo dortsalaren eta sabelaldekoaren konexio aferente eta eferenteak desberdinak dira. Hipokanpo dortsalak objektuei eta tokiei buruzko informazioa kodifikatzen duen bitartean, sabelaldeko hipokanpoak testuinguru

desberdinen arteko bereizketak nabarmentzen ditu (2. irudia). Nabigazio eta oroimen espaziala ere hipokanpo dortsalaren menpe daude *(Fanselow eta Dong, 2010)*. Azkenik, hipokanpo dortsala, baina ez sabelaldekoa, ezinbestekoa da objektu-toki asoziazioetarako *(Barker eta Warburton, 2015)* eta objektu berriei buruzko oroimenak kontsolidatzeko, era goiztiar eta atzeratuan *(Clarke et al., 2010)*. Izan ere, espazioko kokapenak kodifikatzen dituzten leku-zelula gehien hipokanpo dortsaleko CA1 eskualdean daude eta hauek, buruak espazioan duen kokapenari buruzko informazioa bidaltzen dute subikulu eta aurresubikulu dortsalera proiekzio kitzikatzaileen bidez. Hipokanpo dortsaleko CA1 eta subikulutik bidaltzen diren proiekzio nabarmenenak kortex erretroesplenial eta aurreko kortex zingulatura doaz, karraskarietan prozesamendu bisuoespazialarekin lotura zuzena duten kortikexeko bi eremuetara *(Harker eta Whishaw, 2014)*.

Kortex prefrontal mediala (mPFC) garunaren aurrealde dortsalean dagoen horma medialeko zatirik handiena da eta portaera kognitibo konplexuak erregulatzen dituen garuneko eremu nagusietako bat da. Hauen artean funtzio exekutiboak aurkitzen dira, hala nola, plangintza eta erabakiak hartzeko gaitasuna edo sari-bidezko portaera gidatua. Era berean, aipatutako funtzio hauek prozesu kognitibo sinpleagoetan oinarritzen dira, atentzio kontrolean, lan-oroimenean edo malgutasun kognitiboan, besteak beste. Gainera, mPFC-ak ere paper garrantzitsua jokatzen du oroimenen kontsolidazioan eta berreskurapenean *(Euston et al., 2012)*. Irizpide anatomiko eta funtzionaletan oinarrituta, mPFC-a aurreko kortex zingulatua, kortex prelinbikoa eta kortex infralinbikoa deritzen eremuetan banatzen da (4. irudia). Eskualde prelinbikoak atentzioan, lan-oroimenean eta erantzunen aukeraketan parte hartzen du. Aldiz, aurreko kortex zingulatuak portaeraren ordena tenporala eta sekuentzia motoreekin erlazionatutako arauak sortzen ditu. Eskualde infralinbikoaren funtzioa ez da hain ezaguna, baina kontrol autonomikoarekin zerikusia duela uste da, batez ere, beldurrarekin erlazionatutako portaerekin *(Euston et al., 2012)*.



4. irudia. Kortex prefrontaleko eskualde nagusien irudikapen eskematikoa eta bertako informazio sarrera eta irteera nagusiak. ACd, aurreko kortex zingulatu dortsala; PL, kortex prelinbikoa; DP, kortex pedunkular dortsala; VO, sabelaldeko kortex orbitala; LO, alboko kortex orbitala. Euston et al. Neuron 2012-tik hartua.

1.1.1.3. Hipokanpoaren eta kortex prefrontal medialaren aldaketak nerabezaroan

Nerabezaroa haurtzarotik heldutasunerako trantsizioa da. Nerabezaroan zehar, gizarte-jokabidean eta garapen kognitiboan aldaketak agertzen dira eta aldaketa horiek kortex prefrontal medialeko eta hipokanpoko zirkuitu neuronalen garapenarekin irmoki lotuta daude.

Nerabezaroan zehar kortex prefrontaleko sinapsien murrizketa bat gertatzen da, ustez, sinapsi glutamatergikoetan *(Petanjek et al., 2011)*. Bestalde, mielinizazioa areagotzearen ondorioz, lobulu tenporal eta frontalen bolumenak handitu egiten dira *(Benes et al., 1994)*. Gainera, garai honetan sabelaldeko hipokanpotik kortex prefrontalera doazen konexio glutamatergiko berriak sortzen dira. Kontaktu monosinaptiko hauek, kortex prefrontaleko zelula piramidal eta interneurona parbalbumina-positiboak inerbatzen dituzte *(Gabbott et al., 2002)* eta karraskarien jaio ondorengo 50. egunean heltzen dira *(Caballero et al., 2014b)*. Hipokanpoa eta kortex prefrontalaren arteko bidearen osotasuna funtsezkoa da oroimen mota desberdinetarako eta gaixotasun psikiatrikoetan bide honen konexioak ahulak dira *(Godsil et al., 2013)*.

Kortex prefrontalaren inerbazio doparminergikoa prozesu kognitiboetarako beharrezkoa da eta inerbazio hau nerabezaroan zehar heltzen da. Aurreko kortex zingulatuko zuntz dopaminergikoen dentsitatea jaio ondorengo 20. eta 35. egunen artean garatzen da guztiz. Eskualde prelinbikoan, aldiz, jaio ondorengo 60. eguneraino luzatzen da. Dopaminaren D1 eta D2 hartzaileak ere areagotuz doaz heldutasunera iritsi arte. Bestalde, terminal dopaminergikoak zelula GABAergikoen soma eta dendritekin aposizio estuan daudela ikusi da eta nerabezaroan zehar esanguratsuki areagotzen dira kortex prefrontaleko V. eta VI. geruzetan, parbalbumina-positiboak diren interneuronetan batez ere *(Caballero et al., 2016)*. Arratoi gazteetan (15-30. jaio ondorengo eguna) egindako interneurona GABAergikoen erregistroen arabera, D1 motako dopaminaren hartzaileek jaurtiketa-arineko interneuronen kitzikapena areagotzen dute; ez ordea D2 motako hartzaileek. Hala ere, jaio ondorengo 50. egunetik aurrera, interneuronek bai D1 bai D2 hartzaileak adierazten dituzte eta dopaminak kortex prefrontaleko eragin GABAergikoa areagotuko du *(Tseng eta O'Donnell, 2007)*. Beraz, deskribatutakoaren arabera, dopaminarekin erlazionatutako inhibizioaren kontrola kortex prefrontalean GABAren jarduera erraztean datza.

Orain arte aipatutako aldaketez gain, nerabezaroan seinalizazio GABAergikoak eta neurotransmisio glutamatergikoak ere aldaketa garrantzitsuak jasaten dituzte. Aldaketa hauek 1.1.2.2. atalean (Interneurona GABAergikoen heltze-prozesua) eta 1.1.3.2. atalen (NMDA hartzaileen garrantzia heltze-prozesuan, ikasketan eta oroimenean) deskribatuko dira hurrenez hurren.

1.1.2. Interneuronak

Interneuronak ezinbestekoak dira zelula kitzikatzaileen arteko elkarrekintza konplexuak doitzeko. Gaur egungo joeren arabera, interneuronen aniztasunak garunak duen indar konputazionala irudikatzen du, non interneuronek hainbat ataza burutzen dituzten *(Moore et al., 2010)*. Interneuronek ez dute soilik gehiegizko kitzikapena ekiditeko burutzen inhibizioa. Sareko eragiketa konplexuetan parte hartzen dute, hala nola, erantzunaren aukeraketan, irabazi kontrolean edo zelula kitzikatzaileen jaurtiketa patroi espazio-tenporalak doitzen eta garun oszilazioak ere sortzen dituzte. *(Wehr eta Zador, 2003; Roux eta Buzsáki, 2015)*. Garun osasuntsuetan, jarduera guzti hauek dinamikoki egokitzen dira kanpoko eskakizunei behar bezala erantzuteko. Interneuronek

ikasketa eta oroimenean duten inplikazioa ikerketa ugaritan dokumentatu da, nahiz eta azpimota bakoitzak parte hartze ezberdina izan garuneko eskualde eta atazaren arabera.

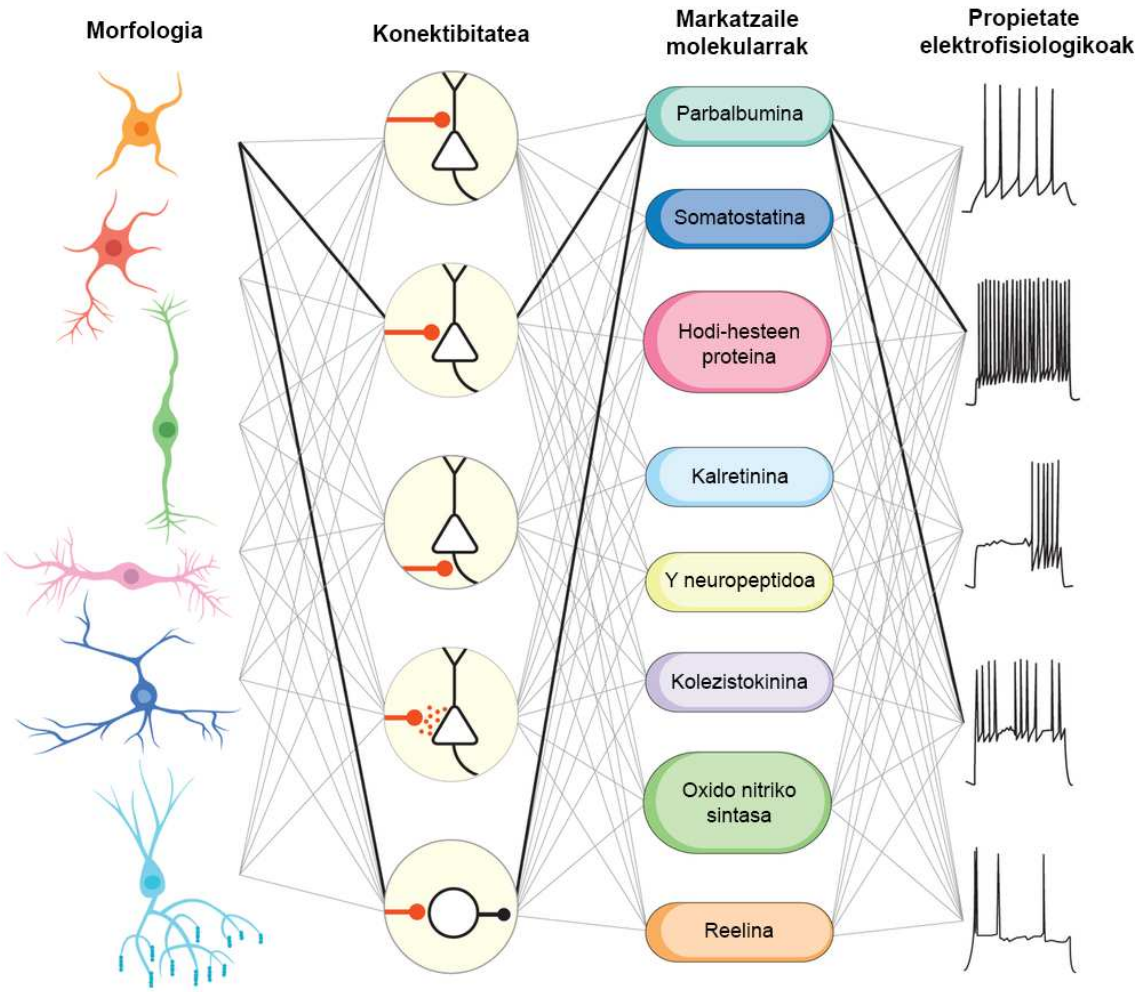
1.1.2.1. Interneuronen sailkapena

Interneurona GABAergikoak nerbio sistema zentraleko inhibizioaren iturri nagusia dira *(Jentsch et al., 2002)* eta neurona guztien %10-25a osatzen dute, garuneko eskualdearen arabera *(Le Magueresse eta Monyer, 2013)*. Interneuronetan fenotipo ugari ezberdintzen dira, hurrengo irizpide hauen arabera sailkatuz: (1) morfologia, (2) propietate elektrofisiologikoak, (3) markatzaile molekularrak: kaltzio-ligatzaileak (parbalbumina, kalretinina edo kalbindina), neuropeptidoak (somatostatina, hodi-hesteen peptidoa, Y neuropeptidoa, reelina) eta hartzaileak (5-HT3R, mGluR1, CB1), (4) zuzentzen diren neuronen domeinu azpizelularra eta (5) jatorri eta transkripzio-kontrolen arabera duten patua (5. irudia) .

Garun-kortex eta hipokanpoko interneuronen jatorria enbrioiaren azpipalioko hiru eskualdeetan dago: isats-gongoil eminentzian (IGE), gongoil eminentzia medialean (GEM) eta zona preoptikoan (ZPO). Kortex eta hipokanpoari dagokionez, IGE-k sortzen ditu interneurona gehien. Hauen artean parbalbumina adierazten duten jaurtiketa arineko saski eta argimutil zelulak (*Chandelier cells*) eta somatostatina adierazten duten interneuronak daude, azken hauek kalretinina (KR), Y-neuropeptidoa (YNP) edo reelina koadierazi dezaketelarik. Isats-gongoil eminentziak kolezistokinina (KZK), kalretinina, hodi-hesteen peptidoa (HHP), reelina eta neuroglia-formako zelulak sortzen ditu, baina ez somatostatina (SST) adierazten duten interneuronak. Sailkapen gisa erabiltzen diren molekulak, hala nola, proteina kaltzio-ligatzaileak (parbalbumina, kalretinina eta kalbindina) edo neuropeptidoak (somatostatina, hodi-hesteen peptidoa eta kolezistokinina) interneuronen azpipopulazioak desberdintzeko oso erabiliak dira ikerketa anatomikoetan eta propietate elektrofisiologikoekin korrelazioa dute.

Parbalbumina (PB) jaurtiketa arineko saski-zelula eta argimutil zeluletan aurkitzen da. Lehenak, soma eta hurbileko dendritak inhibitzen ditu eta azkenak axoien hasierako segmentua. Parbalbuminaren erdiespena neurogarapenak erregulatzen du. Hipokanpoan eta kortexean, parbalbuminaren adierazpenaren areagotze handiena jaio ondorengo lehen eta hirugarren asteen bitartean gertatzen da. Jaiotzean ez da

baina etengabe areagotzen da jaio ondorengo bigarren astean zehar. Neurogarapeneko hirugarren astean, igoera izugarria ematen da eta parbalbuminaren adierazpena apurka-apurka gehituz doa nerabezaroan zehar, 40. joe eta 55. joe bitartean heldu mailak lortzen diren arte *(Caballero et al., 2014a)*. Parbalbumina adierazten duten interneuronek ezinbesteko garrantzia dute inhibizio tonu egokia lortzeko. Inhibizioak eskualde kortikal ezberdinen aldi plastikoak kontrolatzen ditu eta, beraz, kognizioan nahita nahiezko eragina dauka.



5. irudia. Interneuronen sailkapena beraien morfologia, konektibitatea, markatzaile molekular eta propietate elektrofisiologikoen arabera. Kepecs eta Fishell. Nature. 2014-tik hartua eta moldatua.

Kalretinina (KR) adierazten duten interneuronen azpipopulazio GABAergikoak oso heterogeneoak dira. Kalretinina adierazten duten interneuronen gehiengoa zelula bipolarrak dira, bi azpipopulazio ezberdintzen direlarik: hodi-hesteen proteina adierazten duten zelula bipolarrak (HHP) eta somatostatina koadierazten duten Martinotti-bezalako zelulak. Azpipopulazio hauek isats-gongoil eminentziatik eta gongoil eminentzia medialetik datoz, hurrenez hurren. Interneurona KR-positiboak luzaroan ezezagunak izan badira ere, azken ikerketek interneurona HHP-positiboen antzeko ezaugarriak dituztela deskribatu dute *(Cauli et al., 2014)*. Zelula hauen funtzio nagusia "zirkuitu desinhibitzailea"-n zelula inhibitzaileei inhibizioa ematea da *(Gulyás et al., 1996)*. Kortex prefrontalean, zelula KR-positiboak normalean interneurona SST- eta PB-positiboetara zuzentzen dira *(Pi et al., 2013)*. Aitzitik, hipokanpoan interneurona kalbindina-positibo eta beste interneurona KR-positiboetara zuzentzen dira batik bat *(Gulyás et al., 1996)*, ustez, somatostatina ere adierazten dutenetara *(Gulyás et al., 1996; Somogyi et al., 2005)* eta ez dute interneurona PB-positiboekin kontaktatzen eskualde honetan.

Kalbindina (KB)-ren behin behineko adierazpena 21. joe-rako eskuratzen da interneuronetan. Interneurona KB-positiboek ere populazio heterogeneoa osatzen dute eta hauen artean buket-bikoitzeko zelulak (58%), zelula multipolarrak eta neuroglia-formako zelulak (31%) ezberdindu daitezke. Kalbindinaren markaketa ahula erakusten duten zelula batzuk zelula piramidalen morfologia dute neokortexeko II. eta III. geruzetan eta hipokanpoko geruza piramidal eta granularrean.

Neuropeptidoak neuronen kitzikapenaren modulatzaile dira. Neuropeptidoak neurotransmisore klasikoekin alderatuta, tamaina, sintesi eta jarduera mekanismo ezberdinak dituzte. Neuropeptidoak inguruko neuronen egoera modulatzen duten ko-transmisoreak dira. Beraien jarduera neurotransmisore klasikoena (glutamatoa eta GABA) baino askoz ere motelagoa da. Ikerketa farmakologikoek erakutsi dutenez, neokortexean adierazten diren neuropeptidoek emoziozko prozesuetan eta prozesu kognitiboetan parte hartzen dute.

Somatostatina (SST) axoi eta dendritetako besikuletatik askatzen den neuropeptido bat da *(Ludwig eta Pittman, 2003)*. Bere askapenerako frekuentzia handiko seinale aferenteak behar dira *(Kits eta Mansvelder, 2000)*. Somatostatinaren eragin zelular eta sinaptikoak nahikoa ondo ulertzen diren arren, garuneko zirkuituetan, portaeran eta kognizioan dituen ondorioak oraindik argitzeko daude *(Baraban eta Tallent,*

2004; Liguz-Lecznar et al., 2016). Somatostatina adierazten duten interneuronak oso zatikatuta dauden zuhaitz dendritikoetara daude zuzenduta eta integrazio sinaptikoa aktiboki moldatzen dute. Hipokanpoan, SST-k hiperpolarizazio postsinaptikoa eragiten du *(Pittman eta Siggings, 1981)* potasio korronteak areagotuz *(Schweitzer et al., 1998)* eta kaltzio korronteak murriztuz *(Ishibashi eta Akaike, 1995)*. Somatostatinak $GABA_B$ hartzaile presinaptikoen bidez zelula kitzikatzaileetako glutamatoaren askapena inhibitzen du *(Boehm eta Betz, 1997; Urban-Ciecko et al., 2015)*. Alabaina, SST GABAren potentzial postsinaptiko inhibitzailea murrizteko gai da, hau da, GABAren eragin inhibitzailea indargabetu dezake despolarizazio bat sortuz *(Scharfman eta Schwartzkroin, 1989; Leresche et al., 2000)*. Honek, interneurona berdinetik GABA eta SST askatzen direla iradokitzen du, baina baldintza ezberdinetan eta helburu ezberdinetarako. Gainera, SST adierazten duen interneurona azpimota espezifiko bat aurkitu da kortexeko IV. geruzan. Interneurona SST+ hauek ez dira II/III. geruzetan kokatzen diren eta I. geruzako dendrita apikaletara proiekzioak bidaltzen dituzten Martinotti interneuronen berdinak *(Ma et al., 2006)*. Laugarren geruzako interneurona SST-positiboak X-94 zelulen antzeko zelulak dira, jaurtiketa arineko zelulen ezaugarriak dituzte eta dirudienez jaurtiketa arineko interneurona PB-positiboekin kontaktatzen dute era lokalean *(Ma et al., 2006)*. Beraz, zelula SST-positiboen azpimota bat era selektiboan interneuronetara zuzenduta dauden interneurona multzo batek osatzen du eta hauek, seinale GABAergikoak bidaltzen dizkiete interneuronei beraien mintz-potentzialak erregulatzeko eta, ondorioz, aldaketa plastikoak mugatzeko.

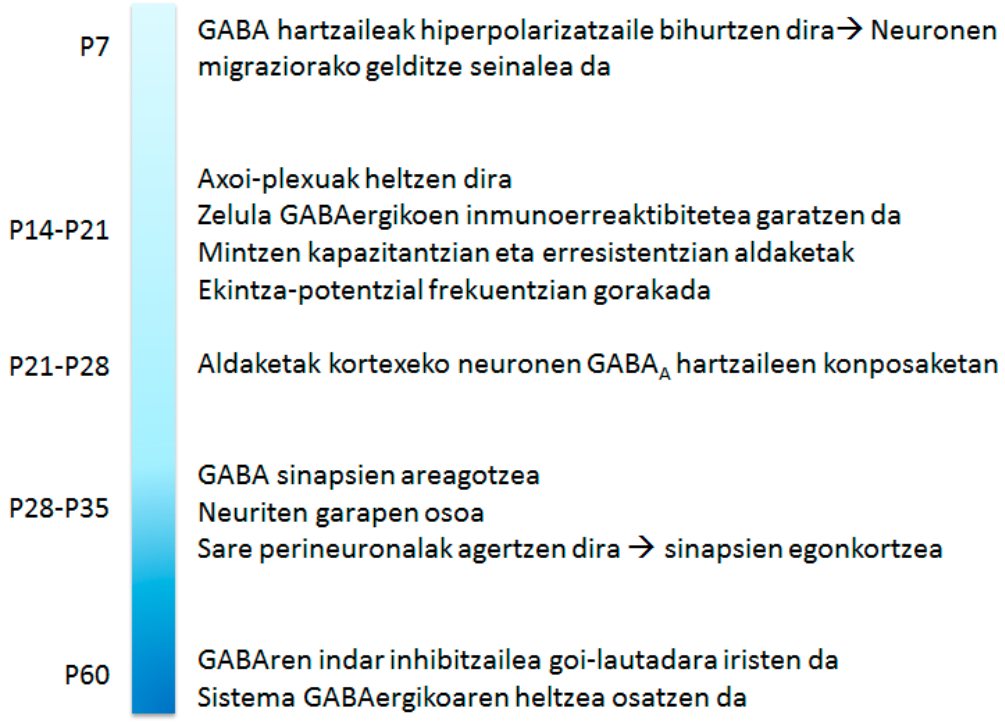
1.1.2.2. Interneurona GABAergikoen heltze-prozesua

Interneurona GABAergikoek zelula piramidalen aktibitatea erregulatzen dute, baina jaiotza aurretiko eta jaiotza ondorengo garapenean plastikotasun kortikalean, lotura sinaptikoan eta oszilazioen sorreran ere rol garrantzitsua jokatzen dute *(Le Magueresse eta Monyer, 2013)*. Zirkuitu GABAergikoen garapenaren ezaugarrietako bat bere iraupen luzea da. Heltze-prozesua enbrioi-sasoian hasten da eta hainbat urrats ematen ditu zelularen berezko ezaugarriak guztiz garatu arte. Aldaketa guzti hauen artean hurrengoak dira aipatzekoak: GABAren askatze eta jasotzearen heltzea, neuronen gaitasuna sinapsiak osatzeko garapeneko garai zehatzetan eta zelulen seinaleztapena erregulatzen duten

proteina berezien adierazpena zelulek behin-betiko propietate elektrofisiologikoak lortzeko.

Neurogarapen goiztiarrean, GABA neurotransmisore despolarizatzailea da, zelula barruan ematen den kloro pilaketagatik. Jaio ondorengo lehen astearen inguruan, potasio-kloro kotransportatzailearen (KCC2) adierazpena izugarri areagotzen da prosentzefaloan eta GABA hiperpolarizatzaile bihurtzen da *(Rivera et al., 1999)*. KCC2-ren adierazpena lekuko neuronen jardueraren menpe dago, beraz, neuronen migrazioa gelditzeko seinale gisa balio du *(Bortone eta Polleux, 2009)*. Jaio ondorengo bigarren eta hirugarren asteetan, hainbat aldaketa gertatzen dira interneurona GABAergikoetan: 1) inmunoerreaktibitate GABAergikoaren heltzea *(Del Rio et al., 1992)*; 2) helduaroko propietate elektrofisiologiak agertzen hasten dira (jaurtiketa frekuentziaren areagotzea, frekuentzia altuko azpiatariko mintz potentzialen oszilazioak eta mintzaren erresistentzian aldaketak) *(Doischer et al., 2008; Le Magueresse eta Monyer, 2013)*; 3) kortexeko interneuronen axoi-plexuen heltzea *(Doischer et al., 2008)*. Parbalbumina adierazten duten zelulek jaio ondorengo 4-5 asteetan zehar guztiz garatzen dituzte neuritak *(Doischer et al., 2008)*. Lehen hilabetean, sinapsi GABAergikoen areagotze bat gertatzen da. $GABA_A$ hartzaileen konposaketa ere aldatuz doa neurona kortikalen garapenean zehar eta helduaroko azpiunitateak 3-4 asteetara ikus daitezke *(Laurie et al., 1992)*. Azpiunitateen truke hori neokortex eta hipokanpoko interneurona PB-positiboen potentzial postsinaptiko inhibitzaileen heltzearekin batera ematen da *(Doischer et al., 2008)*. $GABA_B$ hartzaileak, G proteinei-loturiko hartzaileak dira eta neurogarapenean zehar azpiunitate desberdinak adierazten dituzte *(Fritschy et al., 1999)* (6. irudia). Garapenari lotutako azpiunitateen adierazpenak inplikazio funtzional garrantzitsuak ditu propietate fisiologikoetan.

Interneurona GABAergikoen inhibizioa oso baxua da neurogarapen goiztiarrean eta heldutasuneko ezaugarriak nerabezaro eta heldutasun goiztiarrean lortzen dira *(Le Magueresse eta Monyer, 2013)*. Inhibizio tonuaren areagotzea PB-ren areagotzearekin bat dator *(Caballero et al., 2014a; Caballero et al., 2014b)*. Ikusi denaren arabera, nerabezaroan zehar emari glutamatergiko kitzikatzailea interneurona PB-positiboengan areagotu egiten da *(Caballero et al., 2014a)*, eta hau V-VI geruzetako potentzial inhibitzaile postsinaptiko espontaneoen frekuentziaren handitzearekin batera gertatzen da, hau da, jarduera GABAergikoaren areagotzearekin batera *(Cass et al., 2014)*.



6. irudia. Neurogarapenean zehar sistema GABAergikoan ematen diren aldaketen irudikapen eskematikoa.

1.1.2.3. Interneuronen funtzioa

Oinarrizko mikrozirkuituen funtzioak

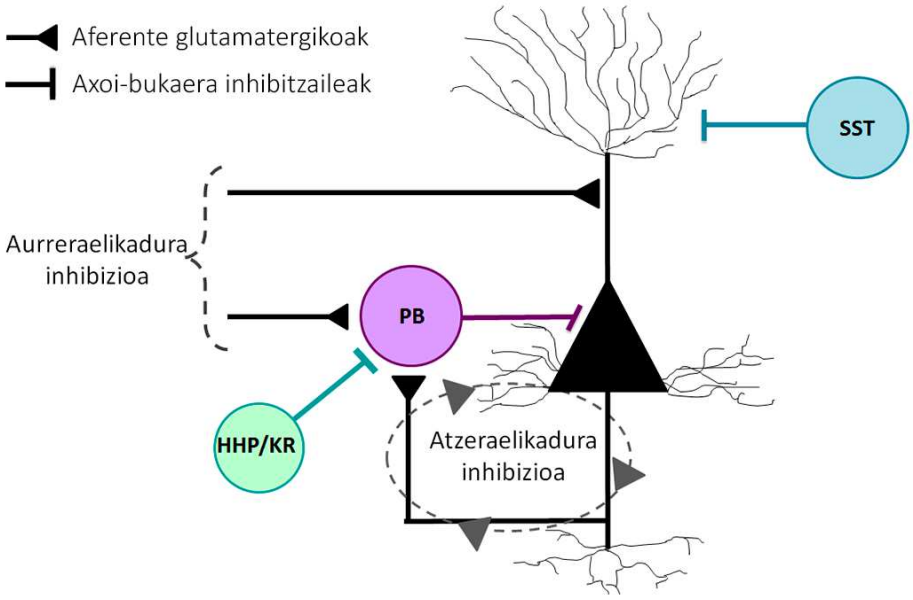
Aurreraelikadura inhibizioak emari kitzikatzaileen aldiberekotasunaren detekzio leihoa laburtzen du neurona piramidaletan. Zelula glutamatergiko aferenteak zelula printzipalekin eta zelula GABAergikoekin paraleloan burutzen dute sinapsia *(Buzsáki, 1984)* (7. irudia). Emari inhibitzailea glutamatoak zelula piramidalengan eragindako despolarizazioa baino lehenago heltzen bada, aurreraelikadura inhibizioak mintz potentziala despolarizazio-atariaren behetik mantenduko du eta zelula kitzikatzaileen ekintza-potentzialak ekidingo ditu. Konduktantzia inhibitzailearen areagotzea eta zelula piramidalaren despolarizazioa ez badira aldiberekoak, milisegundo gutxi batzuk irauten duen desoreka iragankor bat gertatzen da inhibizioak berriz oreka berreskuratu arte. Ondorioz, interneuronek potentzial postsinaptiko kitzikatzaileen batuketa tenporala mugatzen dute eta ekintza-potentzialen sorrera eta kadentzia erregulatzen dute. Aurreraelikadura inhibizioan parte hartzen duten interneurona askok atzeraelikadura inhibizioan ere parte hartzen dute. Dena dela, hipokanpoko zelula GABAergiko batzuk

aurreraelikadurako seinaleztapenean espezializatuta daudela dirudi. Zelula espezializatu hauek, jasotzen duten aferente glutamatergikoen arabera izendatzen dira, hots, geruza molekularreko bide zulatzaileari uztartuta dauden zelulak *(Li et al., 2013)*, granaila zuntzei uztartutako interneuronak (CA3ko *stratum lucidum*-eko interneuronak) *(Vida eta Frotscher, 2000)*, eta Schafferren kolateralei uztartutako interneuronak (CA1 *stratum radiatum*-ean) *(Cope et al., 2002)*. Interneurona guzti hauek aurreraelikadura inhibizioa bideratzen dute adar dendritikoetan. Hala ere, zelula printzipalengan inhibizio perisomatikoa gauzatzen duten interneuronak ere aurreraelikadurako inhibizio iturri garrantzitsua dira. Neokortex eta hipokanpoan, parbalbumina (PB) eta kolezistokinina (KZK) adierazten duten saski-zelulak dira domeinu perisomatikora zuzentzen diren interneurona nagusiak *(Freund eta Katona, 2007; Basu et al., 2013)*. Ondorioz, kontaktatzen duten zelula piramidalen domeinuaren arabera, aurreraelikadura inhibizioak jaurtiketa dendritikoak murriztu ditzake edo zelula kitzikatzaileen seinaleak kontrolatu.

Interneurona GABAergiko ugarik parte hartzen dute atzeraelikadura edo inhibizio errepikakorren begizten sorreran, aurretik aipatutako PB+ eta KZK+ saski-zelulak barne, baina baita PB adierazten duten argimutil zelula axo-axonikoak ere. Hauek, saski-zelula PB positiboen antzera, propietate gisa jaurtiketa arina erakusten dute (7. irudia). Neokortexeko II. eta III. geruzetan, somatostatina adierazten duten Martinotti zelulak zelula printzipalen dendrita apikaletara daude zuzenduta eta atzeraelikadura inhibizioan laguntzen dute. Hipokanpoan, kortexeko Martinotti zelulen antzerakoak diren interneuronen populazio bat dago. Hauen somak *stratum oriens*-en aurkitzen dira eta beraien axoiak *stratum lacunosum-moleculare*-ra zuzentzen dira (OLM: oriens lacunosum-moleculare zelulak). Hiloko bide zulatzailera uztartuta dauden zelulak (HBZ) OLM zelulen baliokideak dira hortz-jiroan, baina zelula granularren dendritetara daude zuzenduta. Atzeraelikadura inhibizio mekanismoetan, zelula kitzikatzaileen jarduerak neurona inhibitzaile postsinaptikoak errekrutatzen ditu eta neurona inhibitzaile hauen deskargak zelula kitzikatzaileen ekintza-potentzialak ekiditen ditu *(Miles, 1990)*.

Jaurtiketa arineko interneurona PB+ bakoitzak milaka neurona piramidalekin burutu dezake sinapsia eta zelula piramidal bakoitzak hainbat saski-zeluletatik jaso ditzake seinale inhibitzaileak. Beraz, jaurtiketa arineko zelula PB-positiboen kokapena funtsezkoa da jomuga-neuronen jarduera erabakitzeko eta hau batez ere interesgarria da zelula printzipalen jaurtiketa erritmoa sinkronizatzeko *(Pinto et al., 2000)*. Bestalde, interneurona SST-positiboak zelula piramidalen urruneko dendrita apikaletara zuzentzen

direnez, dendriten arantzetara heltzen diren seinaleak doitzen edo moldatzen dituzte. Parbalbumina eta SST adierazten duten interneuronen bidez eragindako inhibizioak osagarriak dira. Inhibizio goiztiarra perisomara zuzentzen diren jaurtiketa arineko zelulek eragiten dute, oso sentikorrak direnez, estimuluari arin erantzuten diotelako. Ostera, informazio sentsoriala etengabea denean, interneurona PB-positiboek eragindako inhibizioa murriztuz joaten da. Ondoren, inhibizioa interneurona SST-positiboen esku geratzen da, hauen eragin inhibitzailearen agerpena motelagoa delako eta inhibizio berantiarra dela esan ohi da *(Pouille eta Scanziani, 2004)*.



7. irudia. Mikrozirkuitu inhibitzailearen antolaketaren irudikapen eskematikoa. PB, parbalbumina; SST, somatostatina; HHP: hodi-hesteen proteina; KR: kalretinina

Interneuronak ezinbestekoak dira portaera kognitibo arrunterako

Interneuronen artean aniztasun handia dago, baina PB eta SST adierazten duten interneuronak ikertu dira gehien. Somatostatinak ikasketa eta oroimenean funtsezko rol bat duela jakina da, somatostatina-hartzaileen knock-out (KO) saguek ikasketa espazialean defizita erakusten baitute *(Dutar et al., 2002)*. Horretaz gain, zahartzaroan gizakien eta arratoien kortexean SST gutxiago dagoela ikusi da eta hau ikasteko gaitasunaren murrizketarekin dago korrelazionatuta *(Dournaud et al., 1995)*. Bestalde,

SST-ren injekzio intrahipokanpal eta intrabentrikularrek ikasketa espaziala hobetzen dutela ikusi da hainbat atazetan *(Vécsei et al., 1984; Lamirault et al., 2001)*. Hiloko interneuronen isiltze optogenetikoak (gehienak SST adierazten dute) ikasketa eta oroimen espaziala eten zuten Morrisen ur labirintoan, epe laburreko lan-oroimenean, koordinazio motorean eta jarduera esploratzailean eraginik izan gabe *(Andrews-Zwilling et al., 2012)*.

Interneurona PB-positiboen neurotransmisio inhibitzailearen asaldatzea ere oroimen urritasunarekin dago erlazionatuta. Adibidez, interneurona PB-positiboetatik ziklinaren menpeko kinasa 5-aren erauzketa genetikoak hiperinhibizio batera darama eta, era berean, amigdalaren menpe dauden testuinguru eta pistak eragindako beldur-oroimenak murrizten ditu eta erreferentzia oroimen espaziala kaltetzen du Morrisen ur labirintoan *(Rudenko et al., 2015)*. Gainera, kortex prefrontal medialeko jaurtiketa arineko interneurona PB-positiboak rol garrantzitsua jokatzen dute sari-bidezko portaera gidatuan, lan-oroimenean eta malgutasun kognitiboan *(Murray et al., 2015)*, eta beraien isiltze optogenetikoak atentzio prozesamendua hondatzen du, hau informazio berria eskuratzeko beharrezkoa delarik *(Kim et al., 2016)*. Hipokanpoko CA1eko zelula PB-positiboen desaktibazio funtzionalak interneurona hauek lan-oroimen espazialerako beharrezkoak direla egiaztatu dute *(Murray et al., 2011)*. Are gehiago, M1 azetilkolinaren hartzaile muskarinikoa interneurona PB-positiboen geneetatik ezabatuta duten sagu KO-ek ezagutza memoria urrituta daukate eta, neurri txikiagoan, lan-oroimen espaziala murriztua dute *(Yi et al., 2014)*. Bestalde, interneurona PB-positiboetan NMDA hartzailearen neurotransmisiorik ez duten sagu mutanteak kognizio mailan hutsegite selektiboak erakusten dituzte, hala nola, lan-oroimenean eta ikasketa asoziatiboan *(Carlén et al., 2012)*.

Interneurona mota desberdinak ez daude bata bestearengandik isolatuta, baizik eta zirkuitu-mailan funtzionatzeko konektatuta daude. Gutxi dira interneurona ezberdinen arteko elkarrekintzak ordena-altuko funtzio kognitiboetan duen eragina aztertu dutenak. Kortex prefrontal medialeko jaurtiketa arineko interneurona PB-positiboak helburura-zuzendutako portaera gidatzen duten arren, interneurona SST-positiboek ere parte hartzen dute, bi hauek aurkako jarduera erakusten dutelarik *(Kim et al., 2016)*. Aurreko kortex zingulatuan, saritutako atazen fase ezberdinetan parte hartzen dutela erakutsi zuten Kvitsiani eta lankideek *(Kvitsiani et al., 2013)*, non interneurona SST-positiboak saritutako gunera hurreratzean aktibatzen ziren eta interneurona PB-positiboak saritutako zona uztean erantzuten zuten eta aurreko egonaldiaren denbora kodifikatzen zuten.

Behaketa hauek interneuronak adierazpen kognitibo arrunt baterako beharrezkoak direla nabarmentzen dute eta, dirudienez, kognizio egokia bermatzen duten zirkuitu kortikalen dinamika koordinatzeko, interneurona azpimota bakoitzaren funtzioa espazio-denboran bananduta dago, rol osagarriak betez.

Inhibizioaren aurkako erregulazioa oroimenaren jabetze eta finkatze prozesuetan

Hainbat ebidentziak iradokitzen dute inhibizioaren eta desinhibizioaren arteko elkarrekintzak rol garrantzitsua jokatzen duela ikasketa eta oroimenaren formakuntzan. Iragankorra eta espezifikoa den inhibizioak kausazko inplikazioa du ikasketarekin erlazionatutako plastikotasunean, hain zuzen ere. Lehen ebidentziek SST adierazten duten interneuronen jarduera basala mugimenduan eta sentsazio aktiboan zehar murriztuta zegoela azpimarratu zuten *(Urban-Ciecko eta Barth, 2016)*. Azken urteotan, interneurona PB-positiboen estimulazio farmakologikoek eta optogenetikoek erakutsi dutenez, beraien jarduerak entzumen beldur ikasketa hondatzen du *(Letzkus et al., 2011)*. Froga hauetan oinarriturik, autore batzuk desinhibizioa plastikotasun sinaptikoaren eta ikasketaren mesedegarri den mekanismo orokor bat izan daitekela proposatu dute *(Letzkus et al., 2015)*. Izan ere, Gambino eta Holtmaatek *(Gambino eta Holtmaat, 2012)* zuzenki egiaztatu zuten kortex somatosentsorialean desinhibizioak epe-luzeko potentziazioa errazten zuela. Hipotesi honen euskarri dira baita beste lan batzuetan aurkitutako emaitzak. Denbora luzez jakin da interneurona PB-positiboen inhibizioaren murrizketa iragankor batek edo beraien sare perineuronalak kentzean ikusmen kortexeko aldi kritikoa berriz irekitzen dela begi-dominantziaren plastikotasuna areagotuz *(van Versendaal et al., 2012)*. Aitzitik, interneurona PB-positiboen aurreraelikaduraren aktibazioa beharrezkoa da amigdalaren menpe dagoen beldur ikasketarako *(Wolff et al., 2014)*. Era berean, barril kortexeko IV. geruzan SST adierazten duten interneuronen zenbakia areagotu egiten da baldintzapen klasikoko ikasketa aldian *(Cybulska-Klosowicz et al., 2013)*. Halere, azken kasu hauetan interneuronen jardueraren efektua sarean berdina izango litzateke: zelula piramidalen desinhibizioa. Ustez, amigdalako interneurona PB-positiboek SST adierazten duten interneuronak isiltzen dituztenez eta IV. geruzako interneurona SST-positiboak interneurona PB-positiboetara zuzendutako zirkuitu desinhibitzaileko parte direnez, beraien jarduerak dudagabe zelula piramidalen ekintza-potentzialak bermatuko lituzkete. Honekin bat, sabelaldeko eskualde

tegmentaleko proiekzio GABAergikoak accumbens nukleoko interneurona kolinergikoen aktibitate espontaneoa geldiarazten dute ikasketa asoziatiboa hobetzeko *(Brown et al., 2012)*. Guzti hau kontutan izanik, ikasketan zehar desinhibizioak aldaketa plastikoak errazten dituela esan genezake, zeinek espezifikotasun handiz integrazio sinaptikoa aktiboki moldatzen duen, ziur aski esperientziak eragindako plastikotasun sinaptikorako garrantzitsuak diren frekuentzia-handiko ekintza-potentzialak ahalbidetuz *(Golding et al., 2002; Kampa et al., 2007)*.

Azken behaketek, ikasketa prozesua bukatu ondorengo zirkuituen plastikotasunean inhibizioaren areagotzeak duen rolaren garrantzia azpimarratu dute. Cornus ammonis 1 (CA1)-eko SST-positiboak diren *oriens-lacunosum moleculare* (OLM) zelulek eragindako inhibizio dendritikoa beharrezkoa da beldur-oroimenak eratzeko (*Lovett-Barron et al., 2014)*. Halaber, asoziazio ikasketa burutu eta gero, seinaleztapen GABAergikoaren areagotze bat dago: GABAren kontzentrazio presinaptikoak handitzen dira, gune postsinaptikoetan $GABA_A$ hartzailearen α1 azpiunitatea gorantz erregulatzen da eta korronte postsinaptiko inhibitzaile espontaneoen frekuentzia handitzen da *(Tokarski et al., 2007; Jasinska et al., 2010)*. Interneurona SST-positiboak ere GABA eta SST gehiago adierazten dute ikasketa asoziatiboa eta gero *(Cybulska-Klosowicz et al., 2013)*. Azken pare bat urteetan, ikerketa gutxi batzuk esan dutenez, interneurona PB-positiboen azpimultzo ezberdinak ikasketa eta oroimen prozesuetako fase ezberdinetan dihardute. Esperimentu hauen arabera, interneurona mota bakoitzaren azpimultzo desberdinak espezifikotasun handiz erantzuten diote ataza desberdinei, interneurona mota bakoitzak eragindako inhibizioa are gehiago zatikatu daitekeela iradokiz garunaren gaitasun konputazionala handitzeko. Donato eta lankideek *(Donato et al., 2015)* jaiotze berantiarreko PB-positiboak diren saski-zelulak ataza berrien jabetzean daudela inplikatuta diote eta, berriz, jaiotza goiztiarreko PB-positiboak diren saski-zeluletan aldaketa plastikoak arauen finkapenaren ondoren gertatzen dira. Interneuronen berariazko interneurona HHP-positiboek eragindako inhibizioak jaiotze berantiarreko neurona PB-positiboetan paper garrantzitsua jokatzen du ikasketarekin-erlazionatutako plastikotasun sinaptikoan *(Donato et al., 2015)*. Honekin bat, interneurona PB-positiboen inhibizio farmakologikoak plastikotasun sinaptiko estrukturala hobetu zuen eta antzerako joera ikusi zen labirintoko nabigazioko entrenamendu fasean edo ingurune aberastuan haztean. Bestalde, interneurona PB-positiboen aktibazioak balioztatutako arauen finkapena sustatu zuen *(Caroni, 2015)*. Beranduago, Lager eta lankideek *(Lagler et al., 2016)* PB

adierazten zuten saski-zelulak jaurtiketa patroi ezberdina zuten neurona talde ezberdinetan biltzen zirela erakutsi zuten oroimenez-gidatutako aukeraketa portaeran, zelula mota bakar baten barruan, atazarekin erlazionatuta egon daitezkeen espezializazioak daudela aditzera emanez.

Aurkikuntza hauek ikasketa eta oroimen prozesuetan kitzikapen/inhibizio orekak egiten duen ekarpenaren ulermena hobetzen laguntzen dute. Gero eta ebidentzia fidagarri gehiagok desinhibizioak neurotransmisio kitzikatzailean laguntzen duela eta ikasketarako mekanismo orokorra izan daitekeela adierazten dute. Bestalde, arauen finkapenak konfigurazio inhibitzaile altu baten beharra du *(Donato et al., 2013; Donato et al., 2015)*, ikasketarekin erlazionatutako aldaketa plastikoak eta oroimenaren formakuntza inhibizio mailak zehazten duela iradokiz. Beraz, oreka egoki hau nahasten duen edozein faktorek eragin kaltegarriak izan ditzake kognizioan.

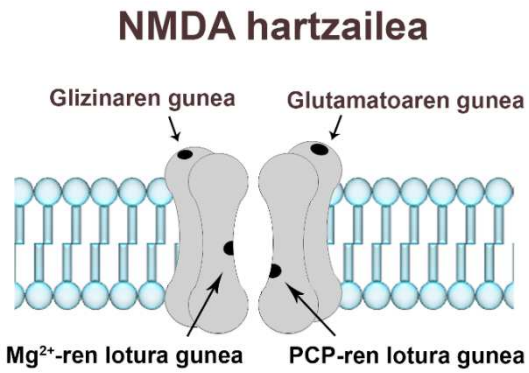
1.1.3. N-metil-D-asparato (NMDA) hartzaileak

1.1.3.1. NMDA hartzaileen estruktura eta sintesia

N-metil-D-aspartato (NMDA) hartzaileak 4 azpiunitate ezberdinez osatuta daude, derrigorrezko NR1 azpiunitateaz eta hautazko NR2 (A, B, C edo D) edo NR3 (A edo B) azpiunitateez, heterotetramero bat osatuz. NR3 azpiunitateak garapen goiztiarrean aurkitzen dira batez ere; NR2 azpiunitateek kanalaren irekitzea erregulatzen dute; NR2A azpiunitatea nerbio sistema zentraleko azpiunitaterik ugariena da; aldiz, NR2B prosentzefaloan eta hipokanpoan da nagusi *(Monyer et al., 1994)*.

NMDA hartzailearen azpiunitateen konbinazioen arabera, NMDA hartzaileen propietate elektrofisiologikoak aldatu egiten dira. NR1-NR2B konbinazioak NR1-NR2A konplexuak baino potentzial postsinaptiko kitzikatzaile luzeagoak ditu *in vitro (Monyer et al., 1994)*. NMDA hartzaileen azpiunitateek plastikotasun sinaptikoan parte hartzen dute baita: hartzaile jakin bateko azpiunitateen adierazpenaren aldaketak hartzailearen propietate funtzionalak aldatu ditzake. Izan ere, NR2Bren txertatzeak sinapsien aldiberekotasunerako denboraldia luzatu dezake, beraz, eraginkortasun sinaptikoa hobetuz eta, ondorioz, oroimenean eraginez *(Yashiro et al., 2008)*.

NMDA hartzailaren azpiunitateak lotune guneetan ere desberdintzen dira: NR1 azpiunitateak glizina lotzeko guneak ditu eta NR2 azpiunitateak glutamatoa lotzeko guneak. Glizinak ko-agonista bezala jokatzen du, hau da, bere lotura beharrezko baldintza bat da NMDA hartzailearen aktibazioa bermatzeko. D-serinak ere ko-agonista bezala joka dezake NMDAR-ren B-glizina guneetara lotzen denean (8. irudia). Mintzeko potentzia atseden-egoeran dagoenean, magnesio ioiak kanalaren poroetan sartzen dira eta ioien fluxua ekiditen dute. Magnesioaren blokeoa aldaratzeko eta, beraz, ioien fluxua bermatzeko, mintzaren despolarizazioa gertatu behar da. Ondorioz, magnesioak sortzen duen blokeoa gainditzea NMDA hartzaileen aktibazioa bermatzeko beste baldintza bat izango da. Heterotetrameroaz gain, NMDA hartzaileek dentsitate postsinaptikoak dituzte, sinapsi glutamatergikoei egituran eta funtzioan egonkortasuna ematen dieten proteina multzoak.



8. irudia. N-metil-D-aspartato (NMDA) hartzailearen irudikapen eskematikoa. Mg2+, magnesio ioiak; PCP: fenziklidina.

1.1.3.2. NMDA hartzailearen garrantzia heltze-prozesu, ikasketa eta oroimenean

Glutamatoak zelula barneko jauziak aktibatzen ditu hartzaile ionotropiko eta metabotropikoen bidez. Glutamatoa neurogarapenerako ezinbestekoa da sinaptogenesia, sareko plastikotasuna, dendriten adarkatzea, eta neurona aitzindarien hedatze eta migrazioa erregulatzen dituelako *(Snyder eta Gao, 2013)*. Jaio ondorengo neurogarapen goiztiarrean NMDA hartzaileak dira glutamatoaren hartzaile bakarrak, AMPA hartzaile funtzionalik ez baitago *(Ben-Ari et al., 1997)*. Hori dela eta, NMDA hartzaileak garuneko heltze-prozesuarekin irmoki lotuta daude. NMDA hartzaileen azpiunitateen adierazpena

eta funtzioa hipokanpoaren garapen egokirako beharrezkoa da. Izan ere, hartzaile hauen erregulazio ezak sinaptogenesian eta zirkuituen heltze-prozesuan akatsak sortzen ditu *(Brigman et al., 2010)*.

NMDA hartzailearen jarduera ere funtsezkoa da interneurona PB-positiboen heltze-prozesuan *(Zhang eta Sun, 2011)*. NMDA hartzailearen seinaleztapena neurogarapen goiztiarrean etenez gero, zelula GABAergikoen jardueraren epe-luzeko murrizketa gertatzen da eta honek, kortex prefrontal medialeko zelula piramidalen desinhibizioa sortzen du *(Belforte et al., 2010; Moreau et al., 2013)*. Kortex prefrontal medialean, tangentzialki migratzen ari diren interneuronen aitzindarietan NMDA hartzaileak adierazten dira *(Soria eta Valdeolmillos, 2002)*. Garuneko zirkuituen heltzea NMDA hartzailearen azpiunitateen aldaketarekin bat dator, gaztaroko prozesamendu neuraletik heldutasunerako trantsizioa markatuz. NR2 azpiunitatearen aldaketa zelula-espezifikoa da kortex prefrontalean: zelula piramidaletan NR2B-ren maila egonkorra da heldutasuneraino, baina jaurtiketa arineko interneuronetan NR2B azpiunitatea pixkanaka NR2A azpiunitatearekin ordezkatzen da, batez ere nerabezaroan *(Wang et al., 2008; Wang eta Gao, 2009)*. Garapenean zehar plastikotasun-atarian gertatzen diren aldaketei azalpena eman diezaiokeen mekanismoetako bat NR2B azpiunitatean gertatzen diren aldaketak dira. Azpiunitate hau duten NMDA hartzaileek zinetika moteleko korronteak erakusten dituzte, kaltzio korronteen batuketa tenporala ahalbidetuz. NR2A azpiunitatea duten NMDA hartzaileak, aldiz, zinetika arinagoa dute, kaltzio seinaleen bereizmen tenporal handiago bat ahalbidetuz. Azpiunitateek jasaten dituzten aldaketen ondorioz, NMDA hartzaileak arrisku faktoreekiko bereziki sentikorrak bihurtzen dira. Hala, ingurumen faktoreek zein faktore genetikoek garun garapen arraunta kaltetu dezakete *(Spear, 2000)*.

NMDA hartzaileek oinarrizko funtzioa betetzen dute garunaren plastikotasunean eta, beraz, ikasketa eta oroimenean. Transmisio sinaptiko kitzikatzaileak erregulatzeko duten ahalmenari esker eragiten dute NMDA hartzaileek ikasketa eta oroimen prozesuetan: epe-luzeko potentziazioaren (ELP) bidez sinapsiak indartu dezakete edo hauek ahuldu epe-luzeko depresioaren (ELD) bidez *(Yashiro et al., 2008)*. NMDA hartzaileen bidezko kaltzio fluxua da ELP eta ELD-ren arduraduna eta, aldi berean, bi mekanismo hauek deskribatu dira ikasketa eta oroimenaren erantzule zelular gisa. Gainera, NMDA hartzaileak jarduera pre- eta postsinaptikoen aldiberekotasuna

nabarmentzeko gai dira eta korrelazio tenporal honek ekintza pre- eta postsinaptikoen denboraren araberako plastikotasuna ahalbidetzen du.

1.2. Eskizofreniaren aurrekari neuropatologikoak

Eskizofrenia ezgaitasun psikiatrikoa da eta bere etiopatogenia oraindik ez dago guzti argituta. Gaur egunera arte, garuneko ikerketak gaixotasun neurologikoetan zentratu dira gehienbat, eta gaixotasun mentalei buruzko ikerketak azken hamarkadetan areagotu dira, batik bat. Informazio gabezia honek eskizofreniaren oinarri neurobiologikoen ulermena eragotzi du eta medikuak eskizofreniaren diagnostikoa sintometan oinarrituta egitera behartuak ikusi dira. Eskizofrenian sintoma positiboak, negatiboak eta kognitiboak agertzen dira *(Saha et al., 2005)*. Sintoma positiboak ohiz kanpoko funtzio mentalen irudi dira, hala nola, haluzinazioak eta eldarnioak. Sintoma negatiboen artean, besteak beste, isolamendu soziala, motibazio eza eta sentipen lautuak daude. Sintoma kognitiboak funtzio exekutibo eskasekin daude erlazionatuta, batez ere, arretaren eta oroimenaren hutsegiteekin. Sintoma hauen agerpen tipikoa nerabezaro berantiar eta heldutasun goiztiarraren artean gertatzen da *(Saha et al., 2005)*, neurogarapeneko prozesuek paper garrantzitsu bat jokatzen duten arren eskizofrenian. Eskizofreniaren etiologiari buruz hainbat teoria proposatu dira, hala nola, joera genetikoa *(Greenwood et al., 2013; Singh et al., 2014)*, jaio aurreko infekzioak *(Labouesse et al., 2015)*, ingurumeneko eraginak *(Pishva et al., 2014)* edo aurretik aipatutako faktoreen konbinazioa *(Uher, 2014)*. Eskizofrenia izateko arriskua areagotzen duten geneak eskizofrenian etenda-1 (DISC-1), disbindina eta neuroregulina-1 (NRG-1) dira, besteak beste *(Le Magueresse eta Monyer, 2013; Salgado eta Sandner, 2013)* (9. irudia).

1.2.1. Gizakietan egin diren aurkikuntzak

Azken bi hamarkadetan eskizofreniaren disfuntzio kognitiboaren jatorria ulertzeko ahalegin asko egin dira. Hori dela eta, prozesu kognitiboek dituzten oinarri neurobiologikoak ulertzeko interes handia dago. Gaur egun, medikamentu antipsikotikoak eraginkorrak dira sintoma positiboak gutxitzeko, baina onura urria dute kognizioan. Arazo kognitiboek gaixo eskizofrenikoei eguneroko bizitzan eragiten diote

eta ezgaitasun kronikoari eta langabeziari oso lotuta daude *(Green et al., 2000)*. Hala ere, gaur egungo tratamenduak edo terapiak ez dira gai sintoma kognitiboak arintzeko. Disfuntzio kognitiboak sakon eragiten du eta beste sintomekiko mendekotasunik ez du erakusten *(Gold, 2004)*. Ezaugarririk nabarmenena lan-oroimen gaitasun murriztua da, batez ere informazio kantitate handiak batera prozesatu behar direnean *(Silver et al., 2003; Barch et al., 2006)*. Kortex prefrontal dortsolaterala (KPFDL) lan-oroimenaren hutsegiteetan dago inplikatuta eta eskaner bidez egindako garun ikerketek behin eta berriz erakutsi dute KPFDL-ren aktibazioan alterazioak daudela eskizofrenia duten gizabanakoetan, batez ere, ataza kognitiboak egiten arin direnean *(Arnsten eta Jin, 2014; Brunoni eta Vanderhasselt, 2014; Wolf et al., 2015)*. Eskizofrenia duten gaixoek ikasketa asoziatiborako gaitasuna ere murriztuta dute eta hau kortex prefrontaleko eta hipokanpoko zirkuituen anatomia eta funtzioarekin lotu dute.

9. irudia. Eskizofreniaren sorreran parte hartzen duten arrisku faktore nagusien sailkapena.

Interneurona GABAergikoen heltze-prozesuaren asaldura baten ebidentziak ere aurkitu dira eskizofrenian. Hainbat ikerketetan garapenean zehar interneuronen migrazio oker bat gertatzen dela ikusi dute. Lehenik, gaixo eskizofrenikoen substantzia zurian interneuronen dentsitatea handiagoa da *(Eastwood eta Harrison, 2003; Joshi et al., 2012)*. Bigarrenik, eskizofrenia jasateko sentikortasunari lotuta dagoen DISC-1 genearen beheratze bat eta gero, gongoil eminentzia medialetik datozen interneuronen migrazio tangentzialean aldaketak nabarmentzen dira *(Steinecke et al., 2012)*. Azkenik, neurorregulina-1 (NRG-1) eta bere hartzaile den ErB4 ere eskizofrenia jasateko arriskuari

lotuta daude eta ErB4 interneurona inhibitzaileetan baino ez da adierazten, batez ere, PB-positiboak diren interneuronetan *(Fazzari et al., 2010)*. Gainera, kitzikapen/inhibizio zirkuituen oreka galdu egiten dela dirudi. Alde batetik, saski-zelula eta zelula piramidalen arteko sinapsietan $GABA_A$ hartzailearen α1 azpiunitatearen adierazpena murriztuta dagoela aurkitu da eta argimutil zeluletan α2 azpiunitatearen areagotzea ikusi da *(Volk et al., 2002)*. Bestalde, sinapsi axo-axonikoak ere murriztuta daude eta sagu-ereduetan ikusi denez, PB-positibo diren saski-zeluletan sinapsi glutamatergiko gutxiago daude *(Zheng et al., 2011)*. Emaitza hauek parbalbumina adierazten duten interneuronen konexioak garapenean zehar ezegokiak direla erakusten dute eta honen ondorioz heldutasunean sarearen aktibitate eta plastikotasun aberranteak eman daitezke. Halaber, nerabezaro berantiarrean sistema GABAergikoan ematen diren aldaketa nabarmenek eta eskizofreniaren sintomen sorrerak adin mendekotasun profil berbera dute.

Post mortem egindako ikerketek kortex prefrontal eta hipokanpoko alterazio GABAergikoak eskizofreniaren sorreran inplikatuta daudenaren ideia are gehiago indartzen dute. Batez ere, parbalbumina eta azido glutamiko deskarboxilasa 67 (GAD67)-aren beherakada da aurkikuntzarik trinkoena gaixo eskizofrenikoen kortex prefrontalean *(Volk et al., 2000; Zhang et al., 2002; Hashimoto et al., 2003)*, aldi berean kalretinina eta kalbindina interneuronen inmunoerreaktibitatea galdu gabe *(Reynolds et al., 2004; Gonzalez-Burgos et al., 2015)*. Parbalbumina eta GAD67-ren adierazpena aktibitate kortikalak erregulatzen duenetik *(Qin et al., 1994; Gierdalski et al., 1999)*, beraien gabeziak gaixo eskizofrenikoen interneurona PB-positiboetan, interneurona PB-positiboen jardueraren gabezia edo beherakada bat adierazten du. Interneurona mota guztietatik beherakada soilik PB duten zeluletara zergatik mugatzen den jakin ez arren, PB-ren adierazpenaren aldaketek garapenean zehar proteina hau duten zelulak bereziki kalteberak direla egiaztatu dute.

Berriki, *post mortem* egindako garunen ikerketek somatostatinaren proteina, somatostatinaren mRNA adierazpenena eta neuropeptido hau duten interneurona GABAergikoak ere murriztuta daudela erakutsi dute gaixo eskizofrenikoetan *(Joshi et al., 2015; Alherz et al., 2017)*. Hashimoto eta lankideek *(Hashimoto et al., 2008)* PCR bidez erakutsi zuten, eskizofrenia zuten gaixoen KPFDL-ean, SSTren mRNAn %44ko beherakada ematen zela kontrol osasuntsuekin alderatuta, eta batezbesteko %57ko beherakada aurreko kortex zingulatuan eta ikusmen kortex primarioan. Zelula mailan, SST-ren adierazpen erlatiboa zelulako ere gutxituta zegoen %31n II/III geruzetan eta

%25ean KPFDL-ko V. geruzan *(Morris et al., 2008)*. Era berean, Konradi eta lankideek *(Konradi et al., 2011)* beherakada esanguratsua aurkitu zuten SST adierazten zuten interneuronen zenbaki eta dentsitatean, eta baita somatostatinaren mRNAren adierazpen mailan eskizofrenian. Emaitza hauek iradokitzen dutenez, interneurona PB-positiboetaz gain, beste interneurona mota batzuk ere egon daitezke eskizofreniari lotuta.

1.2.2. NMDA hartzaileen hipofuntzioa eskizofreniaren substratu patofisiologiko gisa

Eskizofreniari dagokionez, dopaminaren hipotesia izan da ideiarik iraunkorrena psikiatrian. Eredu hori dopamina-hartzaileen antagonistek efektu antipsikotikoak zituztela ikusi ondoren proposatu zuten. Izan ere, gaixo eskizofrenikoen sistema linbikoan hartzaile horien gehiegizko adierazpena aurkitzen da. Hala eta guztiz ere, dopaminaren hipotesiak ez du eskizofreniaren patogenesia guztiz azaltzen, medikamentu antipsikotikoak gaixotasunaren sintoma positiboetarako eraginkorrak baitira, baina sintoma negatiboak eta kognitiboak ez dituztelako hobetzen. Gaur egun, D2 hartzaileen gehiegizko aktibazioa nahasmendu honen sinapsietan ikusten diren desoreka kimiko orokorren efektu bat baino ez dela uste da *(Seeman eta Kapur, 2000)*.

Azken frogek gizabanako eskizofrenikoetan ezohiko transmisio glutamatergikoa eta NMDA hartzaileen hipofuntzio bat dagoela onartzen dute *(Snyder eta Gao, 2013)*. Lehenik eta behin, eskizofrenia izateko arriskua handitzen duten gene askok NMDAR-n bidezko seinaleztapenarekin dute zerikusia *(Moghaddam, 2003; Harrison eta Weinberger, 2005)*. Eskizofrenia garatzeko arrisku geneak neuronen ugaritzea, migrazioa eta sinaptogenesia erregulatzen dute. Bigarrenik, gaixo eskizofrenikoen garunetan NMDA hartzailearen NR1 azpiunitatea murriztua egoteak, NMDA hartzailearen funtzioa asaldatuta dagoenaren idea sendotzen du *(Weickert et al., 2013)*. Hirugarrenik, NMDA hartzailearen adierazpen baxua duten sagu transgenikoek eta NMDA hartzailearen antagonismoko animalia-ereduek eskizofrenia gogorarazten duten sintomak erakusten dituzte. NMDA hartzailearen antagonistek portaera aldaketak sortzeaz gain, eskizofreniarekin zerikusia duten alterazio metaboliko eta neurokimikoak ere eragiten dituzte *(Morris et al., 2005)*. Gainera, gero eta onartuago dago eskizofrenia

neurogarapeneko nahasmendu bat dela eta garuneko garapen goiztiarrari eragiten diola *(Lewis et al., 2008; Nakazawa et al., 2017)*.

1.2.3. Eskizofreniaren MK-801 animalia-eredua

Animalia-ereduak baliagarriak dira gaixotasun ugariren mekanismo fisiopatologikoak argitzeko eta tratamendu berriak garatzeko. Nahasmendu psikiatrikoak ikertzeko animalia-ereduak erabiltzearen oztopo nagusietako bat beraien oinarri neurobiologikoen ezagutza gutxi dugula da. Gainera, eskizofrenia giza nahasmendua dela iradoki dute, batez ere pertzepzioari, pentsamenduari, hizkuntzari eta arretari eragiten diolako. Ezaugarri hauek behe-mailako ugaztunetan modelatzeak zeresana eman du, baina eskizofreniaren prebalentzia handiak (biztanleriaren %1) eta gaixotasunaren ondorio kaltegarriek animalia-ereduak erabiltzearen beharra justifikatzen dute.

Hainbat hurbilketa planteatu dira eskizofrenia karraskarietan modelatzeko eta hiru talde nagusitan bana daitezke: neurogarapeneko ereduak, eredu genetikoak eta eredu farmakologikoak. Neurogarapeneko ereduen artean, haurdunaldian zehar sortutako zailtasunak daude, hala nola, ernaldiko malnutrizioa edo jaio aurretik gripe birusaren eraginpean jartzea *(Salgado eta Sadner, 2013)*. Eragile goiztiarrak ere erabili izan dira neurogarapeneko ereduetan, adibidez, kumeak amarengandik banatzea edo isolamendu soziala *(Jones et al., 1992; Geyer et al., 1993)*, baina normalean eredu genetiko edo farmakologikoekin batera *(Gilabert-Juan et al., 2013)*. Jaioberrien sabelaldeko hipokanpoko lesioak ere anitz erabili izan dira, baina eragindako kaltea eskizofrenian baino askoz ere larriagoa da *(Daenen et al., 2003; Rueter et al., 2004)*. Dena dela, hurbilketa guzti hauen kausazko rola zalantzagarria da. Eskizofrenia oso heredagarria da eta gaixotasunaren osagai genetikoa ezin da alde batera utzi. Geneek eta ingurumen faktoreek elkarri eragiten diote eta elkarreragin horrek nahasmendua azaleratuko den edo ez erabakiko du. Animalia-eredu genetiko batzuk gaixotasuna xehetasun handiz kopiatzen dute, baina eskizofrenian gene ugarik hartzen dute parte eta hauek faktore estokastiko eta ingurumenekoekin duten elkarrekintza konplexua dela eta, eskizofrenia animalia-eredu genetikoen bidez bakarrik errepikatzea ezinezkoa bihurtzen da *(Green et al., 2010)*.

NMDA hartzailearen antagonista ez-lehiakorren animalia-ereduak dira gaur egun hobeen deskribaturiko eredu farmakologikoak. Fenziklidina (PCP) eta ketaminak psikosia eragiteko gai dira gizaki osasuntsuetan eta gaixo eskizofrenikoetan sintoma positiboak areagotzen dituzte *(Lahti et al., 2001)*. Honek eskizofreniaren patofisiologian NMDA hartzaileen parte hartzea iradokitzen du. NMDA hartzaile ez-lehiakorren efektua ez da guztiz ezagutzen, baina neurotransmisio glutamatergiko, dopaminergiko eta GABAergikoarekin interakzio konplexuak izan ditzakeela dirudi *(Grüter et al., 2015)*. Glutamatoaren transmisioan ematen diren aldaketak eskizofrenian ikusten diren sintoma negatibo eta kognitiboekin erlazionatuak izan dira. NMDA hartzaileen antagonistek, dopaminak ez bezala, eskizofreniaren kognizio eta arreta defizitak aztertzeko balio dute *(Marcotte et al., 2001; van der Staay et al., 2009)*. Autore batzuk diotenez, NMDA hartzaileen antagonistek ez dituzte neurogarapeneko prozesuak kontutan hartzen, batez ere dosi akutuak erabili izan direlako eta epe motzeko ondorioak neurtu direlako, epe luzeko efektuak aztertu beharrean. Hala eta guztiz ere, azken urteetan NMDA hartzaileen antagonisten administrazio kroniko eta azpikroniko errepikatuak eman izan zaizkie animaliei jaio ondorengo garai goiztiarrean eskizofreniaren defizit kognitiboak modelatzeko eta epe luzeko portaera aldaketak aztertu dira (nerabezaroan edo heldutasunean) *(Bubeníková-Valesová et al., 2008; Lim et al., 2012)*. MK-801, dizolzipina bezala ere ezagutzen dena, NMDA hartzaile ez-lehiakorren artean farmakorik selektiboena eta indartsuena da eta, beraz, zabal erabili da eskizofrenia animalia-ereduak sortzeko karraskarietan *(Wong et al., 1986)*.

MK-801-ek NMDA hartzailea fisikiko blokeatzen du kanal barruan sartuz, PCP-ren guneetara lotuz eta kanalean zehar katioien fluxua saihestuz. NMDA hartzaileen blokeoak glutamatoaren gehiegizko askapena eragiten du eta bai blokeatutako neuronan bai garuneko beste eskualde batzuetan eragin kaltegarriak izan ditzake. Izan ere, MK-801-k efektu proapoptotikoak ditu farmakoa eman ondorengo epe motzean. Bide apoptotikoak aktibatzeko duen gaitasuna NMDA hartzailearen blokeoaren iraupenaren eta indarraren araberakoa da. Normalean, 0.25mg/kg baino dosi handiagoak dira beharrezkoak atzeraezina den endekapena eta zelularen heriotza eragiteko *(Ikonomidou et al., 1999)*. Zelularen heriotza aktibatzeko mekanismoak ez daude guztiz argi, baina neurogarapen goiztiarrean NMDA hartzailea ERK1/2-CREB bideei lotzea beharrezkoa da bere akzio neurotrofikoetarako eta dirudienez, MK-801-k eragindako zelulen heriotza NMDA hartzailea ERK1/2 seinaleztapen bidetik disoziatzearen ondorioa da *(Lim et al.,*

2012). Ikonomidou eta lankideek *(Ikonomidou et al., 1999)* esandakoaren arabera, MK-801-ek eragindako zelulen heriotzak bakarrik neuronei eragin zien, zelula glialen aktibaziorik gabe. Azken hamarkadako ikerketek, ostera, eskizofrenia duten gaixoetan gliaren narriadura dagoela iradokitzen dute, hala nola, glia zelulen tamaina txikiagoa kortex prefrontalean eta glia zelulen disfuntzioa kortex prefrontal eta hipokanpoan *(Cotter et al., 2001; Kondziella et al., 2007)*. MK-801-ek eragindako kalteen minberatasunak NMDA hartzaileen adierazpen altuarekin eta garunaren hazkundearekin du zerikusia. NMDA hartzailearen antagonismoaren ondoren, glutamatoaren gehiegizko askapena dago eta honek eragindako exzitotoxizitatea bide apoptotikoetatik at doa, hazkuntza konoen aktibitatea eta neuriten luzapen eta adarkatzeari erasanez *(Ringler et al., 2008)*. *Post mortem* aztertutako gaixo eskizofrenikoen garunetan ere lesio neuronalak ikus daitezke, esate baterako, dendriten atrofia *(Garey, 2010)*.

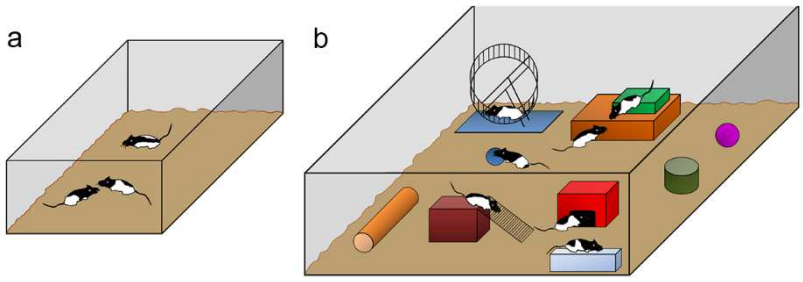
Glutamatoak eragindako exzitotoxizitateak ondorio funtzional handiak ditu. Interneurona GABAergikoak zelula piramidalak baino hamar aldiz sentikorragoak dira NMDA hartzaileen antagonismoarekiko *(Grunze et al., 1996)*. Bereziki, PB adierazten duten jaurtiketa arineko interneuronak dira sentikorrenak, antza, eta haien alterazioa nahikoa da portaeran eskizofreniaren sintomak diruditen antzeko ezaugarriak sortzeko. Hainbat ikerketek frogatu dute MK-801-aren administrazio errepikatuek PB-ren inmunoerreaktibitatea gutxitzen dutela. NMDA hartzaileen blokeo kronikoak arratoi gazte eta helduetan interneurona PB-positiboen dentsitatea murrizten du hipokanpoan, batez ere hortz-jiroan eta CA1 eskualdean *(Rujescu et al., 2006; Braun et al., 2007)*. Neurogarapeneko ereduak ere aurkikuntza hauek errepikatzeko gai izan dira, baina normalean 0.5mg/kg baino gehiagoko dosiak behar dira epe luzeko aldaketa estruktural eta anatomikoak sortzeko *(van der Staay et al., 2011)*. Dosi handi hauek ez dituzte bakarrik hipokanpoko PB dentsitateak aldatzen, baita kortex prefrontal medialeko zelula PB-positiboenak ere, eta eskualde hau orden-altuko funtzio kognitiboetarako beharrezkoa da *(Coleman et al., 2009; Li et al., 2015)*. Are gehiago, Nakazawa eta lankideek *(Nakazawa et al., 2012)* sagu transgenikoak erabiliz, NMDA hartzaileak kortexeko eta hipokanpoko interneurona GABAergikoetatik kentzeak nahikoa zela eskizofrenia-bezalako ezaugarriak agerrarazteko erakutsi zuten. Kaltetutako interneurona gehienek PB inmunoerreaktibitatea zuten, izan ere. MK-801 administratu ondoren jaurtiketa arineko zelula PB-positiboen sentikortasun selektiboaren mekanismoak jakiteke daude oraindik, baina hainbat hipotesi plazaratu dira. Jaurtiketa arineko zelulek potasio kanal berezi bat

dute, hots, Kv1.3 kanala, eta honek mintzaren errepolarizazio arin bat bermatzen du hurrengo ekintza-potentziala azkar sortzeko. Frekuentzia altuko ekintza-potentzialak jaurtitzean NMDA hartzaileen irekitze probabilitatea askoz ere handiago da jaurtiketa arineko zeluletan, frekuentzia baxuagoan jarduten diren beste zelula GABAergikoekin edo zelula kitzikatzaileekin alderatuz. MK-801 farmako ez-lehiakorra denetik, ioi kanala irekita egon behar da hartzailea blokeatu ahal izateko eta, beraz, jaurtiketa arineko NMDA hartzaileak blokeatzeko aukera handiagoa dute. Wang eta Gao-k *(Wang eta Gao, 2012)* beste mekanismo bat deskribatu zuten. Ikerketa elektrofisiologikoen bidez eta MK-801-en dosi azpikronikoak erabiliz zelula piramidaletara edo jaurtiketa arineko interneuronetara zuzendutako terminal glutamatergikoetako NMDA hartzaileak MK-801-en ondorioz ezberdin zeudela aldatuta erakutsi zuten. NMDA hartzaile presinaptikoak oso garrantzitsuak dira neurotransmisoreen askapena errazteko eta modulatzeko. Autore hauen arabera, MK-801-k guztiz blokeatu zituen jaurtiketa arineko interneuronetara zuzenduta zeuden terminal glutamatergikoetako NMDA hartzaileak eta, aldiz, zelula piramidalekin kontaktatzen zuten terminal presinaptikoetan NMDA hartzaile berrien txertaketa ahalbidetu zuen. Datu hauek are gehiago berresten dute NMDA hartzaileen blokeoaren mekanismo sinaptikoak zelula mota desberdinekiko espezifikoak direla. Bi hipotesi hauek ez dira bateraezinak eta ziur aski MK-801-k NMDA hartzaileak hainbat mekanismoren bidez aldatzen ditu, baina guztiak emaitza berdinarekin: interneurona PB-positiboen azpijarduera, kortexeko zelula piramidalen desinhibizio orokor bat eraginez. Bestalde, NR2C eta NR2D azpiunitateak dituzten NMDA hartzaileak bereziki kalteberak izan daitezkeela proposatu dute hauek magnesioarekiko duten afinitate baxua dela eta *(Kotermanski et al., 2009)*. NR2C daukaten NMDA hartzaileak interneurona PB+ eta SST+-etan baino ez dira adierazten eta NR2D azpiunitatea jaurtiketa arineko interneuronetan adierazten da batez ere *(von Engelhardt et al., 2015)*.

1.3. Ingurune aberastua

1.3.1. Ingurune aberastuaren kontzeptua

1940ko hamarkadaren amaieran Hebb izan zen ingurune aberastua (IA) kontzeptu esperimental gisa lehenengo aldiz proposatu zuena. Etxean maskota gisa hezitako arratoiek laborategiko arratoiekin alderatuz portaera hobekuntzak zituztela ikusi zuen *(Hebb, 1947)*. 1960ko hamarkadaren hasieran, Rosenzweig eta lankideek ingurune aberastua testa daitekeen kontzeptu zientifiko bezala proposatu zuten *(Rosenzweig et al., 1962)*. Lehen ikerketek, "garunaren pisua" edo "garunaren proteina totala" bezalako parametroak erabili zituzten ingurumeneko estimuluen efektuak aztertzeko *(Rosenzweig et al., 1969)*. Bestalde, hainbat ikerketek ondorioztatu zuten ingurunearen estimulazioak erantzun plastikoa eragiten zuela, erantzun hau aldaketa biokimikoetan, adarkatze dendritikoan *(Holloway, 1966; Greenough eta Volkmar, 1973)*, gliogenesian *(Altman eta Gas, 1964; Diamond et al., 1966)*, neurogenesian *(Kempermann et al., 1997)* eta ikasketa prozesuan islatzen zelarik.

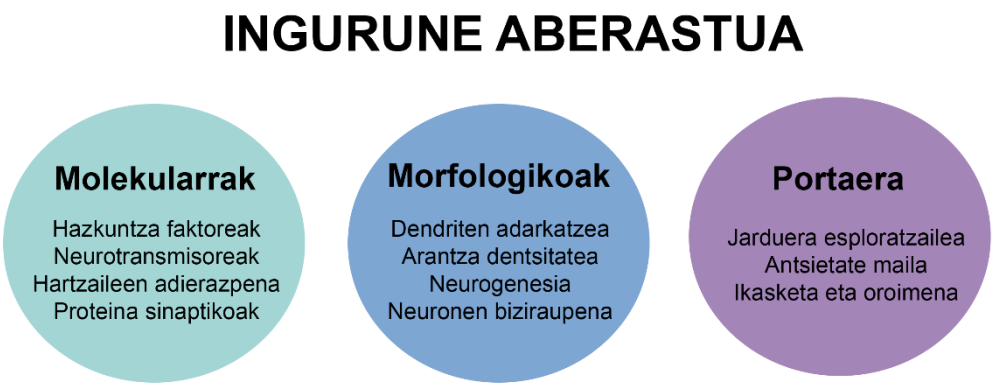


10. irudia. Baldintza estandarren (a) eta ingurune aberastuaren (b) irudikapen eskematikoak. Bengoetxea H et al. Neural Plast. 2012-tik hartua.

Ingurune aberastuaren definizio estandarra hurrengo hau da: "bizigabeko objektuen eta gizarte-estimulazioaren konbinaketa konplexua". Egoera esperimentalean, "aberastutako" animaliak kaiola eta talde handiagotan bizi dira, non harreman sozial konplexuagoak edukitzeko aukera duten. Ingurunea konplexua da eta ikerketak dirauen bitartean, inguruneko ezaugarriak aldatu egiten dira konplexutasuna mantentzeko helburuarekin. Hala, ingurune aberastuko kaiolak tunelez, habia egiteko materialez eta

jolas ezberdinez ornituta daude. Gainera, materialen zein janariaren kokapena maiztasunez aldatzen da. Bestalde, animaliei borondatezko ariketa fisikoa egiteko aukera ematen zaie kaioletan gurpil bat jarriz (10. irudia).

Ingurune aberastuak estimulazio sentsorial, motore eta kognitiboen konbinazio konplexua baimentzen du eta honek molekula, zelula eta portaera mailako aldaketak eragiten ditu (11. irudia). Ingurune aberastuko elementu desberdinek bakarka ere efektu esangarriak izan ditzakete garun plastikotasunean. Adibidez, borondatezko ariketa fisikoak zelulen ugaritzea eta neurona berrien integrazioa bideratzen du hortz-jiroan *(van Praag et al., 1999)*. Bestalde, ingurune berri baten eraginpean jartzeak soilik oroimen espaziala hobetu dezake *(Yang eta Tang, 2011)*. Ingurune aberastuaren definizioan ezin da faktore bakoitzaren eragina erraz isolatu. Izan ere, ingurune aberastuko osagai bakoitzaren efektua besteengandik guztiz banatzea oso zaila da eta neurtzen den parametroaren araberakoa izan daiteke. Hala ere, arrazoi nahikoa daude ingurune aberastuaren funtsezko elementua faktore ezberdinen elkarrekintza dela onartzeko.



11. irudia. Ingurune aberastuan egon ondoren gertatzen diren aldaketa erakusten dituen diagrama.

Ingurune aberastuaren iraupenak eta denboraldiak eragin handia dute lortutako emaitzetan *(Amaral et al., 2008)*. Ikerketa anitzetan, IA garai goiztiarrean hasten da, hala nola, jaiotze egunean edo edoskitzearen amaieran. Kasu hauetan, ingurumen konplexuan hazteak antsietate-bezalako portaera murrizten duela frogatu da, besteak beste *(Hendershott et al., 2016)*. Animali helduak inguren aberastuan ezartzeak ere efektu ditu

(Beauquis et al., 2010; de Witt et al., 2011). Hala ere, gutxieneko IA-ren iraupen bat beharrezkoa da portaera eta aldaketa morfologiak gerta daitezen. Denboraldi hau aste bete eta lau aste bitartean ezarri da, beti ere aztertzen den espeziearen eta parametroaren araberakoa izanik. Esaterako, heldutasunean IA-n bitartekotza motz bat (10 egunekoa) nahikoa da neurogenesia, sare baskularra eta dendriten konplexutasuna hipokanpoan areagotzeko *(Beauquis et al., 2010)*. Bestalde, IA-ko esposizio motzak (6h/eguneko) etengabeko IA-ren hobekuntzen antzerakoak dira funtzio motoreei eta kognitiboei dagokienez *(de Witt et al., 2011)*. Gainera, ingurune aberastuak eragindako onuren iraunkortasuna, IA-ren iraupenaren araberakoa da. Zenbat eta luzeagoa izan IA-ren iraupena, orduan eta denbora gehiagoz irauten dute efektu onuragarriak.

1.3.2. Ingurune aberastuak eragindako aldaketa kognitibo eta estrukturalak

Ingurune aberastua eraginkorra da ikasketa eta oroimen funtzioak hobetzeko eta zahartzaro osasuntsuan ematen den disfuntzio kognitiboa murrizteko gai dela baieztatu da. Ingurune aberastuan hazitako animaliek baldintza estandarretan hazitako animaliek baino hobeto egiten dute espazio-oroimeneko proba Morrisen ur labirintoan. Era berean, IA-n hazitako animaliek, kaioletan isolatuta baina ariketa fisikoa egiteko gurpila duten animaliek baino lan-oroimen espazial hobea dutela egiaztatu da *(Bernstein, 1973)*. Ingurune aberastuak eragiten dituen onura kognitiboak, partzialki honek duen efektu antsiolitikoaren bidez azaldu daitezke. Izan ere, IA-n hazitako sagu zein arratoiek antsietate-bezalako portaera murriztua erakusten dute “igotako gurutze labirintoan” (Elevated Plus Maze) eta eremu irekiko proban, aldi berean jarduera esploratzailea areagotuz *(Hendershott et al., 2016)*.

Ingurune aberastuan bizi diren karraskariek aldaketa estruktural esanguratsuak jasaten dituzte, esateko, garun tamaina eta pisu handiagoa edota kortexeko zelulen nukleoen tamaina handiagoa *(Diamond et al., 1967)*. Ingurune aberastuak hipokanpoko neurogenesia areagotzen du arratoi gazte eta helduetan eta, aldi berean, diferentziatutako zelula granularren biziraupena bermatzen du hortz-jiroan. Ingurune aberastuari buruzko lehen ikerketek erakutsi zutenez, hipokanpoko hortz-jiroko zelula granularrak eta CA1 eta CA3ko zelula piramidalak dendrita gehiago zuten neuronako *(Walsh et al., 1969;*

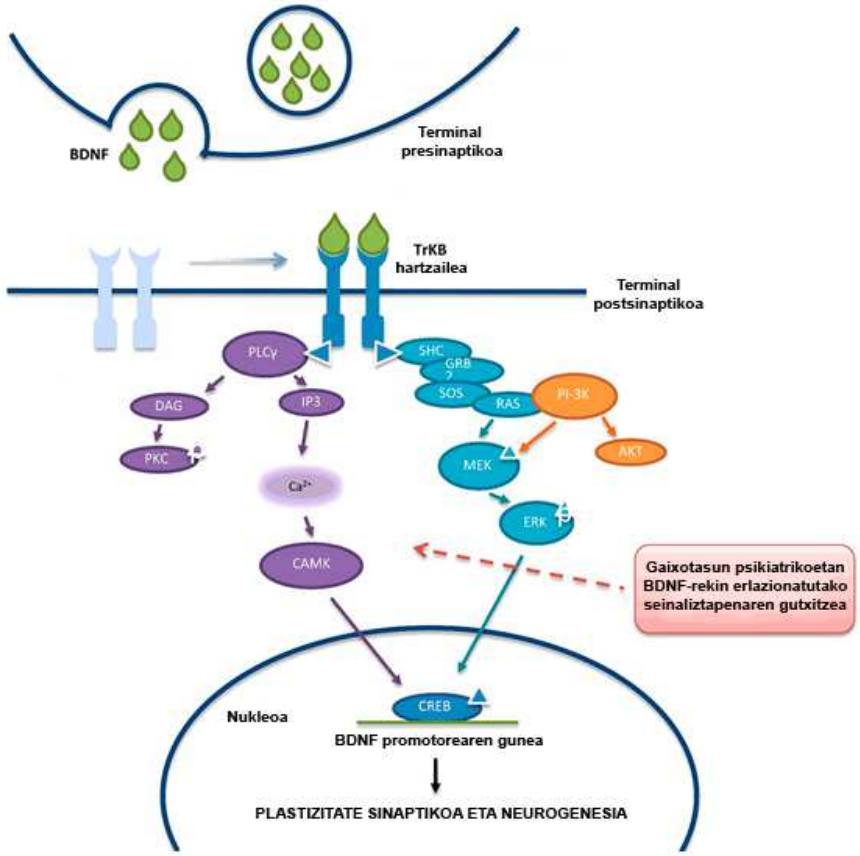
Altschuler, 1979). Beranduago, IA-k dendriten adarkatzea eta arantzen dentsitatearen areagotzea eragiten zuela baieztatu zen *(Globus et al., 1973)*, kontaktu sinaptiko berrien sorrera ondorioztatuz. Hala ere, IA-ren ondoren kortexeko bolumenaren areagotzea ezin zaio soilik neurogenesiari eta sinaptogenesiari atxiki, angiogenesia eta gliogenesia ere ikusi izan baita *(Altman eta Das, 1964; Diamond et al., 1966; Greenough eta Volkmar, 1973)*. Baskularizazioaren areagotzeak glukosaren erabilgarritasuna eta zitobabesleak diren faktoreen askapena erraztuko luke. Ingurune aberastuak plastikotasun sinaptikoarekin erlazionatuta dauden gene-adierazpena modulatzen duela jakin arren, ikasketa eta oroimenean duen efektu onuragarrien mekanismo molekular zehatzak argitzeke daude.

1.3.3. Hazkuntza faktoreak Ingurune Aberastuak eragindako aldaketen bitartekari

Hazkuntza faktoreak neuropeptido familia handi bat dira eta zelula kanpoko seinaleztapenerako garrantzitsuak dira. Hala, nerbio sistema zentraleko zelula aitzindari eta ama zelulen ugalketa eta desberdintze prozesuak erregulatzeko ezinbestekoak dira *(Calof, 1995)*. Baldintza estandarretan hazitako animalietan, garapen garaia edozein dela ere, hazkuntza faktoreek zelulen ugalketa eta biziraupena, axoi eta dendriten hazkuntza eta birmoldaketa, mintzen trafikoa eta fusioa zein sinapsien sorkuntza, funtzioa eta plastikotasuna sustatzen dute.

Ingurune aberastuak gene adierazpena eta hazkuntza faktore desberdinen proteina mailak areagotzen ditu, adibidez, nerbio-hazkuntza faktorea (NGF) *(Mohammed et al., 1990; Pham et al., 1999)*, garunetik eratorritako hazkuntza faktorea (BDNF) *(Falkenberg et al., 1992)*, gliatik eratorritako faktore neurotrofikoa (GDNF) *(Young et al., 1999)* edo hazkuntza faktore endotelial-baskularra (VEGF) *(During eta Cao, 2006)*. Ikerketa asko burutu dira helduen garunetan faktore hauen rola zehazki ezagutzeko. Dirudienez, organismo helduetan faktore hauek neurogenesian, plastikotasun sinaptikoan eta ikasketan parte har dezakete. Adibidez, intsulina-bezalako hazkuntza faktoreen infusio intrazerebrobentrikularrak neurogenesia areagotzen du arratoien hortz-jiroan *(Aberg et al., 2000)*.

Era berean, BDNF-k plastikotasun sinpatikoan eta IA-ri loturiko hobekuntza kognitiboan funtsezko rola jokatzen du *(Novkovic et al., 2015)*. Hipokanpotik BDNF-ren genea ezabatzen denean, "Objektu berrien ezagutza" eta Morrisen ur labirintoan ikasketa espaziala baliogabetzen dira *(Heldt et al., 2007)*. Hazkuntza faktore honek Tropomiosin Kinasa B hartzailea (TrkB) aktibatzen du neuronen diferentziazio, biziraupen eta plastikotasun sinaptikoa eraldatzeko hainbat seinalizazio jauziren bidez, hala nola, ERK edo Akt (12. irudia).



12. irudia. Tropomiosin kinasa B hartzailearen (TrkB) beherantzako seinaleztapen bideek zelulen biziraupena eta diferentziazioa, axoien hazkuntza eta jarduera sinaptikoa erregulatzen duten proteinen sintesia ahalbidetzen dute. Begni et al. Clinical Science. 2016-tik hartua eta moldatua.

2. Hipotesia

Ingurune aberastua (IA) erabilera anitzeko eta balio terapeutiko handiko tresna da. Zahartze osasuntsuan eta neuroendekapeneko gaixotasunetan dituen efektu onuragarriak balioztatuak izan diren arren, gaixotasun neuropsikiatrikoetan duen balio potentziala ez da sakon aztertu. Ingurumen faktoreek eskizofreniaren agerpena ahalbidetu dezakete, beraz, gaixotasunaren sorrera edo areagotzea saihesteko gaitasuna ere badute. Eskizofreniaren inguruko ikerketen erronka nagusietako bat garunean era iraunkorrean kalteturik dauden zirkuituak aldatzen saiatzea da, eskizofrenia neurogarapeneko desoreka bada ere, sintomak nerabezaroan edo heldutasun goiztiarrean agertzen baitira.

Ingurune aberastuak eskizofreniaren sintoma kognitiboengan ondorio onuragarririk duen edo ez aztertzeak interes handia du, bai prebentzio tresna gisa, bai sendabide gisa. Arazo kognitiboengan eragin positiboa izan dezakeen interbentzio ez-farmakologikoa litzateke. Izan ere, ingurune aberastuak eragindako geneen aktibazio eta proteinen sintesiak interneuronen adierazpena eta haien jarduera areagotu dezake. Honek, aldi berean, eragin zuzena izango luke portaeran eta kognizioan. Aldaketa hauek eskizofreniaren galera kognitiboak arintzeko funtsezkoak lirateke.

Beraz, lan honen helburua MK-801 bidez sortutako eskizofrenia animalia-ereduan, bai portaeran zein maila zelularrean eta molekularrean garatutako alterazioak ingurune aberastuaren bidez nola aldatzen diren aztertzea da.

Honenbestez, tesi hau garatzeko honako **hipotesia** landu dugu:

Ingurune aberastuak hazkuntza faktoreen, NMDA hartzaileen, kaltzioari loturiko proteinen eta neuropeptidoen adierazpena modulatzen du bai kortex prefrontal zein hipokanpoan eta guzti hauek aldatuta daude eskizofrenian. Hala, heldutasunaren hasieran animaliak denbora labur batez ingurune aberastuan haziz gero, eskizofreniaren esparruan sor daitezkeen defizit kognitiboak gainditzeko estrategia egokia izango litzateke.

3. Helburua

3.1. Oinarrizko helburua

Neurogarapen goiztiarrean, MK-801 administratu zaien arratoi helduetan ingurune aberastuak alterazio kognitibo eta neurokimikoetan duen eragin positiboa egiaztatzea, eta hobekuntza hauen oinarri molekularrak aztertzea.

3.2. Helburu operatiboak

1. Funtzio kognitibo desberdinak aztertzea: ezagutza memoria "objektu berrien ezagutza" atazaren bidez, memoria asoziatiboa "objektua-tokian" atazaren bidez eta memoria bisuoespaziala "Morrisen ur labirintoaren" bidez.

2. Aldaketa estrukturalak analizatzea: kortex prefrontal medialeko eta hipokanpoko bolumenaren neurketa, eta butiril kolinesteraren aurkako histokimiaren bitartez sare mikrobaskularraren luzeraren estimazioa.

3. Interneuronen azpipopulazio desberdinen eta neurona kopuru totalaren kuantifikazioa: ordenagailuz lagundutako estereologiaren bidez, parbalbumina, kalretinina, kalbindina, somatostatina, azido glutamiko deskarboxilasa eta NeuN proteinen aurkarko inmunohistokimien kuantifikazioa kortex prefrontalean eta hipokanpoan.

4. Aldaketa molekularrak analizatzea: Western Blot teknikaren bitartez, NMDA hartzaileen azpiunitateen, dentsitate postsinaptikoa-95 proteinaren (PSD-95), $GABA_A$ hartzailearen ß2/3 azpiunitatearen, garunetik eratorritako hazkuntza faktorearen (BDNF) eta TrkB-ren adierazpenaren, zein hauekin erlazionatutako zelula barneko seinaleztapen-bideen aldaketa erlatiboen kuantifikazioa.

5. Ingurune aberastuak aurrez izendatutako parametroetan duen onura potentzialen identifikazioa.

4. Material eta metodoak

4.1. Animaliak

Long Evans arratoi emeak kume arrekin erosi ziren Janvier Labs enpresan (Frantzia). Jaiotze eguna, 0 jaio ondorengo eguntzat (joe) hartu zen eta 21. joe izan zen edoskitze amaiera. Animali guztiak argi/ilunpeko hamabi orduko ziklotan hazi ziren (argiak 8etan piztuz) eta ura eta janaria ad libitum izan zuten esperimentu guztian zehar. Prozedura guztiak 2007/526/EE Europako Gomendioarekin bat zetozen, eta Euskal Herriko Unibertsitateko (UPV/EHU) Animaliekin egiten diren Esperimentaziorako Etika Batzordearen oniritziarekin egin ziren (CEEA/410/2015/LAFUENTE SANCHEZ).

4.2. Prozedura farmakologikoak

4.2.1. MK-801-aren administrazioa

MK-801 [(5S,10R)-(+)-5-Metil-10,11-dihidro-5H-dibenzo[a,d] ziklohepten-5,10-imina hidrogeno maleatoa, Dizozilpina hidrogeno maleatoa] Sigma-Aldrichen erosi zen (Erref: M107, St. Louis, MO, EEBB). Aurretiaz egindako ikerketetan oinarrituta, 0.5mg/kg-ko dosia aukeratu genuen, dosi honek portaera alterazio iraunkorrak eragiten baititu *(van der Staay et al., 2011)*. MK-801 kloruro sodikoa %0.9an (NaCl) diluituz eta injekzio intraperitonealaren bidez administratu zitzaien arratoi kumeei 10. joe-tik 20. joe-ra. Animali kontrolak NaCl-aren bolumen berdina jaso zuten (VH: eramailea soilik daramaten animalien taldea). Injekzioak goizeko 9etan ematen zitzaizkien. Tratamenduak iraun bitartean, animaliak egunero pisatu ziren eta hortik aurrera bi astetik behin. MK-801-aren stock soluzioa NaCl-tan prestatu eta -20ºC-tan gorde zen, 2.5mg/ml-ko kontzentrazioan. Stock soluzioa erabiltzerako orduan NaCl-tan diluitu zen 0.5mg/ml-ko kontzentraziora.

4.2.2. BrdU-ren administrazioa

Talde bakoitzeko 6 animalik timidinaren analogoa den 5'bromodesoxiuridina-ren (5'BrdU, Sigma-Aldrich, Espainia) injekzio intraperitoneal bakarra jaso zuten NaCl-tan. Bihotzean zeharkako perfusioa burutu baino 24h arinago eman zitzaien 200mg/kg-ko dosi batean.

4.3. Talde esperimentalak

Lau talde esperimental desberdin erabili ziren (n=10-12) (13. irudia):

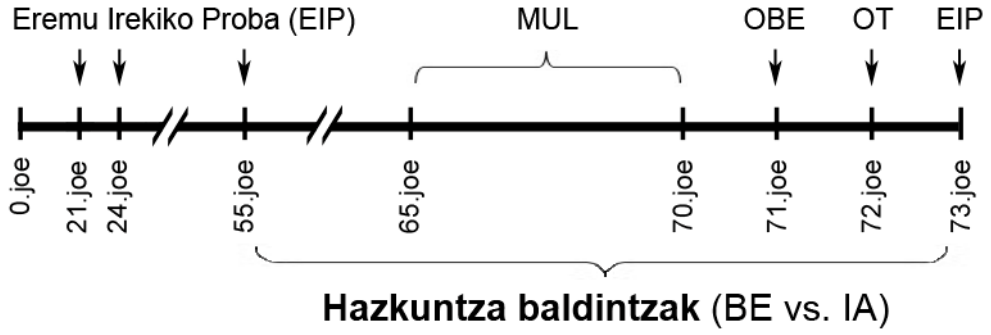
a) **VH:** Laborategiko baldintza estandarretan hazi eta 10. joe-tik 20. joe-ra NaCl jaso zuten arratoiak.
b) **MK-801**: Laborategiko baldintza estandarretan hazi eta 10. joe-tik 20. joe-ra MK-801 (0.5mg/kg) jaso zuten arratoiak.
c) **MK-801+EE:** 0. joe-tik 55. joe-ra laborategiko baldintza estandarretan eta 55. joe-tik 73. joe-ra ingurune aberastuan hazitako animaliak. Talde honetako animaliek 10. joe-tik 20. joe-ra MK-801-n injekzioak (0.5mg/kg) jaso zituzten.
d) **VH+EE:** 0. joe-tik 55. joe-ra laborategiko baldintza estandarretan eta 55. joe-tik 73. joe-ra ingurune aberastuan hazitako animaliak. Talde honetako animaliek 10. joe-tik 20. joe-ra NaCl-aren injekzioak jaso zituzten.

13. irudia. Tratamendu farmakologiko eta hazkuntza baldintzen kronograma. Joe, jaio ondorengo eguna; IA, ingurune aberastua (EE, Enriched Environment).

Laborategiko baldintza estandarretan, 2-3 animali hazi ziren 500 x 280 x 140 mm tamainako kaioletan. Ingurune aberastuko kaiolak 720 x 550 x 300 mm-ko tamaina daukate eta 6 animali hazi ziren kaiola bakoitzean. Kaiola hauek tamaina eta kolore desberdinetako hainbat objektu dituzte (tunelak, aterpeak, jolasak, etab.) eta hauek bi egunetik behin aldatzen zaizkie. Horrez gain, ingurune aberastuko kaioletan borondatezko ariketa fisikoa egiteko gurpil bat zuten erabilgarri. Baldintza desberdinetako prozeduren sailkapena 13. irudian ikus daiteke.

4.4. Portaera proba eta ataza kognitiboak

Portaera atazetarako 10-12 animali erabili genituen taldeko. Ingurune aberastuan zeuden animaliak, baldintza berdinetan mantendu ziren atazen iraupenean zehar. Animaliak 21. joe eta 24. joe-etan Eremu irekiko proba (EIP) burutu zuten, MK-801-aren epe laburreko efektuak ikusteko. 55. joe-ean eta 73. joe-ean testatu ziren berriz EIP-n antsietate-bezalako portaera eta lokomozioa neurtzeko. Morrisen ur labirintoa (MUL) 65. joe eta 70. joe-en bitartean burutu zen. Ondoren, Objektu berrien ezagutza (OBE) eta Objektu-tokian ataza (OT), 71. joe-ean eta 72. joe-ean egin ziren, hurrenez hurren (14. irudia).

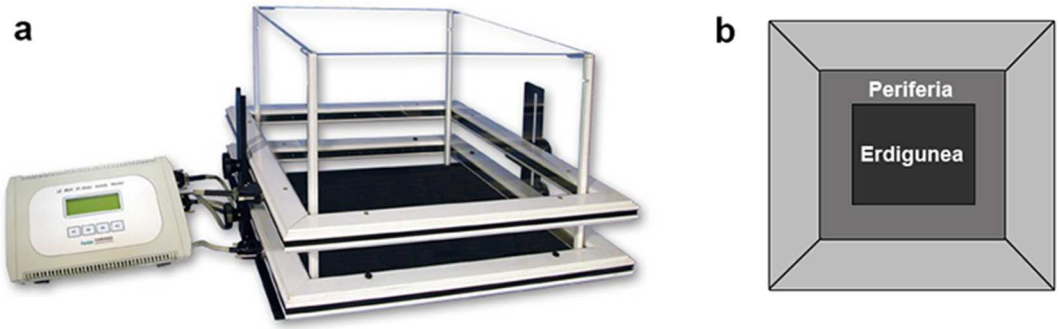


14. irudia. Portaera atazen kronograma. EIP, Eremu irekiko proba; MUL, Morrisen ur labirintoa; OBE, Objektu berrien ezagutza; OT, Objektu-tokian ataza; BE, baldintza estandarrak; IA, ingurune aberastua. Joe, jaio ondorengo eguna.

4.4.1. Eremu irekiko proba

Berezko lokomozio-jarduera Eremu Irekian neurtu genuen (15. irudia). Jardueraren detekzioa erabat automatizatua zegoen Actitrack diseinu pertsonalizatutako softwarearen bidez (Panlab, Espainia). Eremu irekia plexiglasez egindako eremu karratu bat zen (44 x 44 x 35 cm), animalien detekzio egokia lortzeko 16 x 16 cm-ko infragorrizko bi habe paraleloz inguratuta zegoelarik. Esparrua bi zatitan bananduta zegoen, erdialdeko gunea eta gune periferikoa. Arratoi bakoitza esparruaren erdian jarri eta bere jarduera 10 minutuz erregistratu zen. Erdian eta periferian egindako desplazamendu horizontalak neurtu ziren. Saiakera bakoitzaren ondoren, esparrua %96ko alkoholarekin garbitzen zen. Animali bakoitzak lau sesio egin zituen jaio ondorengo 21., 24., 55. eta 73. egunetan.

Esparruaren erdialdean egindako distantzia erlatiboa formula honen bidez lortu zen: 100 x [erdialdean mugitutako distantzia / periferian mugitutako distantzia].

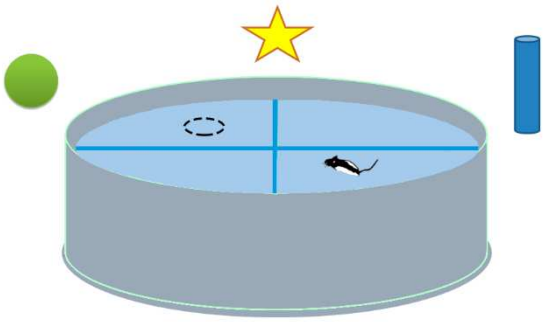


15. irudia. Eremu irekiko proba. (a) Eremu irekiko esparrua eta Actritrak tresna. (b) Esparruko erdigunearen eta periferiaren goiko ikuspegiaren irudikapen eskematikoa.

4.4.2. Morrisen ur labirintoa

Morrisen ur labirintoa karraskarien ikasketa eta oroimen espaziala neurtzeko erabiltzen da eta hauek inguruan dauden aztarnak erabiliz ur azpian dagoen plataformara nabigatzeko duten gaitasunean oinarritzen da. Esperimentu honetarako, 175 cm-ko diametroko igerilekua 22 ± 1ºC tenperaturako urez bete eta pintura zuri ez-toxikoarekin ura opaku bihurtu zen. Jomuga den plataforma zuria uretan murgildu zen, uraren gainazaletik 2 cm-ra. Lau ikusmen-aztarna jarri ziren igerilekua inguratzen zuten errezeletan (16. irudia). Arratoiak lau puntu kardinaletatik askatzen ziren egunero, eta abiatze-puntu horiek egunero aldatzen ziren probak iraun bitarteko 5 egunetan ausazko modu batean. Plataforma, aldiz, toki berdinean mantendu zen proba guztian zehar. Saiakera bakoitzean, arratoiak paretari begira askatzen ziren eta 120 segundoz uzten zitzaien igeri egiten plataformarekin topo egin arte eta ondoren, 30 segundoz uzten ziren bertan. Arratoiak 120 segundotan plataforma aurkitzeko gai ez baziren, ikertzaileak gidatzen zituen animaliak bertara. Saioen arteko tartea 20 minutukoa zen. SMART Bideo Jarraipen Softwarea (Panlab. SL, Bartzelona) erabili zen ibilbideak grabatzeko. Software honen bidez, ihes-latentzia, ibilbidearen luzera, igeri-abiadura eta jomugarako batezbesteko distantzia parametroak neurtu ziren saiakera bakoitzeko. Bost eguneko

ikasketa prestakuntza eta gero, seigarren egunean plataforma kendu eta erreferentzia oroimena aztertzeko jomugarako batezbesteko distantzia (JBD) parametroa neurtu zen. JBD hurbiltasun neurri bat da eta parametro hau ohiko eta ezagunagoak diren "helburuko koadrantean egindako denbora" edo "plataforma gurutzatze zenbakia" bezalako parametroak baino sentikorragoa eta fidagarriagoa da *(Maei et al., 2009)*. Arratoiei azken azterketa bat egin zitzaien ageriko plataforma saioan, arazo bisual eta motoreak izanez gero, animaliak analisitik baztertzeko.

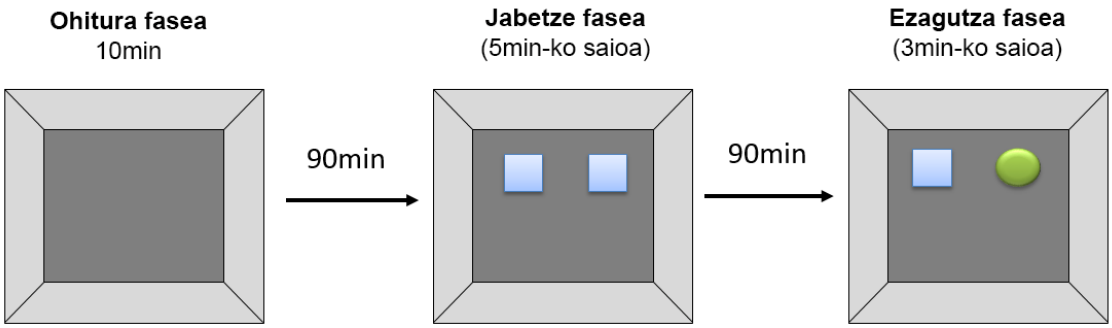


16. irudia. Morrisen ur labirintoaren irudikapen eskematikoa. Ezkutatutako plataforma lerro etenen bidez adierazten da. Lau aztarna bisual jarri ziren plataforma inguruan, koadrante bakoitzean bana, animaliak orientatzeko.

4.4.3. Objektu berrien ezagutza

Objektu berrien ezagutza (OBE) atazak animaliek objektu berriak antzemateko duten gaitasuna neurtzen du eta karraskariek inguruneko objektu berriak esploratzeko duten berezko joeran oinarritzen da. Ataza honek 3 fase ditu: ohitura, jabetze eta ezagutza faseak. Ohitura fasean, arratoiak banan-banan jarri ziren 90 x 90 x 40 cm-ko esparru huts batean 10 minutuz ingurunea askatasun guztiz miatu zezaten. 90 minutu igaro ondoren, animaliak jabetze fasea burutu zuten. Fase honetan, plastikoz egindako bi objektu berdin (Legoko piezez eginak) jarri ziren eremuan eta objektu bakoitza esploratzen igaro zuten denbora neurtu genuen 5 minutuko iraupeneko proban. Arratoiak paretari begira askatu ziren hertsapena ekiditeko asmoz. Saio arteko 90 minutuko tartea eta gero, arratoiak berriz itzuli ziren eremura 3 minutuko ezagutza probarako, non objektu ezagunetako bat objektu berri batengatik aldatu zen (17. irudia). Objektu berria objektu ezagunaren

material berdinarekin zegoen eginda. Proba honetan ez genuen aztarna bisualik erabili.



17. irudia. Objektu berrien ezagutzarako atazaren irudikapen eskematikoa. Ezkerretara, esparru hutsa ohitura faserako. Erdian, jabetze fasean erakusten zaizkien bi objektu berdinak. Eskuinean, ezagutza fasea, non objektu ezagun bat berri batengatik aldatu den.

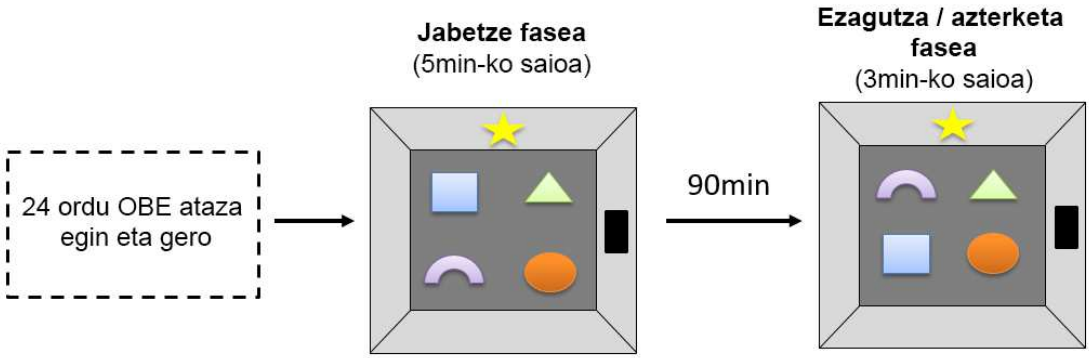
4.4.4. Objektua-tokian ataza

Objektua-tokian ataza (OT) Objektu berrien ezagutza atazaren oso antzekoa da. Ataza hau aukera bakarreko ezagutza oroimena ebaluatzeko proba bat da eta ez du prestakuntzarik edo indargarririk behar. Objektua-tokian asoziazio proba bat da, non animaliek objektu bat aurretiaz ikusitako toki konkretu batekin erlazionatu beharko duten. Objektu berrien ezagutzarako erabilitako esparru eta material berdinak erabili ziren ataza honetan. Jabetze fasean, altura berdintsuko lau objektu erabili ziren. Bi ikusmen aztarna jarri ziren esparruko paretetan orientazio espaziala hobetzeko (18. irudia). Arratoiak esparruaren erdian jarri eta objektuak esploratzeko 5 minutu eman zitzaien berriz kaiolara bueltatu aurretik. 90 minututako atxikitze tartea igaro ondoren, ezagutza fasean, bi objektu lekuz aldatu ziren. Hala, 3 minututako proban zehar mugitutako eta ez-mugitutako objektuak esploratzen emandako denbora neurtu zen. Objektu-toki asoziazio-oroimen egoki bat izanez gero, animaliak denbora gehiagoz ariko dira mugitutako objektuak esploratzen, mugitu ez diren objektuekin alderatuz.

Bai OBE bai OT atazetan, hiru minutuko proba nahikoa sentikorra da azterketa edo ezagutza faserako. Iraupen luzeagoa duten probek objektuen ezagutza gutxitu dezakete, objektu berria gero eta ezagunagoa bihurtzen baita denbora pasa ahala. Animali batzuk objektu edo toki konkretu batzuk nahiago izan ditzaketela kontutan hartuta, objektuak eta

beraien kokapena aldatzen joan ginen animali desberdinen artean. Argi ahul batek argitzen zuen esparrua argi-isladak ekidinez. Usain aztarnak saihesteko asmoz, esparrua %96ko alkoholarekin garbitzen zen saiakera bakoitzaren ondoren.

Bi ataza hauetan, animaliek objektuak usaindu, aurreko zangoekin ukitu edo zentimetro batetik behera zuzenki begiratzen ziotenean hartzen genuen esploraziotzat. Gutxienez, 5 minututako jabetze fasean zehar 12 segundoz esploratu behar zituzten objetuak animalia analisian sartu ahal izateko. Bereizketa indizea hurrengo formula hau erabiliz lortzen da: (DB - DE) / (DB + DE) [DB = objektu berria edo mugitutako objektuak esploratzen eman duen denbora, DE = objektu ezaguna edo mugitu gabeko objektuak esploratzen eman duen denbora].



18. irudia. Objektua-tokian atazaren irudikapen eskematikoa. Ezkerretara, Objektu berrien ezagutza eta Objektua-tokian atazaren artean igarotako denbora. Erdian, jabetze fasean aurkeztutako lau objektuak koadrante bakoitzean kokatuta. Paretetako bi ikusmen aztarnek informazio espaziala eskuratzen laguntzen dute. Eskuinean, azterketa edo ezagutza proban bi objektu lekuz aldatuta daude.

4.5. Inmunohistokimia

Analisi inmunohistokimikoa talde bakoitzeko 6 animalitan burutu zen. Pentobarbital anestesikoaren farmako-gaindosi bat erabiliz sakrifikatu ziren animaliak. Ondoren, bihotzean zeharreko perfusioa burutu zitzaien, lehenengo serum fisiologikoarekin (kloruro sodikoa %0.9an ur destilatuan) odoleztatze-sistemako hematiak garbitzeko asmoz eta garbiketaren ondoren, ehunaren finkatzea burutu zen

paraformaldehidoa %4 gatz fosfato tanpoian (PBS) 0.1M (pH 7.4) erabiliz. Garbiketa eta finkatze prozesuak presio konstanteko perfusio bonba baten bidez egin ziren, 12 mmHg presiopean. Ehunen finkatzea animalia hil ostean ehuna ez endekatzeko egiten da, honela arratoiaren ehuna bizi egoeraren antzerako egoera batean gordetzeko. Finkatzeak zelula barneko elementuen insolubilizazioa bideratzen du eta ehunari gogortasun puntu bat ematen dio errazago maneiatu ahal izateko.

Perfusioaren ostean, garuna garezurretik atera eta finkatzerako erabili zen substantzia berdinean post-finkatu zen 24 orduz 4ºC-tan. Ondoren, garun osoak sakarosa %30ean duen PBS 0.1M soluzioan sartu ziren kriobabesteko. Materiala kriobabestutakoan, 50 µm lodierako ebaketa koronalak egin ziren kriotomoan (Leica, Wetzlar, Germany). Flotazioko sekzioei 5 minutuko 2 garbiketa egin zitzaizkien eta 20 minutuz %3ko hidrogeno peroxidotan inkubatu ziren ehunaren berezko peroxidasak neutralizatzeko. Hiru garbiketa burutu eta gero, lotura gune ez-espezifikoak blokeatzeko asmoz, zaldiaren seruma %5ean erabili zen PBS 0.1M eta %0.5 Triton X-100-etan (PBS-TX) diluituta ordubetez. Gero, flotazioko ebaketak antigorputz primarioarekin inkubatu ziren blokeo soluzioa erabiliz, 4ºC-tan gau osoan zehar (1. taula). GAD67-ren inmunohistokimiarako TX alde batera utzi zen pausu guztietan. Hurrengo egunean, beste 3 garbiketa egin ondoren, sekzioak bigarren antigorputzarekin inkubatu ziren (1. taula) giro-tenperaturan ordu betez eta PBS-TX-etan diluituta. Berriz, PBS 0.1M-etan 5 minutuko 3 garbiketa egin ondoren, mozketak abidina-biotina-peroxidasa konplexuarekin (Vectastain Elite ABC kit, Vector Laboratories) inkubatu ziren. Ondoren, 3,3´-diaminobenzidina (DAB, erref: D5637, Sigma-Aldrich, Espainia) kromogenoa erabiliz errebelatu ziren mozketak, peroxidasen substratu bezala H_2O_2 erabiliz. Azkenik, sekzioak portetan jarri, 48 orduz deshidratatu eta Xiloletan 2 orduz garbitu ondoren, DPX-ekin estali ziren (Erref: 06522, Sigma-Aldrich, Espainia).

Kalbindina, somatostatina eta NeuN-en inmunodetekzioarako antigenoen azaleratzea burutu zen. Horretarako, lehen pausu bezala mozketak sodio zitratoarekin (pH 6.0) 10 minutuz berotu ziren 95ºC-tara. Ondoren, ebaketak bi orduz inkubatu ziren blokeo soluzioan eta antigorputz primarioa jarri zitzaien gau osoan zehar (1. taula). Hurrengo egunean, ebaketak garbitu eta antigorputz sekundarioarekin beste 2 orduz inkubatu ziren. Kalbindina eta somatostatinaren aurkako inmunohistokimietan antigorputz sekundarioa 1:200-eko kontzentrazioan erabili zen eta NeuN-en

inmunohistokimian, aldiz, 1:1000-ko kontzentrazioan. Azkenik, mozketak portetan jarri, 48 orduz deshidratatu eta Xiloletan 2 orduz garbitu ondoren, DPX-ekin estali ziren (Erref: 06522, Sigma-Aldrich, Espainia).

1. taula. Antigorputzen kontzentrazioak eta erreferentziak

Antigorputz primarioak		
Sagu anti-NeuN	1:2000	Erref: MAB3377, Merck Millipore, Alemania
Sagu anti-kalbindina	1:2000	Erref: CB D-28k 300, Swant, Suitza
Untxi anti-somatostatina	1:5000	Erref: T-4103, Peninsula Laboratories, San Carlos, CA
Sagu anti-parbalbumina	1:5000	Erref: PV 235, Swant, Suitza
Sagu anti-kalretinina	1:2000	Erref: 6B3, Swant, Suitza
Sagu anti-GAD67	1:10000	Erref: MAB5406, Merck Millipore, Alemania
Antigorputz sekundarioak		
Zaldi anti-sagu IgG	1:200	Erref: PK-6102, Vectastain ABC Kit, Vector Laboratories, EEBB
Zaldi anti-untxi IgG	1:200	Erref: PK-6200, Vectastain ABC Kit, Vector Laboratories, EEBB

4.6. Inmunofluoreszentzia bikoitza

Parbalbumina eta GAD67-ren inmunofluoreszentzia bikoitza burutzeko, lehenik mozketak bi aldiz garbitu ziren PBS 0.1M-etan 5 minutuz eta gero, %5eko behi-albumina seruma (BSA) zuen PBS 0.1M-etan blokeatu ziren ordu betez giro-tenperaturan. Segidan, ebaketak GAD67-ren aurkako antigorputzarekin inkubatu ziren (1:10000, Erref: MAB5406; Merck Millipore, Alemania) %0.5 BSA zuen PBS 0.1M-etan diluituta 4ºC-tan gau osoan zehar. PBS 0.1M-ekin 5 minutuku 3 garbiketa egin ondoren, ebaketak saguaren aurkako Alexa Fluor 488 (1:300, Erref: A-11029, Invitrogen, Carlsbad, CA, EEBB) antigorputz sekundarioarekin inkubatu ziren ordu betez. Ondoren, mozketei 5 minutuko 3 garbiketa egin eta gau osoan zehar untxian egindako parbalbumina antigorputz primarioan (1:5000, Erref: PV 25, Swant, Suitza) inkubatu ziren, PBS 0.1M, %0.1 Triton X-100 eta %0.5 BSA-tan zuen soluzioan diluituz antigorputza. Hurrengo egunean, untxiaren aurkako Alexa Fluor 568 (1:300, Erref: A-11036, Invitrogen,

Carlsbad, CA, EEBB) antigorputz sekundarioa gehitu zitzaien. Bost minutuko 3 garbiketa eta gero, mozketak Vectashield (Vector Labs, Peterborough, EEBB) erabiliz muntatu ziren. Azkenik, sekzioak Apotome erantsita duen fluoreszentzia mikroskopioan (Zeiss) aztertu ziren.

4.7. BrdU-ren inmunohistokimia

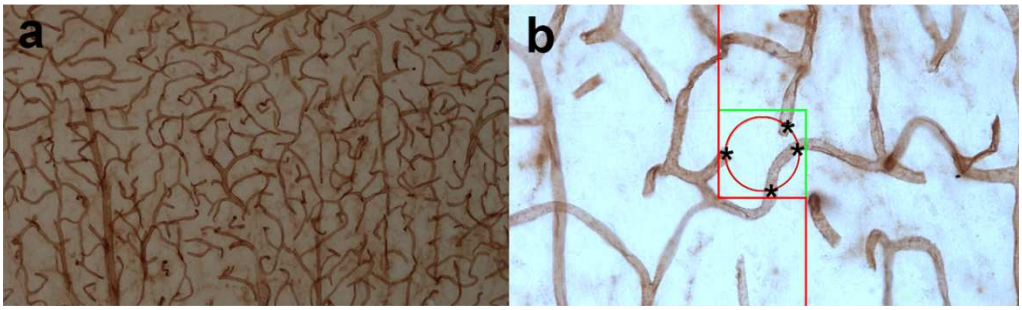
BrdU-ren inmunohistokimiarako erabilitako protokoloa aurretik deskribatutako inmunohistokimia protokoloaren oso antzekoa da. Desberdintasun bakarra aurretik DNA-ren desnaturalizazioa burutu beharra dagoela da. Horretarako, garun sekzioak %50 formamidaz gatz sodio zitratotan (pH 7) aurretratatuak izan ziren 2 orduz 65ºC-tan. Ondoren, DNA 2N HCl-z baliatuz desnaturalizatu zen 37ºC-tan 30 minutuz eta ebaketak PBS 0.1M-etan (pH 7.4) neutralizatu ziren 5 minutuko 3 garbiketa eginez.

Puntu honetatik aurrera, aurretik deskribatutako era berean jarraitu zuen protokoloak. Laburtuz, ebaketen peroxidasak neutralizatu, ordu betez zaldiaren serum normalean blokeatu eta, ondoren, antigorputz primarioan inkubatu (sagu anti-BrdU, 1:200, Erref: B2531, Sigma-Aldrich, Espainia) ziren. Hurrengo egunean, sekzioak antigorputz sekundarioan inkubatu ziren (saguaren aurkako zaldi IgG-a, 1:200, Erref: PK-6102, Vector Laboratories, EEBB) ordubetez eta abidina-biotina-peroxidasa konplexuan (Vectastain Elite ABC kit, Vector Laboratories, EEBB) beste ordubetez, DABrekin errebelatu aurretik (Erref: D5637, Sigma-Aldrich, Espainia). Azkenik, ebaketak portetan jarri, 48 orduz deshidratatu eta Xiloletan 2 orduz garbitu ondoren, DPX-ekin estali ziren (Erref: 06522, Sigma-Aldrich, Espainia).

4.8. Butiril kolinesterasaren histokimia

Butiril kolinesterasaren histokimia sare kapilarraren bistaratze egoki bat ahalbidetzen duen teknika bat da. Flotazioko ebaketak bi aldiz garbitu ziren Tris Maleato tanpoian (pH 6.0) 20 minutuz. Gero, azetil kolinesterasaren jarduera inhibitu zen Tris Maleato tanpoia eta 1.416mg BW284CS1 (1,5-bis (4-allildimetilamoniofenil)-pentan-3-bat dibromida; Erref: A-9013, Sigma-Aldrich, Espainia) 20 minutuz erabiliz. Mozketak

gau osoan zehar inkubatu ziren inkubazio soluzioan (1. eranskina), giro-tenperaturan eta aluminioarekin estalita. Hurrengo egunean, 10 minutuko 2 garbiketa egin ziren Tris Maleato tanpoiarekin. Azkenik, ebaketak portetan jarri eta 48 orduz ilpunpean deshidratatu ondoren, Xiloletan 2 orduz garbitu eta DPX-ekin (Erref: 06522, Sigma-Aldrich, Espainia) estali ziren (19. irudia).



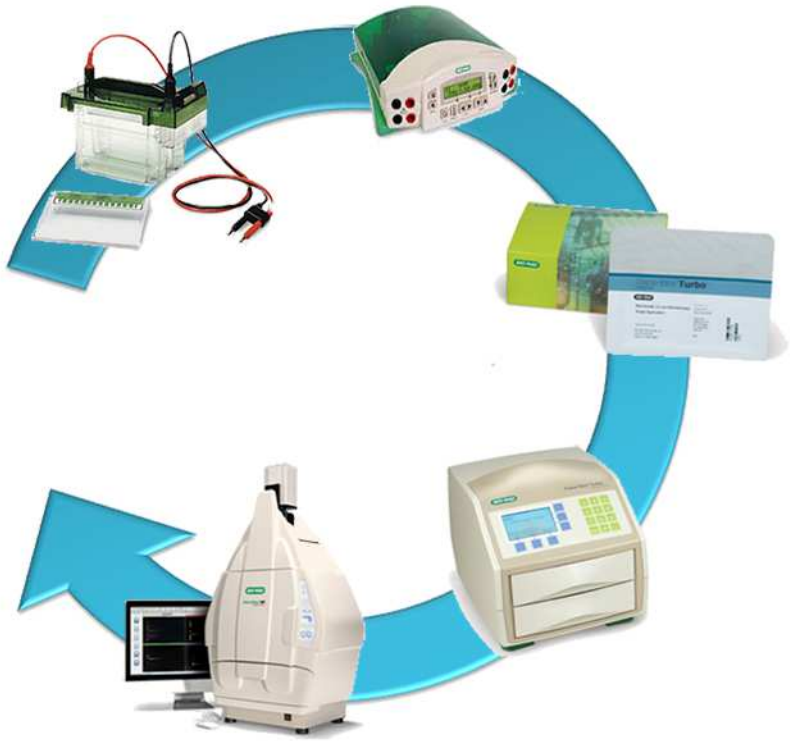
19. irudia. Butiril kolinesterasaren histokimiak kortexeko mikrobaskularizazioa ikusarazten du. (a) Baskularizazioaren adarkatzearen irudi orokorra. (b) Irudiaren erdialdean dagoen disektore esferikoa odol-hodien intersekzioak kontatzeko erabili da.

4.9. Western Blot

Proteina lisatuen prestaketa

Arratoiak anestesiatu ondoren, burua moztu zitzaien gillotina baten laguntzaz. Garunak garezurretik atera ziren kraniektomia bidez eta ondoren bisturi eta lupa baten laguntzaz, bi hemisferiotako kortex prefrontalak eta hipokanpoak moztu genituen. Hipokanpo osoa moztu eta gorde arren, hipokanpo dortsala baino ez dugu erabili Western Blot analisietarako. Kortex prefrontala ebakitzeko orduan, alde orbitalak kendu egin ziren. Ehuna polipropilenozko mikrotutuetan jarri eta hauek segidan -80ºC-tako izozkailuan gorde ziren. Ehuna homogeneizatzeko, gutxi gora behera 60 µl lisi tanpoi erabili ziren ehun miligramo bakoitzeko. Lisi tanpoi mililitro bakoitzari 15 µl proteasen inhibitzaile (Erref: P3840, Sigma-Aldrich, Espainia) gehitu zitzaion. Behin ehuna homogeneizatu eta gero, lisatua izotzetan mantendu zen 30 minutuz eta ondoren

zentrifugatu egin zen 15 minutuz 13000 b/min abiaduran 4°C-tan. Gainjalkina jaso eta mikrotutu berrietan gorde zen.



20. irudia. Western Bloteta egiteko erabilitako materialak.

Bradford entsegua proteinen kuantifikaziorako

Gainjalkin bakoitzean dagoen proteinen kontzentrazioa ezagutzeko, Bradford proteinen entsegua burutu zen. Horretarako, ur distilatua eta proteina laginak mikrotutu desberdinetara pipeteatu ziren, 2. taulan adierazten den bezala. Ondoren, koloratzaile erreaktiboa gehitu zitzaien laginei (Bio-Rad proteinen entsegua, Erref: 5000006, Bio-Rad, Hercules, CA, EEBB) eta 5 minutuz giro-tenperaturan mantendu ziren. Mikrotutu bakoitzeko edukia mikroplakako 3 putzutan banandu zen (250µl putzu bakoitzean) eta lagin ezagun eta ez-ezagunen absorbantzia mailak neurtu ziren fluorimetroan (Erref: Sinergy HT, Biotek Instruments, Inc., UK) ordubete igaro aurretik.

Laginak prestatzea eta gela korritzea

Lagin bakoitzetik beharrezkoa zen proteinen kantitatea jakiteko Bradford entseguaz baliatu ginen. Laginak 5µl 4x lagin tanpoiarekin nahastu eta ur distilatua gehitu zitzaien 20 µl-ko azken bolumena lortu arte. Laginak 10 minutuz irakin ziren eta zentrifugazio motz baten ondoren (30s), lagin kantitate berdinak kargatu ziren poliakrilamidazko geletan (Criterion™ TGX™ Precast Gels, Bio-Rad, Espainia). Laginekin batera, kaleetako baten, pisu molekularraren markatzailea (Fisher BioReagents™ EZ-Run™ Prestained Rec Protein Ladder, Fisher Scientific, Espainia) jarri zen. Gelak 200V-tan korritu ziren. Pisu molekular handiko proteinen (>150KDa) detekzioarako, 45 µg proteina kargatu genituen %4-15 geletan. Aldiz, 150 KDa baino txikiagoak diren proteinen detekzioareko 10-20 µg kargatu ziren %12ko geletan.

2. taula. Ezagunak eta ez-ezagunak diren proteinen kontzentrazioak zehazteko beharrezkoak diren lagin kantitateak.

	H_2O_d	Koloratzaile erreaktiboa	BSA
Hutsa	800 µl	200 µl	--
4	796 µl	200 µl	4 µl
8	792 µl	200 µl	8 µl
12	788 µl	200 µl	12 µl
16	784 µl	200 µl	16 µl
20	780 µl	200 µl	20 µl
Lagina	800 µl	200 µl	Laginaren µl 1

Proteinen transferentzia geletik mintzera

Pisu molekular baxuko proteinen detekziorako (<150KDa) transferentzia erdi-lehorra egin zen Trans-Blot® Turbo™ Transfer System (Bio-Rad, Hercules, CA, EEBB) aparatu komertziala erabiliz. Zazpi minutuko transferentzia burutu zen, intentsitate konstantean (2.5A), 25V-ko tentsio maximo batekin eta PVDF mintzak erabiliz (Trans-

Blot® Turbo™ Midi PVDF Transfer Packs, Erref: 1704157, Bio-Rad, Hercules, CA, EEBB).

Transferentzia hezerako, nitrozelulosazko mintzak (Erref: 10600012, GE Healthcare, Espainia) erabili ziren eta hauek, filtro papera eta zuntzezko kuxinekin batera transferentzia tanpoian murgilduak izan ziren 15 minutuz. Proteinak tentsio konstantean (30V) transferitu ziren 16 orduz, 4ºC-tan. Tranferentzia mota hau 150KDa baino pisu molekular handiagoko proteinak detektatzeko erabili zen.

Markaketa antigorputzekin

Mintzak bi ordu t'erdiz inkubatu ziren hauts-esnetan lotura ez-espezifikoak blokeatzeko. Horretarako hauts-esnea TBST-tan diluitu zen %5ean. Antigorputz primarioan inkubazioa (3. taula) TBST + %5 BSA-tan egin zen gau osoan zehar 4ºC-tan. Hurrengo egunean, mintzak TBST tanpoiarekin 3 aldiz garbitu ziren eta antigorputz sekundarioan inkubatu ziren blokeo soluzioan eta %1 BSA erabiliz ordu betez giro-tenperaturan. Horren ondoren, 5 minutuko 3 garbiketa egin eta seinalea errebelatzeko kimioluminiszente bat erabili zen (Erref: 34076, SuperSignal® West Dura Extended Duration Substrate, Fisher Scientific, Espainia).

Datuen analisia

Errebelatutako mintzen argazkiak ChemiDoc™ XRS+ Imaging System (Bio-Rad, Hercules, CA, EEBB) eskanerraren bidez lortu ziren (20. Irudia). Argazki hauek Image Studio Digits 3.1 (LI-COR Biotechnology, Cambridge, UK) programan ireki ziren dentsitate optikoak kuantifikatzeko. Horretarako, laukizuzen bat marraztu eta bertako seinalearen intentsitatea neurtu zen. Goi eta beheko intentsitateen batezbestekoa erabili zen atzeko seinale gisa.

Banda bakoitzaren seinalea proteina bakar batekiko normalizatu zen, hala nola, aktina (lagin guztietan maila berean agertzen den proteina) edo proteina kantitate guztiarekiko. Azken kasu honetan, intereseko proteina detektatu eta gero (proteinen egitura fosforilatuak, batik bat), mintzak garbiketa tanpoian (Restore Western Blot stripping buffer, Erref: 21059, Thermo Fisher Scientific, Espainia) inkubatu ziren 15 minutuz, berriro antigorputz primarioan inkubatu ahal izateko. Proteinen ugaritasun erlatiboa lortzeko, banden dentsitate optikoak animali kontrolekiko normalizatu ziren.

Emaitza zehatzak ziurtatzeko, intereseko proteinak eta hauen kontrolak mintz berean detektatuak izan ziren, eta kontrolekiko normalizazioa mintz berberean zeuden banden artean burutu zen bakarrik. Datu guztiek, beraz, eramailea daramaten animaliekiko areagotze edo murrizte erlatiboak adierazten dute.

3. taula. Antigorputzen kontzentrazioak eta erreferentziak

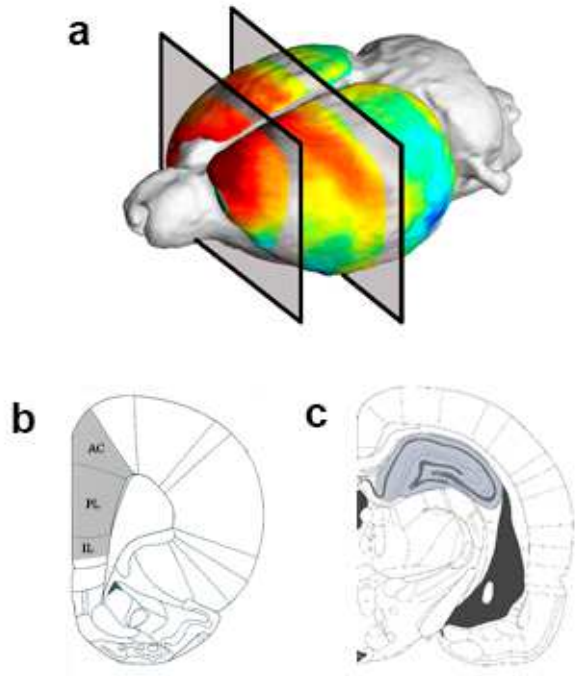
Antigorputz primarioak		
Sagu anti-NR2A	1:1000	Erref: MAB5216, Merck-Millipore, EEBB
Untxi anti-NR2B	1:1000	Erref: AB1557, Merck-Millipore, EEBB
Sagu anti-NR1	1:1000	Erref: 05432, Merck-Millipore, EEBB
Untxi anti-PSD95	1:1000	Erref: Af628, Frontier Institute, EEBB
Untxi anti-phospho-Akt (Ser 473)	1:1000	Erref: 9271, Cell Signaling Technology Inc, EEBB
Untxi anti-Akt	1:1000	Erref: 9272, Cell Signaling Technology Inc, EEBB
Untxi anti-phospho-p44/42 MAPK (Erk 1/2) (Thr 202/204)	1:1000	Erref: 9101, Cell Signaling Technology Inc, EEBB
Untxi anti-p44/42 MAPK (Erk 1/2)	1:1000	Erref: 9102, Cell Signaling Technology Inc, EEBB
Untxi anti-phospho-TrkB (Tyr 706)	1:250	Erref: sc-135645, Santa Cruz Biotechnology Inc, Espainia
Untxi anti-TrkB (794)	1:1000	Erref: sc-12, Santa Cruz Biotechnology Inc, Espainia
Untxi anti-BDNF	1:2000	Erref: NBP1-46750, Novus Biologicals, EEBB
Untxi anti-aktina	1:2000	Erref: A2066, Sigma-Aldrich, Espainia
Antigorputz sekundarioak		
Anti-untxi IgG HRP-konjugatua	1:20000	Erref: A-6154, Sigma-Aldrich, Espainia
Anti-sagu IgG HRP-konjugatua	1:20000	Erref: A-9044, Sigma-Aldrich, Espainia

Soluzioei eta tanpoiei buruzko informazio zehatzagoa 1. Eranskinean aurki daiteke.

4.10.Zelulen kuantifikazioa

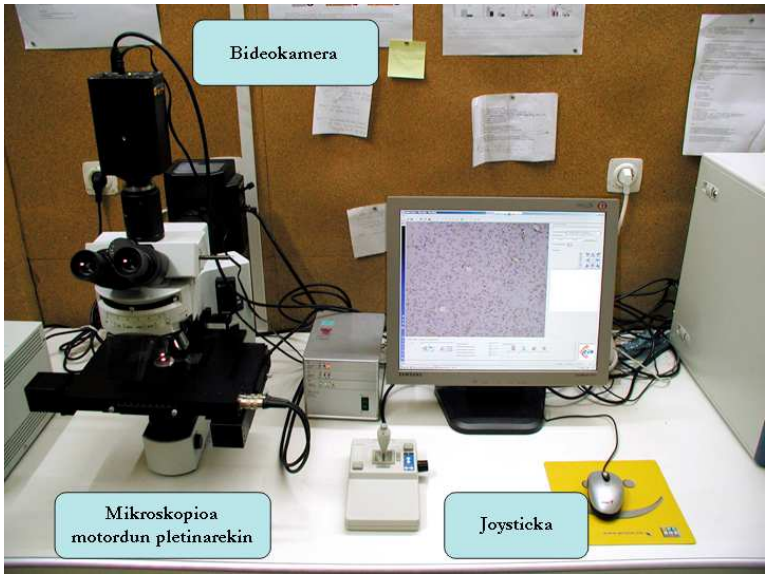
4.10.1. Estereologia ez-alboratua

Kortex prefrontal medialean (kortex prelinbikoa eta aurreko kortex zingulatua) eta hipokanpoan (hortz-jiroa eta cornus ammonis 1) zelula inmunoerreaktiboen estimazioak burutzeko estereologia inpartzialaz baliatu ginen. Kortex prefrontal medialaren laginketa Bregmatik 4.68 mm eta 1.92 mm bitartean egin zen. Bestalde, hipokanpoan Bregmatik -2.40 mm eta -5.76 mm bitartean egin zen (21. irudia). Kortex prefrontal medialaren eta hipokanpoaren mugak zehazteko, Paxinos eta Watsonen atlas estereotaxikoa erabili zen. Desberdintasun zitoarkitektonikoak ez zirenez erraz nabarmentzen, batez ere zati prelinbiko eta infralinbikoaren artean, beheko muga bezala aurreko burmuin azigo arteria ezarri genuen. Hori dela eta, lan honetan zati prelinbikoari buruz ari garenean, kontutan hartu behar da zati infralinbikoaren alderdi dortsalena ere nolabait barneratuta dagoela.



21. irudia. (a) Planoek arratoiaren garuneko intereseko eskualdeak adierazten dituzte. (b) Kortex prefrontal medialeko ebaketa koronalak. Grisez, intereseko eskualdeak. (c) Hipokanpoko ebaketa koronala. Grisez, intereseko eskualdea.

Mercator Irudien Analisi sistema (Explora-Nova, La Rochelle, Frantzia) erabili genuen kamera digital bat eta 3-ardatzetako plaka motorizatua atxikita duen Olympus BX51 mikroskopioarekin batera (22. irudia). Zelula inmunopositiboak 40x aldiz handiagotzen duen objektiboarekin zenbatu ziren Zatitzaile Optikoaren planteamendua erabiliz. Zatitzaile Metodoa, Zorizko Laginketa Sistematikoan (ZLS) oinarrituz, zelula zenbaki totala estimatzeko gai da interesezko eskualde konkretu batean. Horretarako, eremu osoa X, Y eta Z norabideetan distantzia uniformea duten kontaketa espazio birtualen multzo batez estaltzen da. Zelula kopuru totala zehazteko hurrengo formula hau erabiltzen da: $N=\sum Q \times 1/slz \times 1/elz \times 1/alz$, non Q-k espezimen bakoitzean kontatu diren zelula kopurua adierazten duen eta N-k estimatutako zelula kopurua. Ikerketa honetan erabilitako sekzio laginketa zatikia (slz) 1/8 da kortex prefrontalerako eta 1/10 hipokanporako.



22. irudia. Estereologia ez-alboraturako erabilitako estazio estereologikoa.

Intereseko eremuak 4x handiagotzeko objektiboarekin mugatu ziren. Lauki eta kontaketa markoaren (disektorea) tamaina desberdinak erabili ziren intereseko eremu bakoitzean (4. taula) eta %5eko zaintza zona bat aukeratu zen. Kontaketak estereologiaren kontaketa arauen arabera burutu ziren. Hipokanpoko interneuronen kantitatea zenbatesteko, elz (eremuaren laginketa zatikia) eta alz (alturaren laginketa zatikia)-ri 1

balorea ezarri genien. Orokorrean, animali bakoitzeko gutxi gora behera 7-8 sekzio kontatu ziren intereseko eremu bakoitzean. Bolumenen estimazioak Cavalieri-ren metodoa erabiliz lortu ziren.

4. taula. Zatitzaile Metodoan erabilitako elz baloreak neuronen zenbaki kopurua eta sare mikrobaskularraren luzera zenbatesteko.

	Eremua		Disektorearen tamaina(µm)	Disektoreen arteko tartea (µm)
NeuN	mPFC	PL, AC	25x25	350x350
	HPC	Geruza molekularra (HJ)	50x50	75x75
		Geruza granularra (HJ)	-	-
		Geruza polimorfikoa (HJ)	25x25	75x75
		CA1 (str.oriens eta str.lacunosum)	50x50	150x150
BCh	mPFC	PL, AC	r=50	200x200
	HPC	CA1, CA3, HJ	r=50	200x200
GAD67	mPFC	PL, AC	80x80	270x270
	HPC	CA1, CA3, HJ	80x80	270x270
IN (PB, KR, KB)	mPFC	PL, AC	80x80	220x220
SST	mPFC	PL, AC	80x80	270x270
	HPC	CA1, CA3, geruza polimorfikoa (HJ)	50x50	50x50

elz, eremuaren laginketa zatikia; mPFC, kortex prefrontal mediala; HPC, hipokanpoa; NeuN, neuronen nukleoaren markatzailea; BCh, butiril kolinesterasaren histokimia; IN, interneuronak; PB, parbalbumina; KR, kalretinina; KB, kalbindina; GAD67, azido glutamiko deskarboxilasa 67; SST, somatostatina; PL, kortex prelinbikoa; AC, aurreko kortex zingulatua; CA1, hipokanpoko cornus ammonis 1; CA3, hipokanpoko cornus ammonis 3; HJ, hipokanpoko hortz-jiroa; r, disektore esferikoaren erradioa.

Somatostatina adierazten duten interneuronak hortz-jiroko geruza polimorfikoan baino ez direnez agertzen, eskualde honetako geruza hau bakarrik kuantifikatu zen. Era berean, CA1 dortsaleko zelula parbalbumina- eta somatostatina-positiboen

estimaziorako, *stratum oriens* eta *stratum pyramidale* bakarrik hartu ziren aintzat. Are gehiago, CA1-eko geruza piramidala eta hortz-jiroko geruza granularra baztertuak izan ziren zelula kalbindina-positiboen estimazioetatik, bertan zelula kitzikatzaileek ere kalbidina adierazten dutelako. Halaber, kalbindinaren aurkako antigorputzak kortex prefrontaleko II/III. geruzetan dauden zelula piramidalak ere orbaintzen dituenez, geruza sakonak baino ez dira irudikatzen kalbindinaren estimazioetan.

Odol-hodien luzera zenbatesteko, Zatitzaile Metodoa erabili zen baina disektore optikoak espazio-baloiengatik (disektore esferikoak) aldatuz. Laginketa irizpideak 4. taulan azaltzen dira.

Kontaketa parametroak zehazteko errore koefizientearen (CE) bariantza osorako ekarpena %20a baino txikiagoa zela frogatu genuen atariko proban, hurrengo formula honen bidez: CE^2/CV^2, non CE^2 zenbateste estereologikoen bariantza den eta CV^2 bariantza biologikoa. Normalean, zatikia 0.5 baino txikiagoa denean zenbateste estereologikoak nahikoa zehatzak direla esan ohi da eta estimazioak ontzat ematen dira. Honen arabera, esan genezake gure laginketa prozedura zuzena izan dela.

4.10.2. Parbalbumina eta GAD67 ko-adierazten duten zelulen kuantifikazioa

Kasu honetan, kuantifikazioa mPFC-eko 6 animaliren 4 sekziotan egin zen eta hipokanpoan 3 animaliren 3 sekzio desberdinetan. Sekzioak 400 µm-tan banandu ziren mPFC-ean eta 500 µm-tan hipokanpoan. Irudiak 20x-ko objektiboa erabiliz atera ziren ApoTomea atxikita zuen Zeiss fluoreszentziako mikroskopian. Irudiak berreraiki ondoren, Fiji-ko zelula kontatzaile tresnarekin zenbatu ziren. mPFC-ean 150x150 µm-ko karratu bat erabili zen bai parbalumina bakarrik zein parbalbumina eta GAD67 ko-adierazten zuten interneuronak kontatzeko. Karratu horietako bat eremu linbikoaren parte dortsalean (area prelinbikoan, batik bat) zegoen zentratuta eta bestea aurreko kortex zingulatuaren erdian. Hipokanpoan, sekzio osoak erretratatu ziren eta fotomuntaien bidez berreraiki. Hipokanpoko CA1, CA3 eta HJ eskualde osoak mugatu ziren eta bertako PV eta PV-GAD67 ko-adierazten zuten interneurona guztiak zenbatu ziren.

Dentsitateak PV eta GAD67 adierazten duten soma kopurua mm^2-ko bezala ematen dira. Ostean, PV-GAD67 ratioak PV eta GAD67 adierazten duten soma kopurua zati PV+ interneurona zenbaki orokorra bezala azaltzen dira.

4.11.Analisi estatistikoa eta irudien prestaketa

Animalien pisuaren aldaketak eta "Eremu irekiko proba"-ko portaera 21., 24. eta 55. jaio ondorengo egunetan Student T-testaren bidez aztertu ziren normaltasunaren arabera banandutako datuen kasuan. Aldiz, datuak normaltasunaren arabera bananduta ez zeuden kasuetan, Mann-Whitney-ren U testa erabili zen. Datu bolumetrikoak eta PV-GAD67ren ko-adierazpeneko datuak bi bidetako bariantzaren analisia (ANOVA) erabiliz alderatu ziren, faktore nagusiak tratamendua (sodio kloruroa edo MK-801) eta hazkuntza baldintzak (estandarra edo ingurune aberastua) izanik. Neurketa errepikatuen ANOVA Morrisen ur labirintoko ikasketa fasea analizatzeko erabili zen. Gainontzeko portaera datu guztiak eta datu histologiko eta molekularrak bide bakarreko ANOVAren bidez aztertu ziren, estimazio estereologikoak eta Western Blotak barne. Neurketa errepikatuen ANOVAren bidez analizatu ziren datuetan, lehenik eta behin datuen normaltasuna eta esferikotasuna aztertu zen, Shapiro-Wilks-en eta Mauchly-ren proben bidez, hurrenez hurren. Proba hauek datuen normaltasuna berretsi zuten, baina datu batzuek esferizitatearen hipotesia hausten zutenez, Greenhouse-Geisser-en zuzenketa erabili zen F estatistika zuzentzeko eta aldaketa esanguratsuak aztertzeko. ANOVAren bidez analizatu ziren datuetan normaltasuna eta bariantzaren homogeneitatea aztertzeko, Shapiro-Wilk eta Levene-ren probak erabili ziren, hurrenez hurren. Bariantza berdineko datuak Bonferroni-ren Post Hoc probarekin aztertu ziren eta Levene-ren proba hausten zuten datuen kasuan, Tamhane-ren T2 proba erabili zen ANOVAren esangura aztertzeko. Interneurona desberdinen zenbakiak ataza kognitiboetako portaera aurresan dezakeen ikusteko, erregresio linealaren analisia erabili zen.

Konputazio guztiak SPSS softwarea (23.0 bertsioa, IBM, Espainia) erabiliz burutu ziren eta 0.05 baino baxuago ziren p baloreak esanguratsutzat hartu ziren. Emaitzak batezbesteko ± BEK (batezbesteko errore koadratikoa) bezala adierazten dira. Estereologiako analisiaren batezbesteko baloreak eta desbiderapen estandarrak 2. Eranskinean aurki daitezke.

Ehunen sekzioen argazkiak Olympus BX41 mikroskopioarekin atera ziren eta Adobe Photoshop 6.0-ren bidez prestatu. GraphPad Prism 4 softwarea (GraphPad, La Jolla, CA, EEBB) erabili zen grafika guztiak sortzeko.

5. Results

5.1. Body weight gain is affected by MK-801 during treatment period

It has been previously described that MK-801 administration decreases body weight gain in rodents *(Li et al., 2015)*. We therefore measured body weight between P10 and P20 on a daily basis and every two weeks thereafter. Student's T-test revealed significantly lower body weight gain during treatment period in MK-801-treated rats that was observed from P12 to treatment cessation (Figure 23), but no differences were found at P31 or later on.

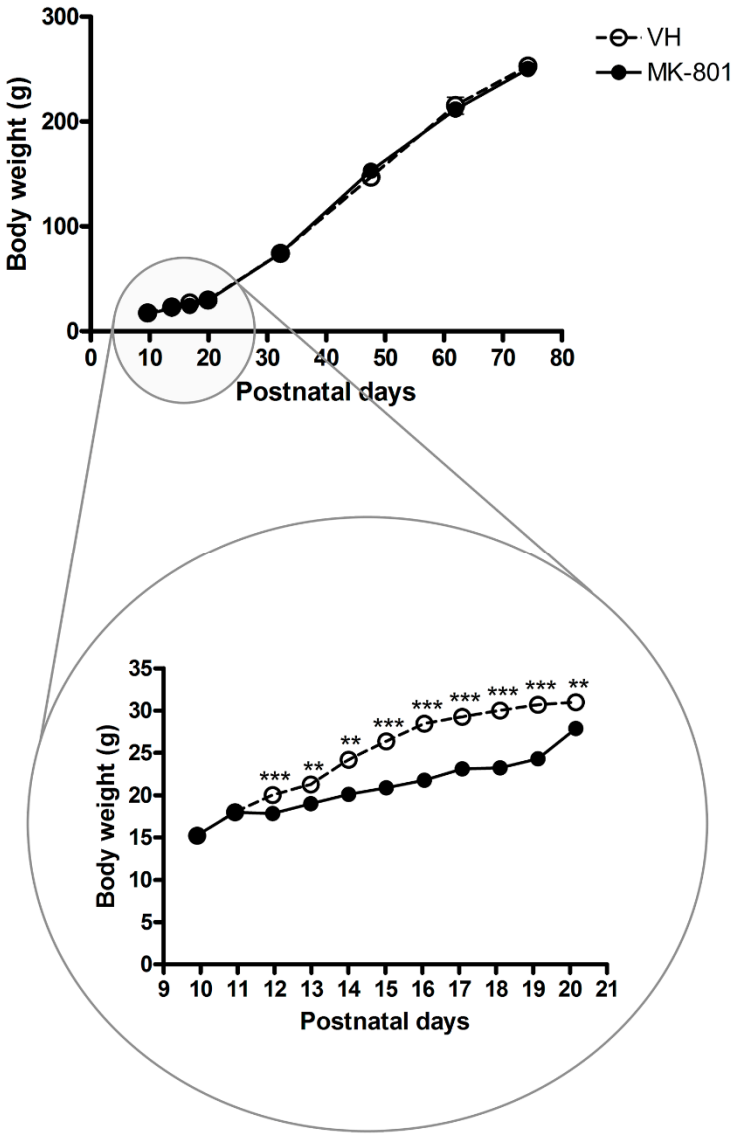


Figure 23. Effect of neonatal MK-801 administration on body weight gain across development. Data are represented as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001 (T-test; vehicle group vs. MK-801 group).

5.2. Locomotor activity in Open-Field Test

In the Open Field Test (OFT), the total distance traveled was significantly shorter in MK-801-treated rats than in saline controls 24-hours after last injection (Mann-Whitney U test, $p < 0.001$; Figure 24a). In addition, the relative distance moved in the center of the arena was also significantly shorter (Mann-Whitney U test, $p < 0.001$; Figure 24b). On P24, these differences were absent ($p=0.378$) and normal locomotor activity was preserved until P55 ($p=0.403$).

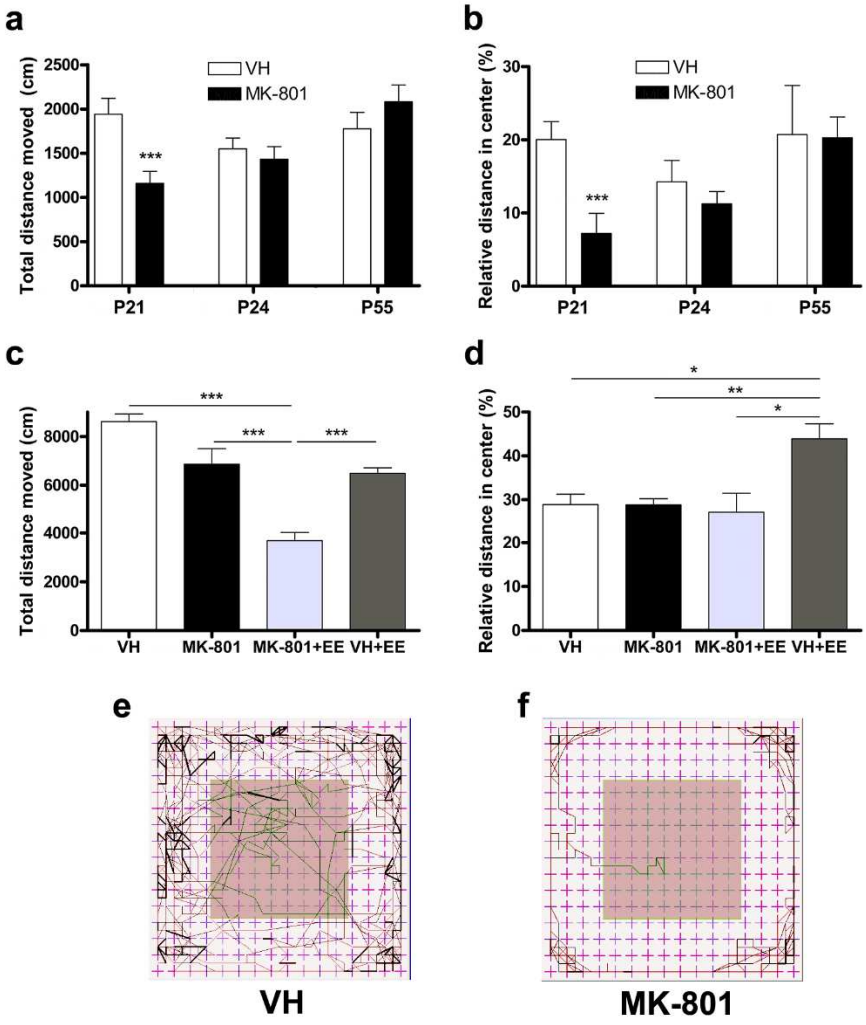


Figure 24. Effects of chronic MK-801 treatment on locomotor activity in the Open Field Test at (a,b) postnatal days (P) 21, P24 and P55 and (c,d) after exposure to enriched environment on P73. Data in (a,c) represent the total distance traveled whereas (b,d) show the percentage of distance moved in center vs. periphery. (e,f) Representative tracks of locomotor activity in Actitrack at P21 in (e) vehicle group and (f) MK-801 group. Data are plotted as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001.

After a short exposure to EE in early adulthood, rats exhibited significant changes in locomotor activity in terms of total distance moved ($F(3,35)=22.31$, $p<0.0001$) and the relative distance spent in the center ($F(3,34)=7.31$, $p<0.001$). Post Hoc analysis revealed that there was not any difference between standard environment raised animals in either parameter on P73. However, MK-801+EE showed significantly less activity than MK-801 group ($p<0.0001$; Figure 24c). Comparisons of relative distance in center showed that VH+EE traveled about one-third as much distance in center compared to the other 3 groups (Figure 24d). The tendency to avoid the periphery of the OFT is widely used to assess anxiolytic-like effects of treatments or interventions.

5.3. EE restores spatial learning and recognition memory impairment induced by MK-801

Morris Water Maze

To determine the presence of cognitive deficits after neonatal MK-801 treatment, and to evaluate the potential therapeutic effects of EE, we tested the animals in Morris Water Maze from P65 to P70. Repeated-measures ANOVA showed significant differences of performance during the acquisition phase in Morris Water Maze in terms of escape latencies ($F(3,42)=7.877$, $p<0.0001$, Figure 25a) and path length ($F(3,42)=7.942$, $p<0.0001$, Figure 25b). Post Hoc comparisons showed that MK-801-treated rats showed a significant increase in both time and distance to find the hidden platform when compared to VH (time $p<0.0001$; distance $p=0.01$) and VH+EE rats (time $p=0.005$; distance $p=0.001$) (Figure 25a and b), representing major impairment of spatial learning. This impairment in spatial learning of MK-801-treated rats allowed dissecting the role of EE in cognitive enhancement. Indeed, MK-801+EE rats showed normal spatial learning (MK-801+EE vs. VH - time and distance $p=1.00$; MK-801+EE vs. VH+EE - time $p=1.000$, distance $p=0.365$). Bonferroni Post Hoc test showed that MK-801+EE group spent less time to locate the platform compared to MK-801 rats in standard conditions ($p=0.004$), and traveled less distance ($p=0.044$), which strongly suggests improvement in spatial learning abilities.

All rats showed a significant improvement in the acquisition phase of Morris Water Maze. Escape latency and distance to the platform decreased over the 5 days of training ($F(4,39)=43.858$, $p<0.001$; ($F(4,39)=26.908$, $p<0.0001$), respectively), indicating that animals spent less time to reach the platform as the training proceeded. Representative traces of the swimming paths during last day of training are shown in Figure 25c.

After 5 days training, probe trial was performed with the platform removed to evaluate reference memory. The parameter "mean distance to target" (MDT) provided by SMART software was used, which is a measure of proximity and directionality to the location of the platform. Some studies have concluded that this parameter is consistently more sensitive at detecting group differences than more commonly used parameters, like percentage of time or number of crossing of target quadrant *(Gallagher et al., 1993; Maei et al., 2009).* Swimming speed was measured throughout the test, and significant difference was observed in the mean swimming speed between groups in probe trial ($F(3,39)=8.211$, $p<0.0001$). Nevertheless, MDT is minimally affected by changes in velocity. We found significant differences in reference memory between groups ($F(3,35)=11.135$, $p<0.0001$; Figure 25d). Bonferroni Post Hoc test showed that MK-801 treatment during neurodevelopment did not affect reference memory in the Morris Water Maze (VH vs. MK-801, $p=1.00$). However, rats that had experienced EE showed lower values of MDT, suggesting improved reference memory (EE groups vs. VH, $p<0.0001$; MK-801+EE vs. MK-801, $p=0.01$; VH+EE vs. MK-801, $p=0.007$; Figure 25d).

In the visible platform test there were no differences between groups in time ($F(3,39)=2.425$, $p=0.08$) or distance ($F(3,39)=1.324$, $p=0.28$), which indicates that the differences in performance in spatial learning acquisition may not be ascribed to differences in general sensorimotor skills (Figure 25a and b, "cue").

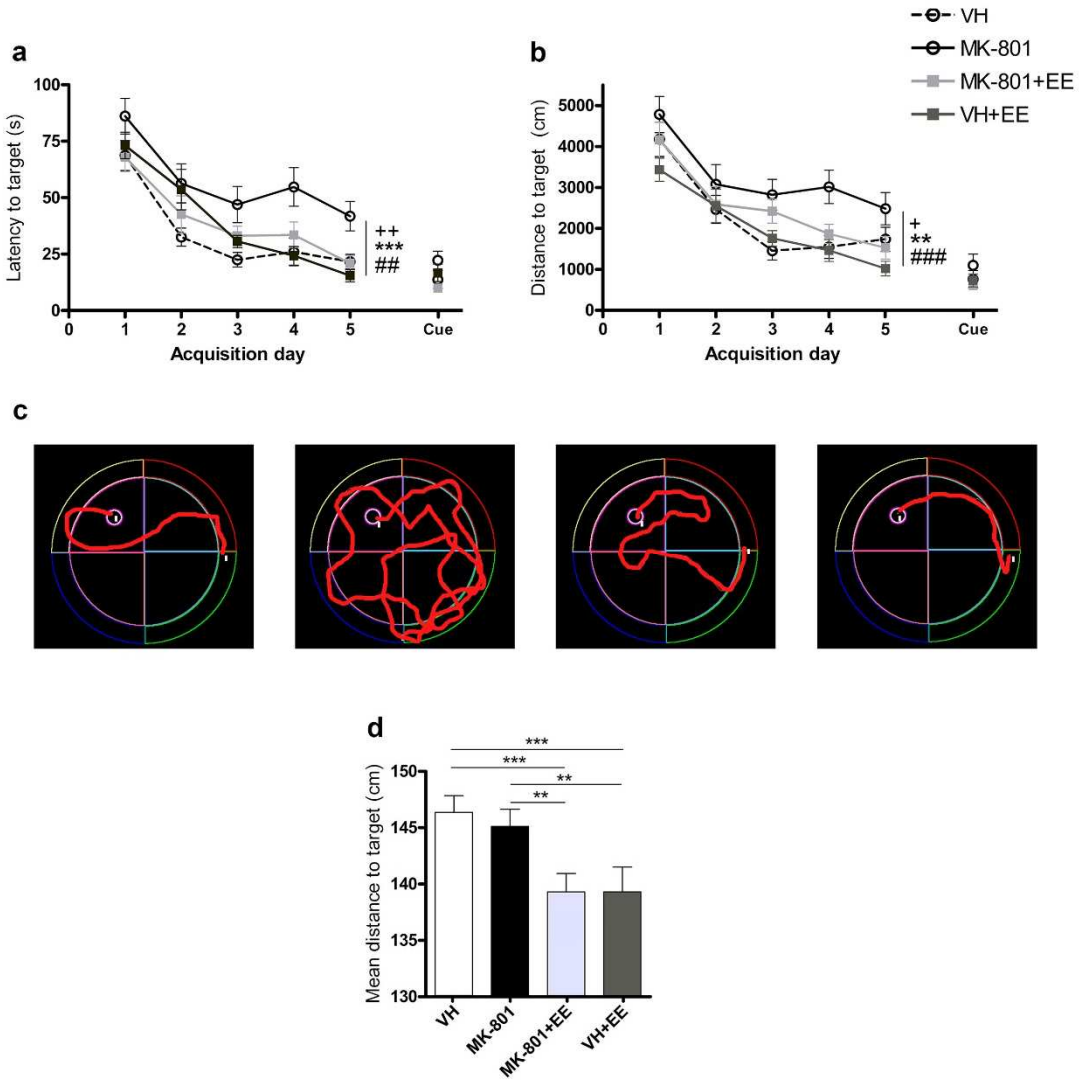


Figure 25. Spatial learning and memory in the Morris Water Maze. MK-801 animals were less efficient in finding the platform, as demonstrated by increased (a) average time and (b) distance during acquisition trials, and this was reverted by adult short-term exposure to EE. (c) Tracks are depicting schematic representation of swimming paths on Morris Water Maze on day 5 in each group. (d) Mean distance to target during Morris Water Maze probe trial showed no significant differences between standard condition animals, but enriched animals presented improved reference memory. Results are presented as mean ± SEM. In a and b, *significance MK-801 vs. VH (**$p < 0.01$; ***$p < 0.001$); $^{+}$significance MK-801 vs. MK-801+EE ($^{+}p < 0.05$; $^{++}p < 0.01$); $^{\#}$significance MK-801 vs. VH+EE ($^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$), using Repeated-Measures ANOVA. In d, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (one-way ANOVA).

Novel Object Recognition task

Novel Object Recognition (NOR) test is widely used to assess cognitive function in animal models. In the acquisition phase, the total exploration time was comparable between groups ($F(3,27)=1.930$, $p=0.149$). However, there was a significant difference in in the test trial ($F(3,26)=15.75$, $p<0.0001$; Figure 26). Post Hoc analysis showed that early MK-801 treatment led to cognitive dysfunction, as discrimination index was significantly lower in MK-801 group compared to saline controls ($p=0.04$).

MK-801+EE group presented normal recognition memory (vs. VH, $p=0.412$), and discrimination index was significantly augmented when compared to MK-801 group ($p=0.012$), representing major memory improvement. Rats in VH+EE group showed the greatest discrimination index, but not statistically different from MK-801+EE group (VH vs. VH+EE, $p=0.001$; MK-801 vs. VH+EE, $p<0.0001$; Figure 26).

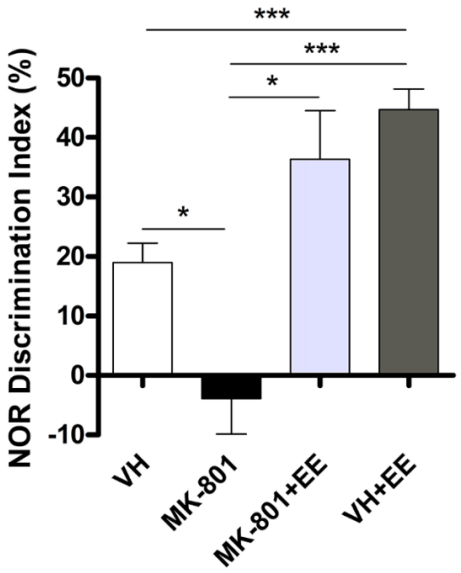


Figure 26. Discrimination Index in Novel Object Recognition task performed on P71. MK-801 group exhibited impaired object recognition memory that was reverted by EE. Data are represented as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001 (One-way ANOVA).

5.4. Associative recognition memory deficit is partially reversed by EE

Object-in-Place is a type of object-place paired-associative task that highly relies on the interplay between hippocampus and mPFC. During the sample trials in the Object-

in-Place tests, the interaction time with objects was comparable across groups ($F(3,37)=1.768$, $p=0.17$). In the test trial, one-way ANOVA showed differences between groups ($F(3,35)=7.016$, $p<0.0001$; Figure 27). Neonatal MK-801 exposure significantly reduced discrimination index (VH vs. MK-801, $p=0.047$), and enrichment produced a 3-fold increase in MK-801 animals, although it was not significantly different from MK-801 group. However, the average discrimination index of MK-801+EE group (15.2%) was not statistically different from the VH group either (22.02%, $p=1.00$), showing a partial recovery of associative memory. VH+EE showed the highest preference index (30.5%), doubling the index of MK-801+EE rats.

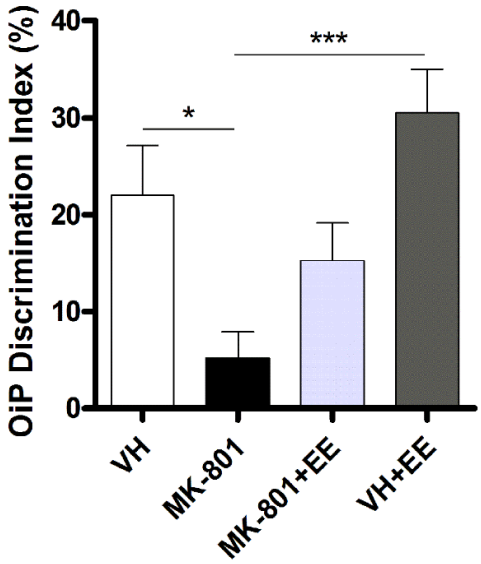


Figure 27. Graph shows the discrimination index in Object-in-Place (OiP) task performed on P72. MK-801 group exhibited impaired object recognition associative memory that was partially reverted by EE. Data are represented as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001 (One-way ANOVA).

Since we failed to find significant differences between MK-801 and MK-801+EE groups, we performed a two-way ANOVA analysis to unravel the effects of MK-801 and EE as independent variables. We found that both MK-801 ($F(1,35)=14.714$, $p=0.001$) and EE ($F(1,35)=4.921$, $p=0.033$) had a significant effect on discrimination index in Object-in-Place task.

5.5. Volume of medial prefrontal cortex and hippocampus

Medial prefrontal cortex (mPFC) and hippocampal volumes were measured in the same sections used for NeuN quantification. Two-way ANOVA showed a significant decrease in prelimbic ($F(1,20)=8.32$, $p=0.009$) and anterior cingulate ($F(1,20)=8.19$, $p=0.01$) volume associated with early life MK-801 administration (Figure 28). Similarly, the treatment significantly reduced hippocampal CA1 volume ($F(1,20)=9.08$, $p=0.007$), but not that of dentate gyrus ($F(1,20)=1.48$, $p=0.237$). Contrarily, EE significantly increased mPFC volume (prelimbic cortex $F(1,20)=7.02$, $p=0.015$; anterior cingulate cortex $F(1,20)=11.57$, $p=0.003$), and the volume of DG ($F(1,29)=4.99$, $p=0.04$). No significant changes were detected in CA1 of hippocampus caused by EE (Figure 28).

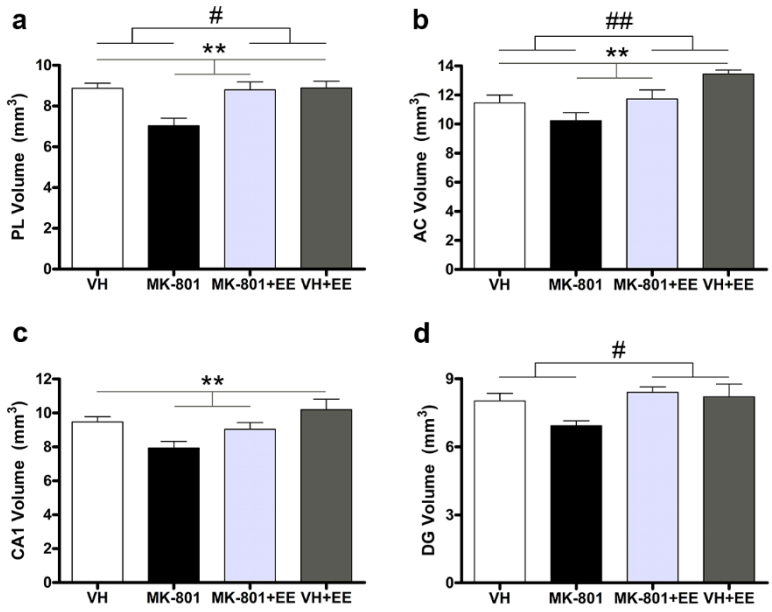


Figure 28. Estimates of mPFC and hippocampal volumes by Cavalieri's method. Histograms show the estimated volumes of the (a) PL, prelimbic cortex, (b) AC, anterior cingulate cortex, (c) CA1, cornus ammonis 1, and (d) DG, dentate gyrus of hippocampus in different experimental groups. Values represent mean ± SEM. *Significance of treatment (*$p<0.05$; **$p<0.01$), $^{\#}$significance of housing ($^{\#}p<0.05$; $^{\#\#}p<0.01$).

5.6. Vascular changes

Neurons, astrocytes, and blood vessels are organized in a functional unit, termed as "neurogliovascular unit", in which the vasculature can affect neuronal and glial activity and, in turn, dynamically adjust to their changes. Micro- and macrovascular abnormalities have direct implications on the pathophysiology of schizophrenia *(Katsel et al., 2017).* For assessing if MK-801 or EE induced vascular changes, endothelial cells of vessels were revealed by butyryl cholinesterase histochemistry, and total vessel length was estimated by stereological means in mPFC and hippocampus. We failed to find differences in mPFC, although one-way ANOVA was near significance ($F(3,20)=2.965$, $p=0.057$; Figure 29). However, we found significant changes in hippocampus ($F(3,19)=7.039$, $p=0.002$), mainly due to decreased microvascular length in MK-801-treated animals (MK-801 vs. VH, $p=0.031$; MK-801 vs. MK-801+EE, $p=0.013$; MK-801 vs. VH+EE, $p=0.003$).

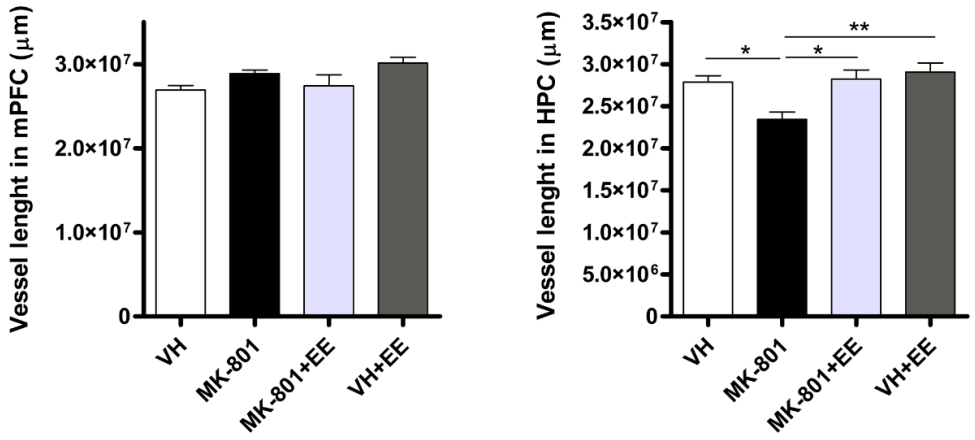


Figure 29. Estimated vessel length by stereology using Space Ball (spherical probe) in medial prefrontal cortex (left) and hippocampus (right). Post Hoc analysis showed that EE reverted MK-801-induced loss of vasculature in hippocampus. mPFC, medial prefrontal cortex; HPC, hippocampus. Graphs show mean ± SEM. *p<0.05; **p<0.01; ***p<0.001.

To further assess whether MK-801 and EE could have significant effects as independent variables, we performed two-way ANOVA in hippocampus and mPFC. Similar to the results obtained in one-way ANOVA, no effect of either factor was found in mPFC [MK-801 ($F(1,19)=0.006$, $p=0.937$); EE ($F(1,19)=0.023$, $p=0.88$)]. However, in hippocampus, we found that MK-801 had a significant effect ($F(1,19)=7.420$, $p=0.013$),

as well as EE (F(1,19)=9.394, p=0.006), but their interaction was not significant (F(1,19)=3.395, p=0.081).

5.7. Cell proliferation and survival in subgranular zone of dentate gyrus

It has been previously described that MK-801 administration in adult animals increases cell proliferation in the subgranular zone of dentate gyrus. This seems to result from dysregulated control of proliferation. EE has been shown to increase cell proliferation as well. We aimed to test if early life MK-801 administration would induce long-lasting increase in cellular proliferation, and how EE would affect in that case. For that, we injected BrdU at high doses (200mg/kg) 24 hours before transcardial perfusion. 200mg/kg is almost a saturating dose, which is recommended for quantification assays.

We found significant differences between groups (F(3,21)=3.882, p=0.029; Figure 30). Increased cell proliferation was observed in MK-801 and MK-801+EE groups compared to control animals (VH vs. MK-801, p=0.035; VH vs. MK-801+EE, p=0.043). Even though the mean density of BrdU+ cells in VH+EE was higher than in VH group, one-way ANOVA failed to find significant differences.

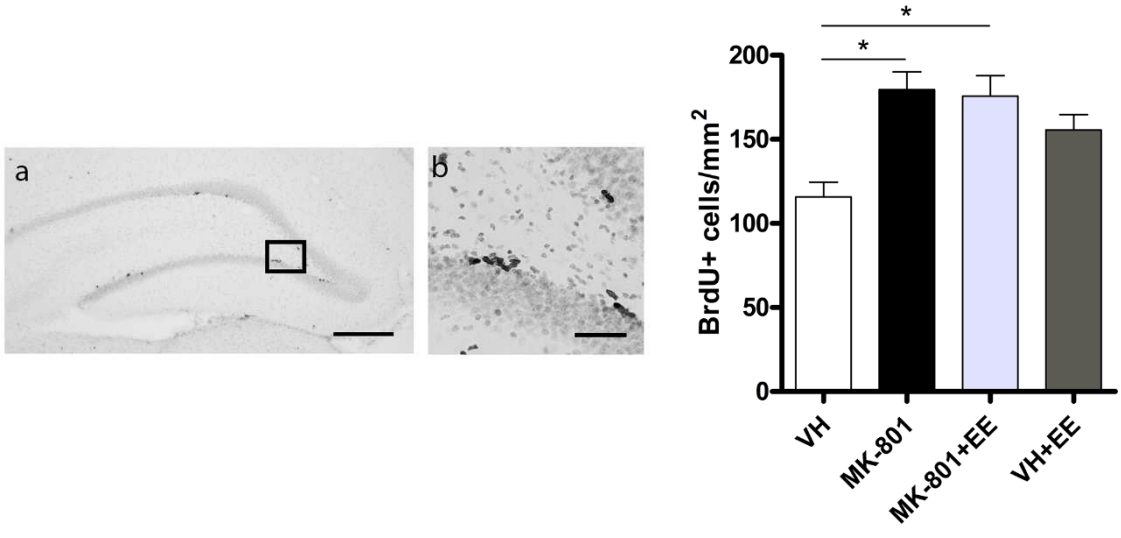


Figure 30. BrdU+ cells in subgranular zone of dentate gyrus in MK-801 animals using (a) 4X magnification and (b) 20X magnification. Histogram shows that the density of proliferative cells was significantly increased in MK-801-treated animals, independently of the housing condition. Data are represented as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001. Scale bar (a): 300 µm; Scale bar (b): 50 µm.

In adult hippocampal neurogenesis, immature neurons transiently express CR before differentiating into dentate granule cells and fully integrating in the hippocampal circuit. To establish differences in neuronal differentiation and survival, the number of CR-expressing cells was estimated in the granule cell layer of dentate gyrus. The total number of CR-expressing cells was increased in granule cell layer of VH+EE group when compared all other groups ($F(3,20)=4.97$, $p<0.01$; Figure 31).

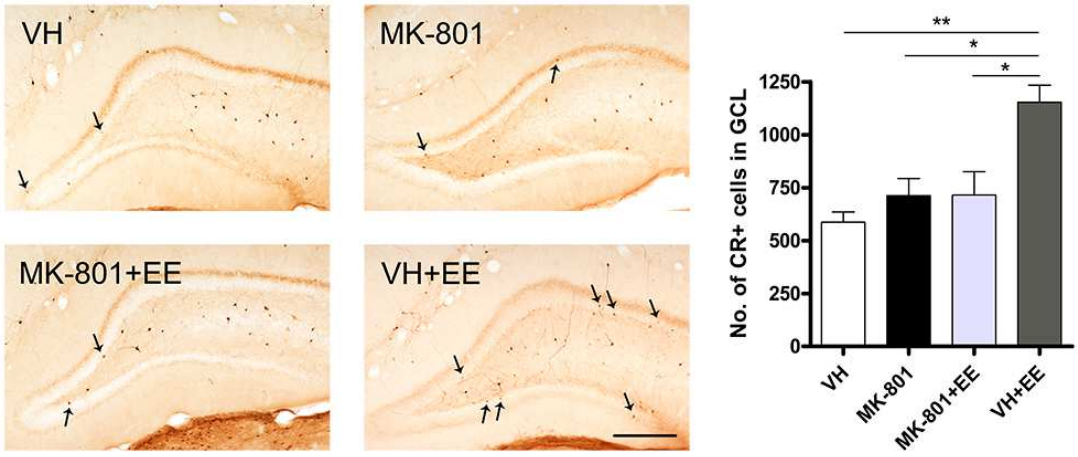


Figure 31. Images show the calretinin-positive (CR+) cells in the granular layer of dentate gyrus of different experimental groups indicated by arrows. Histogram shows the differences in the total number of calretinin-expressing cells between groups. GCL, granular cell layer of dentate gyrus. Data are represented as mean ± SEM. *p<0.05; **p<0.01. Scale bar: 300 µm.

5.8. Number of calcium-binding protein and somatostatin-expressing interneurons in hippocampus and medial prefrontal cortex

The total number of different populations of interneurons was estimated by unbiased stereology in prelimbic cortex and anterior cingulate cortex of mPFC, and in CA1 and dentate gyrus of hippocampus. Interneurons were identified upon their calcium-binding protein expression, i.e. parvalbumin (PV) (Figure 33 and 34), calretinin (CR) (Figure 35 and 36), and calbindin (CB), and upon their somatostatin expression (Figure 37 and 38).

One-way ANOVA showed that there was a significant difference in the number of PV and CR-expressing interneurons, but not in CB-expressing ones. PV+ differences

were present in all analyzed regions [prelimbic ($F(3,20)=11.77$, $p<0.0001$); anterior cingulate ($F(3,20)=11.66$, $p<0.0001$); CA1 ($F(3,20)=10.40$, $p<0.0001$); and DG ($F(3,20)=7.14$, $p=0.02$)]. Similar differences were found in CR-expressing interneurons [prelimbic ($F(3,20)=5.97$, $p=0.004$); anterior cingulate ($F(3,20)=14.13$, $p<0.0001$); CA1 ($F(3,20)=3.64$, $p=0.03$), except for DG ($F(3,20)=2.30$, $p=0.11$)].

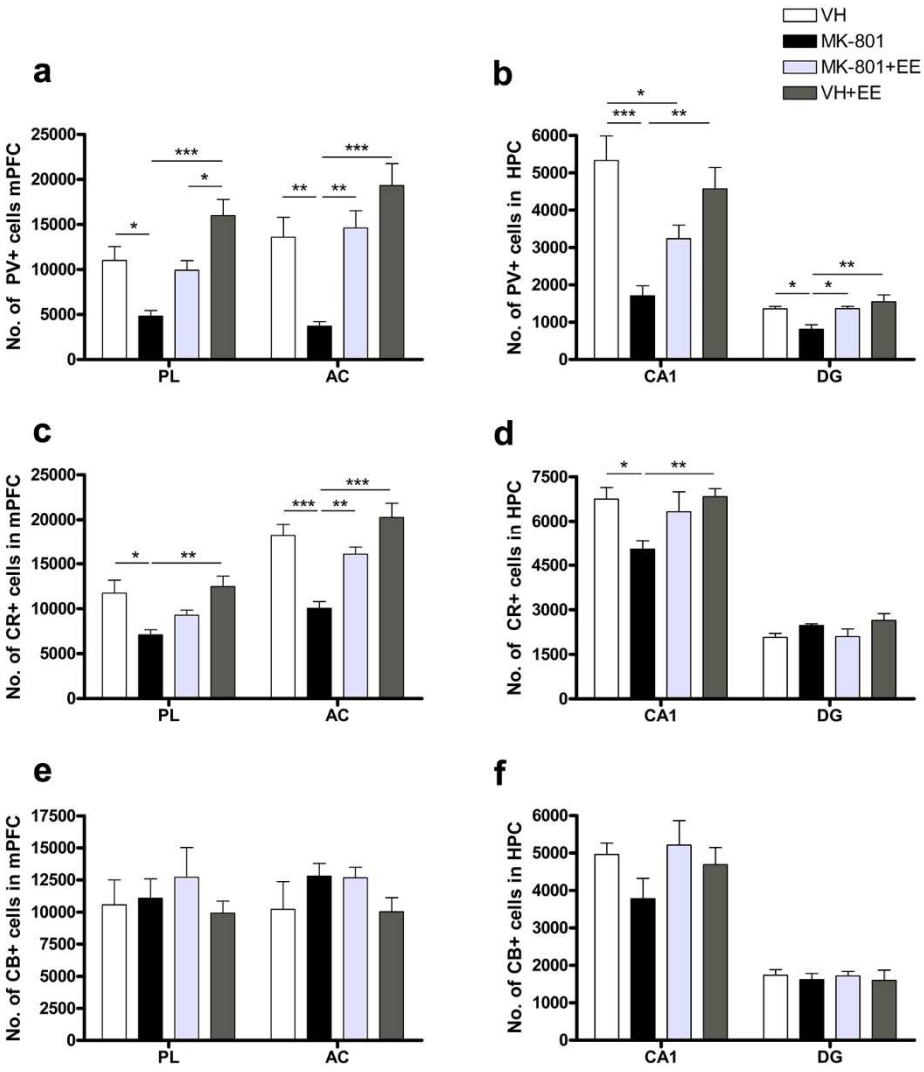


Figure 32. Effects of prenatal exposure to MK-801 and enriched environment on the number of different interneuron immunoreactivity in (a,c,e) medial prefrontal cortex (mPFC) and (b,d,f) hippocampus. Interneurons are identified by their calcium-binding protein expression: (a,b) parvalbumin, (c,d) calretinin, and (e,f) calbindin. PL, prelimbic cortex; AC, anterior cingulate cortex; CA1, cornus ammonis 1; DG, dentate gyrus of hippocampus. Data are represented as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001.

In mPFC, MK-801-treated rats showed a dramatic decrease in the number of PV+ cells in prelimbic ($p=0.022$) and anterior cingulate ($p=0.01$) cortices compared to vehicle group (Figure 32a and 33). Regarding the hippocampal PV+ cell estimates, a significant decrease was also observed in CA1 ($p<0.0001$) and DG ($p=0.024$) (Figure 32b and 34). These deficits in PV+ interneuron expression were partially recovered by a short exposure to EE, as the number of PV+ cells in MK-801+EE group was not statistically different from the control group in either region, except for CA1 ($p=0.042$). The number of PV+ interneurons in MK-801+EE was different from MK-801-treated rats without environmental intervention in anterior cingulate cortex ($p=0.004$) and DG ($p=0.023$), but not in prelimibic cortex ($p=0.081$) or CA1 ($p=0.244$). VH+EE group showed augmented mean PV+ interneurons in mPFC compared to all other groups (Figure 32a), but it was only significantly higher than MK-801+EE group in prelimbic region ($p=0.027$). Similar to vehicle group, VH+EE was significantly different from MK-801 group in all regions that were analyzed (prelimbic, $p<0.0001$; anterior cingulate, $p<0.0001$; CA1, $p=0.003$; DG, $p=0.002$) (Figure 32a and b).

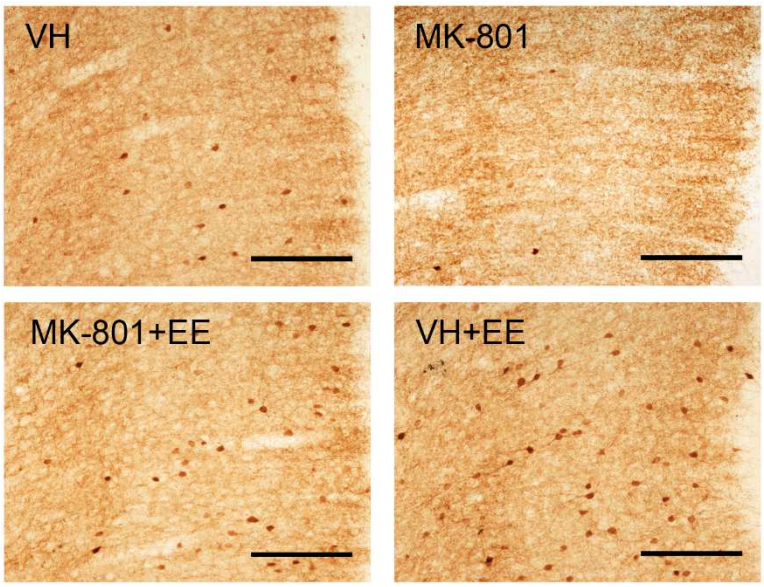


Figure 33. Parvalbumin-positive interneurons in prelimbic region of medial prefrontal cortex. Scale bar: 200 µm.

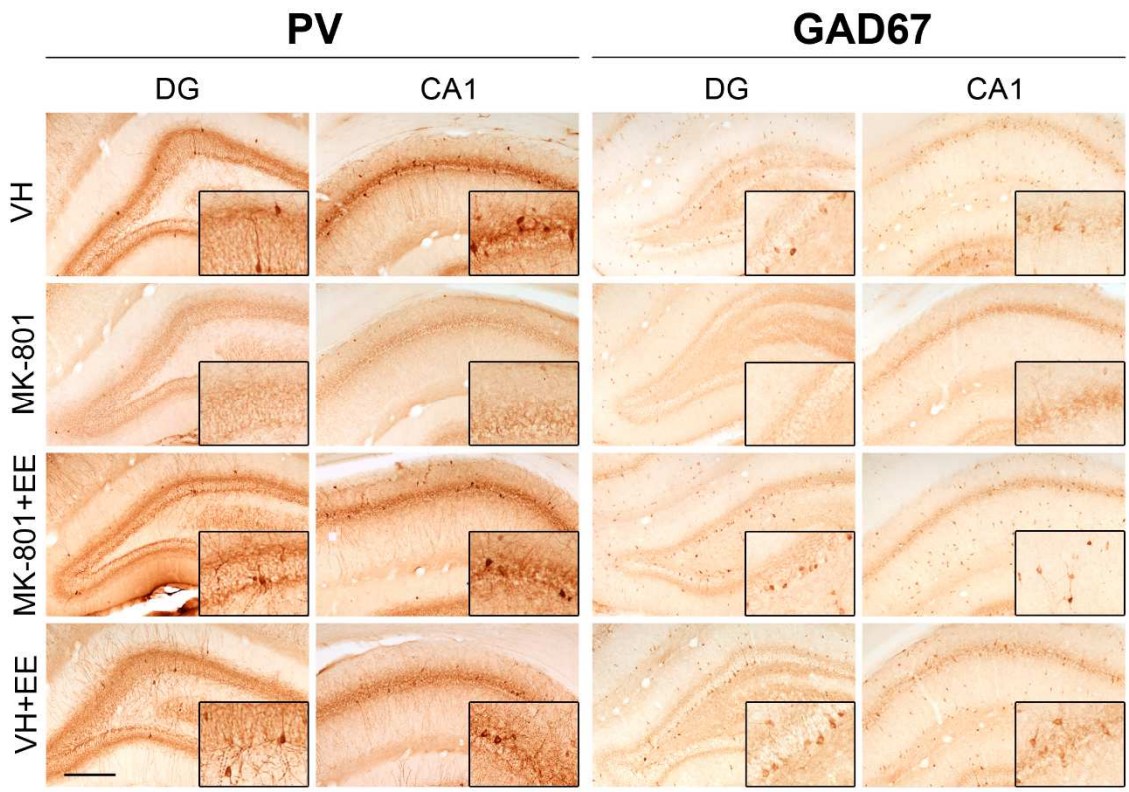


Figure 34. Representative images showing distinct expression of parvalbumin (PV) and glutamic acid decarboxylase-67 (GAD67) somata in hippocampal cornus ammonis 1 (CA1) and dentate gyrus (DG) of different experimental groups. Scale bar: 300 µm.

The decrease in CR-expressing interneurons in MK-801 group was less evident, still significantly different from vehicle animals in both regions of mPFC (prelimbic cortex, p=0.023; anterior cingulate cortex, p=0.001) and in CA1 of hippocampus (p=0.035) (Figure 32c, 32d and 35). Similar to what happened with PV+ interneurons, EE partially reversed the loss of CR-immunoreactivity (MK-801+EE vs. VH; prelimbic p=0.59; anterior cingulate, p=1.0; CA1, p=0.1) (Figure 32c, 32d and 36).

No significant differences were found in either region of mPFC or hippocampus of CB-expressing interneurons [prelimbic ($F(3,20)=0.87$, p=0.48), anterior cingulate ($F(3,20)=2.23$, p=0.12), CA1 ($F(3,20)=1.52$, p=0.24), and DG ($F(3,20)=0.14$, p=0.94); Figure 32e and f]. It is worth to mention that only deep layers of mPFC were considered for CB+ interneuron estimations to avoid confounding effects of CB-expressing pyramidal cells of LII/III.

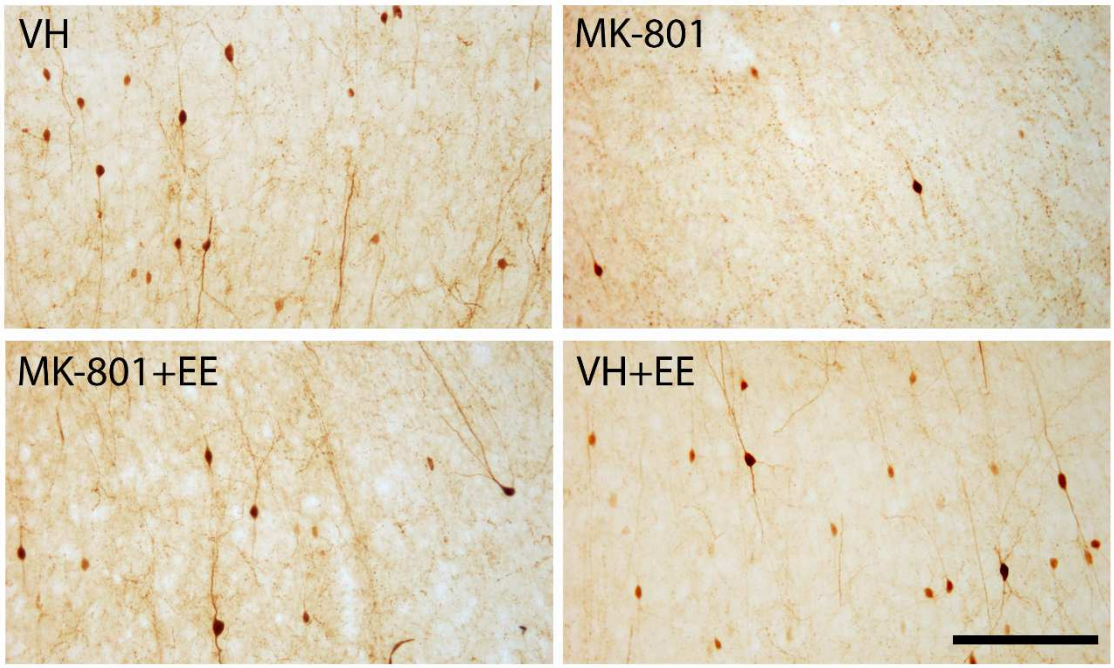


Figure 35. Calretinin-positive interneurons in prelimbic region of medial prefrontal cortex. Scale bar: 150 µm.

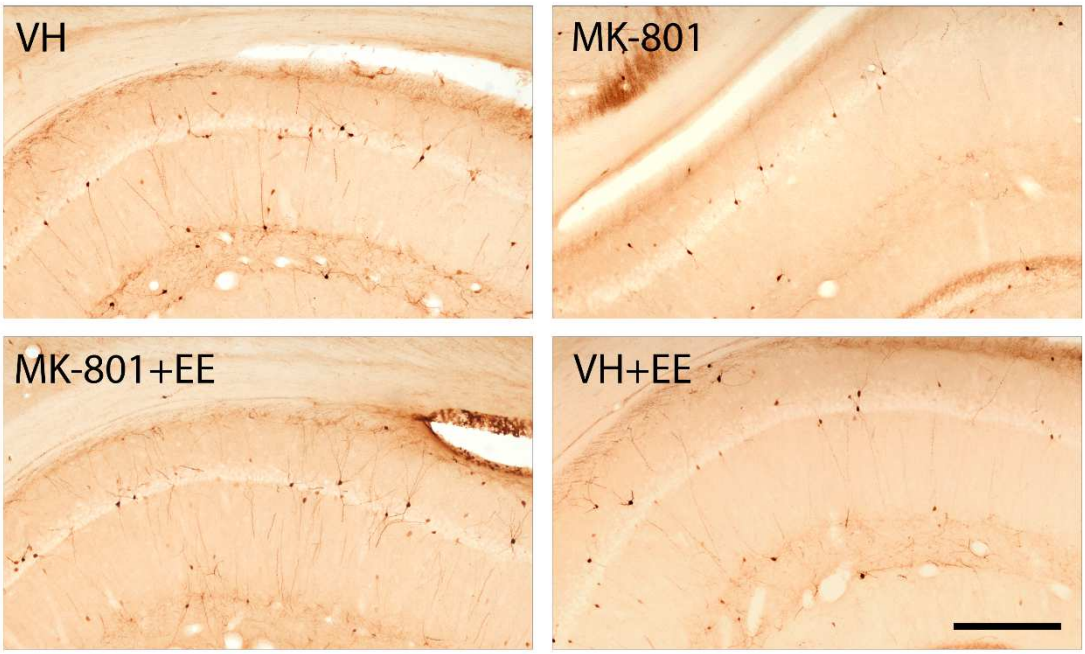


Figure 36. Calretinin-positive interneurons in cornus ammonis 1 (CA1) region of hippocampus. Scale bar: 150 µm.

Regarding the number of somatostatin (SST)-expressing interneurons in mPFC and HPC (Figure 37), we found statistically significant differences in all regions, except for dentate gyrus [prelimbic ($F(3,20)=7.355$, $p=0.002$); anterior cingulate ($F(3,20)=3.233$, $p=0.044$); CA1 ($F(3,20)=7.043$, $p=0.002$); CA3 ($F(3,20)=11.016$, $p<0.0001$); DG ($F(3,20)=2.405$, $p=0.098$); Figure 38].

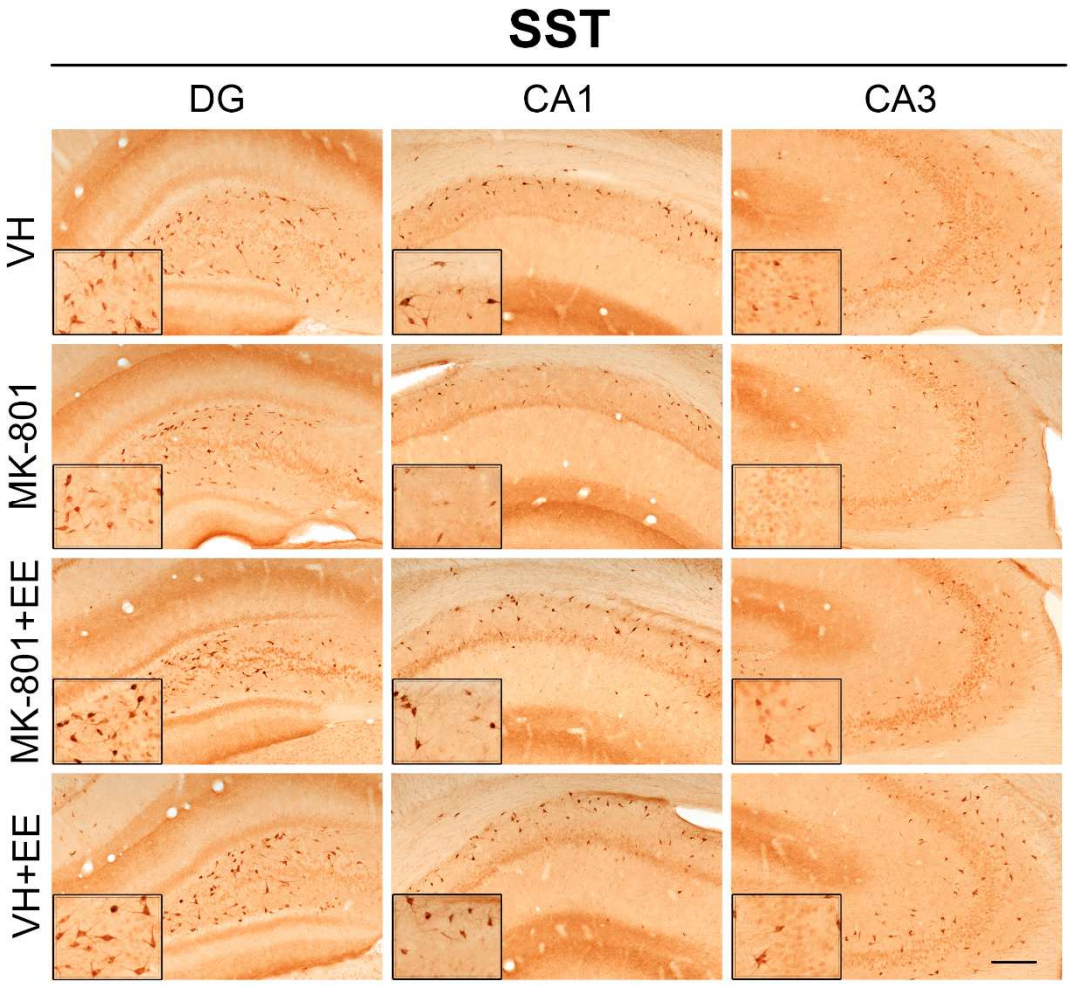


Figure 37. Representative images showing differences in somatostatin-expressing interneurons in distinct hippocampal sections. DG, dentate gyrus of hippocampus; CA1, cornus ammonis 1; CA3, cornus ammonis 3. Scale bar: 300 µm.

MK-801 treatment during neurodevelopment decreased the number of SST+ interneurons in prelimbic region ($p=0.004$), CA1 ($p=0.002$), and CA3 ($p=0.001$) compared to VH group. EE was effective in increasing the number of SST+ interneurons in MK-801+EE group in the affected three regions, but those changes only reached statistical significance in CA3 ($p=0.001$) when compared to MK-801 group. In the remaining two regions, i.e. prelimbic cortex and CA1, EE partially reverted SST-

expression deficits. Contrarily, environmental enrichment did not alter the number of SST+ interneurons in any region of VH+EE animals compared to VH, suggesting that EE only promoted SST-expression in MK-801+EE group.

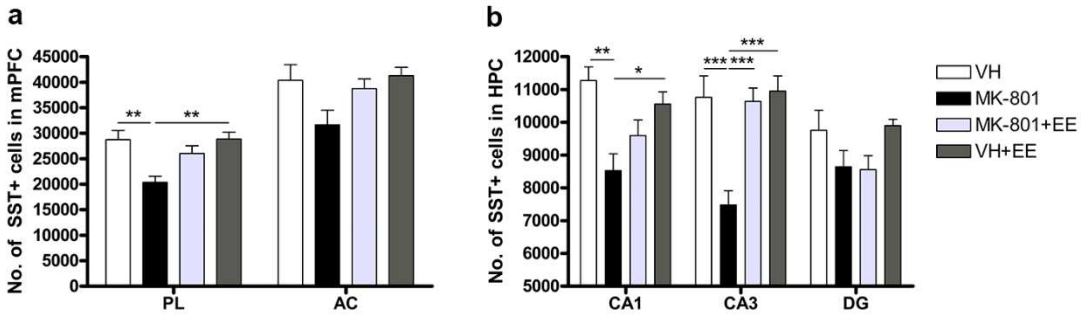


Figure 38. Effects of postnatal exposure to MK-801 and enriched environment on the number of interneurons immunoreactive to somatostatin in (a) medial prefrontal cortex (mPFC) and (b) hippocampus. PL, prelimbic cortex; AC, anterior cingulate cortex; CA1, cornus ammonis 1; CA3, cornus ammonis 3; DG, dentate gyrus of hippocampus. Data are represented as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001.

5.9. Lack of hippocampal inhibition in MK-801-treated rats without overall cell loss

One-way ANOVA on NeuN immunoreactivity showed no differences between groups in the total number of cells in mPFC and hippocampus [NeuN: prelimbic ($F(3,20)=2.35$, $p=0.10$), anterior cingulate ($F(3,20)=0.38$, $p=0.77$), CA1 ($F(3,20)=0.13$, $p=0.94$), and DG ($F(3,20)=1.63$, $p=0.21$); Figure 39a and b]. Contrarily, the number of GAD67-expressing GABAergic interneurons was significantly changed in all regions of mPFC [prelimbic cortex ($F(3,20)=4.94$, $p=0.01$); anterior cingulate cortex ($F(3,20)=5.88$, $p<0.005$)] and hippocampus [CA1 ($F(3,17)=28.16$, $p<0.0001$); DG ($F(3,19)=6.96$, $p=0.002$); Figure 39c and d)].

In prelimbic cortex, the number of GAD67 immunoreactive cells in VH+EE group was significantly increased compared to MK-801 ($p=0.015$) and MK-801+EE ($p=0.033$) groups. In anterior cingulate cortex, VH+EE only differed from MK-801 group ($p=0.015$). In hippocampal CA1, a relevant reduction of GAD67 could be found between

vehicle and MK-801 rats ($p<0.0001$) that was recovered by EE (MK-801 vs. MK-801+EE, $p=0.006$) (Figure 34). VH+EE group presented the greatest number of GAD67+ cells in CA1 (VH vs. VH+EE, $p=0.036$; MK-801 vs. VH+EE, $p<0.0001$; MK-801+EE vs. VH+EE, $p=0.001$). Only the animals exposed to EE (MK-801+EE and VH+EE) presented significantly increased GAD67 immunoreactivity in DG compared to MK-801-treated rats (MK-801 vs. MK-801+EE, $p=0.001$; MK-801 vs. VH+EE, $p=0.02$) (Figure 39).

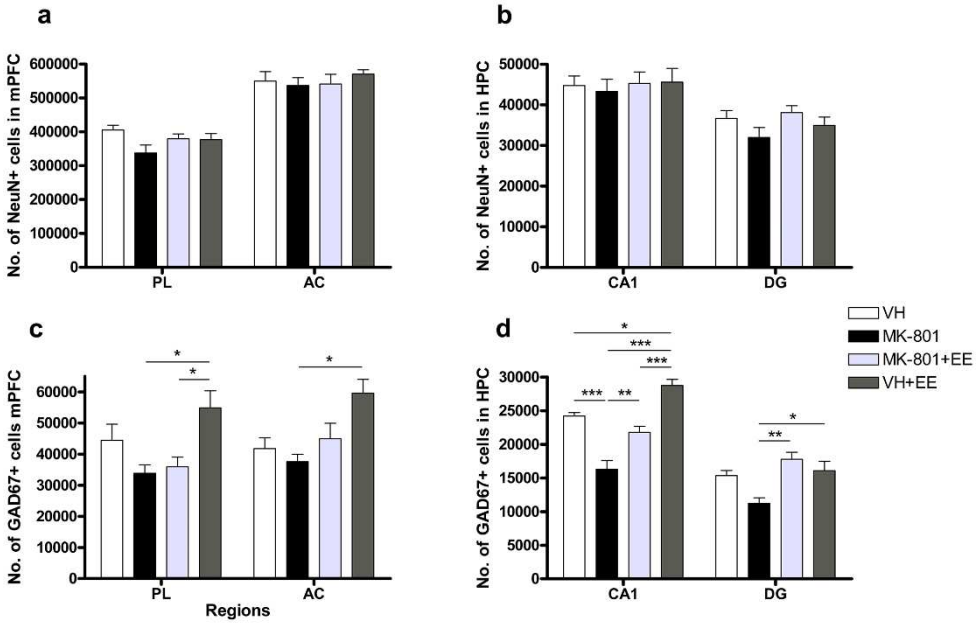


Figure 39. Number of (a,b) NeuN and (c,d) glutamic acid decarboxylase 67 (GAD67) immunoreactive cells in different regions of medial prefrontal cortex (mPFC) and hippocampus. PL, prelimbic cortex; AC, anterior cingulate cortex; CA1, cornus ammonis 1; DG, dentate gyrus of hippocampus. Graphs show mean ± SEM. *p<0.05; **p<0.01; ***p<0.001.

5.10. EE increases the number of PV+ interneurons co-expressing GAD67

Based on stereological counting of PV and GAD67-expression separately, we found no differences in GAD67-immunoreactivity in mPFC between VH and MK-801 groups. To further explore the possibility of a selective downregulation of GAD67 in specific interneurons populations, we labeled PV+ interneurons that co-expressed GAD67. Two-

way ANOVA revealed that there was no interaction between MK-801 and EE conditions. MK-801 treatment decreased the density of PV and GAD67 co-expression that was significant in prelimbic region ($F(1,20)=14.172$, $p=0.001$), CA1 ($F(1,8)=17.8$, $p=0.003$) and CA3 ($F(1,8)=13.167$, $p=0.007$) (Figure 40a). In anterior cingulate cortex, MK-801 induced a decrease in GAD67-expression in PV+ interneurons was near significance ($F(1,20)=4.013$, $p=0.059$). 18 days of adult EE were sufficient to invariably increase the density of PV interneurons co-expressing GAD67 in all regions [prelimbic ($F(1,20)=11.942$, $p=0.002$); anterior cingulate ($F(1,20)=4.568$, $p=0.045$); CA1 ($F(1,8)=8.586$, $p=0.019$); CA3 ($F(1,8)=15.119$, $p=0.005$); DG ($F(1,8)=7.355$), $p=0.027$)] (Figure 40b).

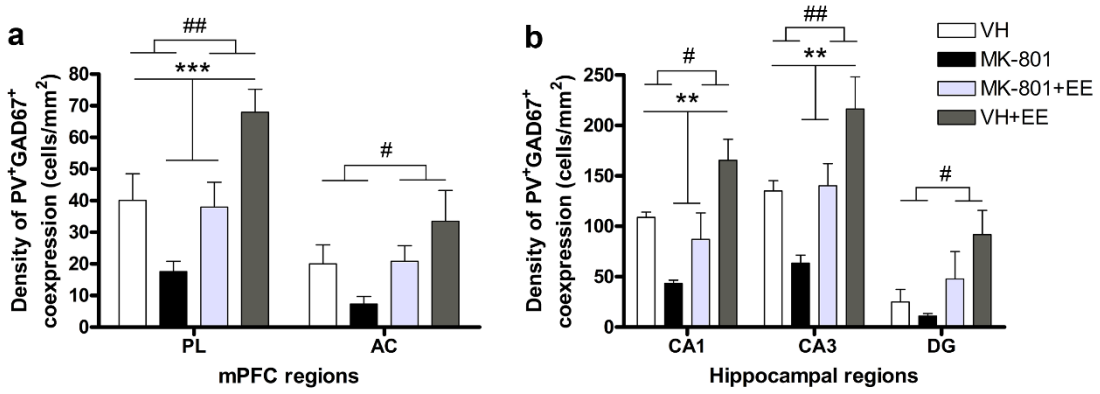


Figure 40. Density of PV and GAD67 coexpressing interneurons in (a) mPFC and (b) dorsal hippocampus expressed as number of somata per mm^2. PL, prelimbic cortex; AC, anterior cingulate cortex; CA1, cornus ammonis 1; CA3, cornus ammonis 3; DG, dentate gyrus of hippocampus. Graphs show mean ± SEM. *significance of treatment (**$p<0.01$; ***$p<0.001$); #significance of housing (#$p<0.05$; ##$p<0.01$).

However, decreased PV and GAD67 co-expression densities could be partially accounted for MK-801-induced PV+ interneuron deficiency. In an attempt to avoid this bias, we further analyze the data, and looked for differences in the relative number as a function of cells co-expressing PV and GAD67 divided by the total number of PV+ interneurons in the selected region (PV and GAD67 co-expression / total number of PV+ interneurons). Two-way ANOVA revealed that in mPFC, EE promoted GAD67

expression in PV+ interneurons [PL (F(1,23)=31.953, p<0.0001) and AC (F(1,23)=10.283, p=0.004)], but MK-801 had no effect [PL (F(1,23)=0.13, p=0.91) and AC (F(1,23)=0.597, p=0.449)]. In hippocampus, MK-801 decreased GAD67 content in PV+ interneurons in CA1 region (F(1,11)=7.121, p=0.028) and in dentate gyrus (F(1,11)=6.289, p=0.036), and was near significance in CA3 (F(1,11)=4.543, p=0.066). On the other hand, EE significantly increased it in CA1 (F(1,11)=13.249, p=0.007) and CA3 (F(1,11)=9.651, p=0.015), and almost in dentate gyrus (F(1,11)=4.52, p=0.066). There was no interaction between MK-801 and EE in any region (Figure 41).

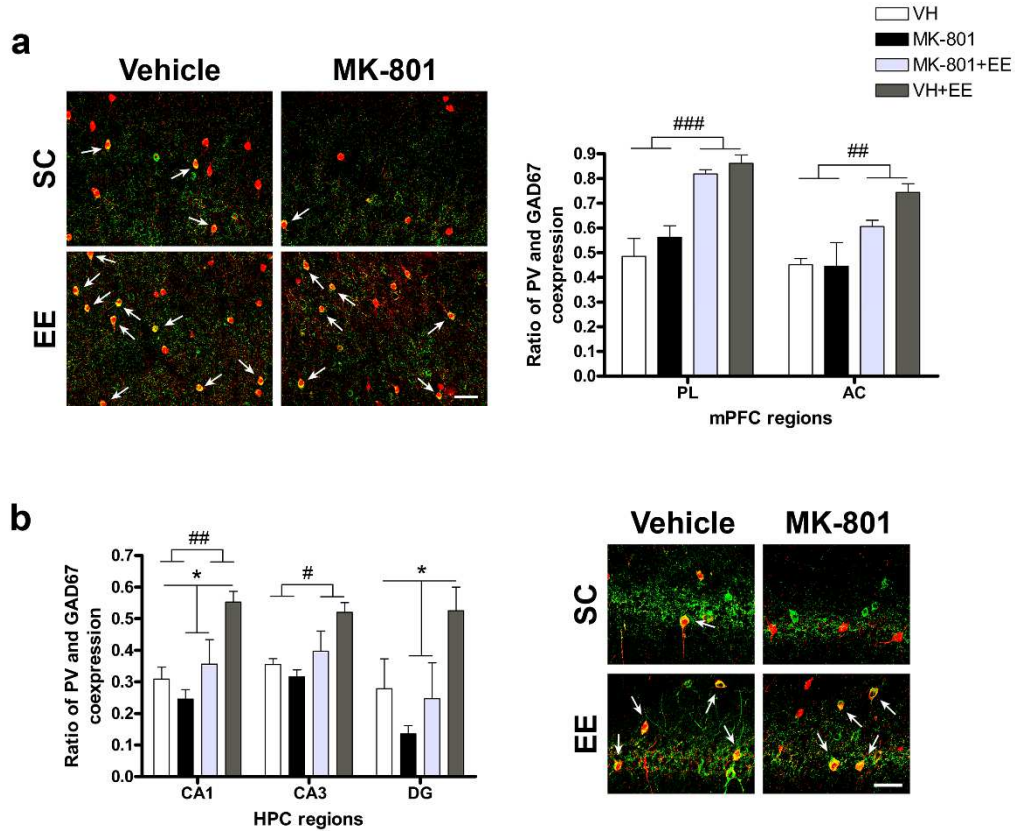


Figure 41. Representative images of PV (red staining) and GAD67 (green staining) co-expressing (yellow) interneurons in (a) medial prefrontal cortex (mPFC) and (b) dorsal hippocampus, indicated by arrows. (a) Histograms show increased relative co-expression of PV and GAD67 in mPFC in rats housed in EE during adulthood. (b) Perinatal MK-801 administration disturbs GAD67 expression in PV+ interneurons in DG and CA1 regions of hippocampus. SC, standard conditions; EE, enriched environment; mPFC, medial prefrontal cortex; HPC, hippocampus; PV, parvalbumin; GAD67, glutamic-acid decarboxylase 67; PL, prelimbic region; AC, anterior cingulate; CA1, cornus ammonis 1; CA3, cornus ammonis 3; DG, dentate gyrus. *significance of treatment (*$p < 0.05$), #significance of housing (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$). Scale bar: 50 µm.

5.11. Relationship between cognitive function and number of interneurons

The observed alterations of PV+ and SST+ cells in mPFC and hippocampus, as well as the differences in GAD67 expression in hippocampus could affect on cognitive tests. Pearson's correlation analysis revealed that the number of PV+ and SST+ interneurons tended to be positively correlated with the performance in Novel Object Recognition and in Object-in-Place tasks. This correlation was from moderate to high, but we failed to find significant linear correlation between interneuron markers and spatial memory in Morris Water Maze. Table 5 shows the Pearson's correlation coefficients (r) and significance (p).

Table 5. Significance of correlations between cognitive tasks and the number of interneurons.

		NOR		OiP		Spatial memory	
		r	*p*	*r*	*p*	*r*	*p*
PV	PL	0,515**	0,010	0,622***	0,001	-0,249	0,241
	AC	0,641***	0,001	0,612***	0,001	-0,153	0,475
	CA1	0,418*	0,042	0,401	0,052	-0,181	0,398
	DG	0,636***	0,001	0,447*	0,028	-0,288	0,172
GAD67	PL	0,205	0,335	0,543**	0,006	-0,030	0,891
	AC	0,445*	0,029	0,624***	0,001	-0,158	0,461
	CA1	0,586**	0,003	0,477*	0,019	-0,144	0,503
	DG	0,667***	0,000	0,259	0,222	-0,178	0,406
SST	PL	0,406*	0,049	0,764***	0,000	-0,212	0,319
	AC	0,410*	0,047	0,324	0,122	-0,148	0,490
	CA1	0,179	0,401	0,656***	0,001	-0,087	0,685
	CA3	0,566**	0,004	0,535**	0,007	-0,084	0,697
	DG	0,053	0,804	0,459*	0,024	-0,177	0,408

NOR, Novel Object Recognition test; OiP, Object-in-Place task; PV, parvalbumin; GAD67, glutamic acid decarboxylase-67; SST, somatostatin; PL, prelimbic cortex; AC, anterior cingulate cortex; CA1, cornus ammonis 1 of hippocampus; CA3; cornus ammonis 3 of hippocampus; DG, dentate gyrus of hippocampus. r: Pearson's correlation coefficient; p: significance of the correlation. *p<0.05, **p<0.01, ***p<0.001. Significant correlations are highlighted in light gray.

Furthermore, simple regression analysis with stepwise method manifested that the number of PV+ cells in anterior cingulate cortex and in DG predicted the performance in

NOR, whereas SST+ interneurons from prelimbic region predicted the discrimination index in Object-in-Place task.

5.12. Adult exposure to EE upregulates BDNF-TrkB signaling after postnatal MK-801 administration

BDNF is involved in several molecular cascades related to synaptic plasticity. It is the most studied neurotrophic factor and the binding of BDNF to its receptor TrkB is thought to be the main responsible of environmental-induced changes. Therefore, protein expression levels of BDNF were examined in the mPFC and hippocampus.

We observed slight changes in mPFC ($F(3,17)=18.729$, $p<0.0001$). BDNF levels were significantly increased only in VH+EE animals (VH vs. VH+EE, $p=0.022$; MK-801 vs. VH+EE, $p=0.004$), and the average increase in MK-801+EE group was not statistically different from VH group (Figure 42a). A small but still significant increase in BDNF protein expression levels was found in the hippocampus of EE housed rats compared to their standard housing littermates in both vehicle and MK-801 rats. More precisely, one-way ANOVA ($F(3,20)=26.882$, $p<0.001$) showed that the levels of BDNF in MK-801+EE animals were statistically significant compared to the standard housing animals ($p<0.0001$). Although the increase in BDNF levels in VH+EE animals was significantly lower than in MK-801+EE ($p=0.038$), the changes were still significant compared to VH ($p<0.001$) and MK-801 animals ($p=0.002$) (Figure 42d).

To gain more insight into the downstream effects of BDNF modulated by EE, we sought to investigate whether EE-induced upregulation of BDNF was accompanied by a parallel upregulation of its receptor TrkB. Here we show that TrkB protein levels were significantly changed in mPFC ($F(3,19)=6.560$, $p=0.003$; Figure 42b), but unchanged in hippocampus ($F(3,20)=0.198$, $p=0.897$; Figure 42e). MK-801 animals showed decreased TrkB receptor in mPFC compared to VH animals ($p=0.008$) and VH+EE group ($p=0.008$). MK-801+EE animals presented increased TrkB receptor, but we failed to find statistical differences compared to MK-801 animals.

Regarding the phosphorylation levels of TrkB receptor, one-way ANOVA revealed significant differences in mPFC ($F(3,21)=10.457$, $p<0.0001$). MK-801-treated rats

showed decreased TrkB phosphorylation (VH vs. MK-801, p=0.028) that was increased to normal values after EE, although no statistical significance was reached. As expected, VH+EE animals showed a notorious increase in pTrkB (MK-801 vs. VH+EE, p=0.025) (Figure 42c).

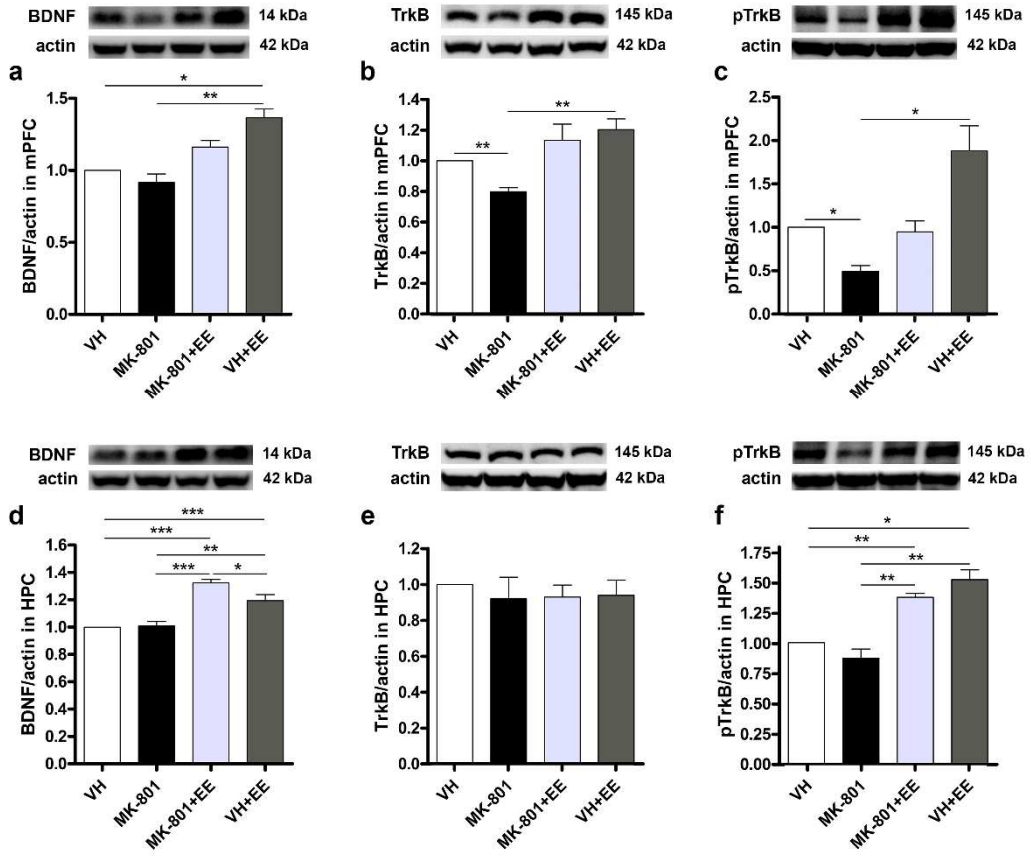


Figure 42. Protein levels for assessing BDNF-TrkB signaling (n=6 per group) in (a,b,c) mPFC and (d,e,f) dorsal hippocampus. (a) Perinatal MK-801 injections (0.5mg/kg, i.p.) had no effect on prefrontal BDNF levels of adult animals, (b) but decreased the expression of its high-affinity receptor TrkB and (c) its phosphorylation. Adult intervention with EE promoted (a) BDNF and (b) TrkB expression and (c) its phosphorylation. In hippocampus, (d) BDNF levels were significantly increased in MK-801+EE animals, (e) without relative changes in TrkB expression between groups, and (f) upregulated TrkB phosphorylation after EE. Histograms represent optical densities obtained for each primary antibody compared to actin, expressed as the ratio relative to VH animals. Data are represented as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; One-way ANOVA with Tamhane's Post Hoc test was used to obtain significances.

However, in hippocampus ($F(3,21)=30.063$, $p<0.001$) EE-housed animals showed increased TrkB receptor phosphorylation (VH vs. MK-801+EE, p=0.002; VH vs. VH+EE, p=0.044; MK-801 vs. MK-801+EE, p=0.003, MK-801 vs. VH+EE, p=0.003; Figure 42f) without any differences between standard housed animals.

Changes in the protein levels of BDNF, TrkB and phospho-TrkB in mPFC were not as significant as in hippocampus. Some concerns were raised when we failed to find statistically significant differences between MK-801 and MK-801+EE groups in the above-mentioned parameters. Therefore, a two-way ANOVA analysis was performed to determine if MK-801 and EE had significant effects as independent factors and we provide the statistical power of each of these factors (represented as partial eta squared) in mPFC (Table 6).

Table 6. Main effects of MK-801 and EE on the expression of BDNF, TrkB and phospho-TrkB in mPFC.

Factor	Protein	F	Significance	Eta squared
MK-801	BDNF	5.843	0.029*	0.280
	TrkB	4.099	0.057	0.177
	pTrkB	13.452	0.002**	0.473
EE	BDNF	32.686	<0.0001***	0.685
	TrkB	15.797	0.001***	0.454
	pTrkB	10.579	0.005**	0.414

As shown in the above table, both MK-801 and EE had significant effects on BDNF and pTrkB levels in mPFC, and EE had also an effect on the expression of TrkB receptors in mPFC. Overall, the effect size of EE was from moderate to large, whereas the size effect of MK-801 was relatively small. There was no interaction between these two factors.

5.13. Activation of Akt and ERK pathways in mPFC and hippocampus

The PI3K-Akt and MEK-ERK pathways are the principal pathways that contribute to neuronal survival, differentiation and synaptic plasticity. Both pathways are known to be common downstream signal transduction systems to the NMDA and growth factor

receptors. To address whether experience of vehicle and MK-801-treated rats in EE upregulated the activity of these pathways, the relative ratio of phosphorylated Akt (pAkt) to total Akt and phosphorylated ERK (pERK) to total ERK was calculated by Western Blot analysis in the mPFC and hippocampus.

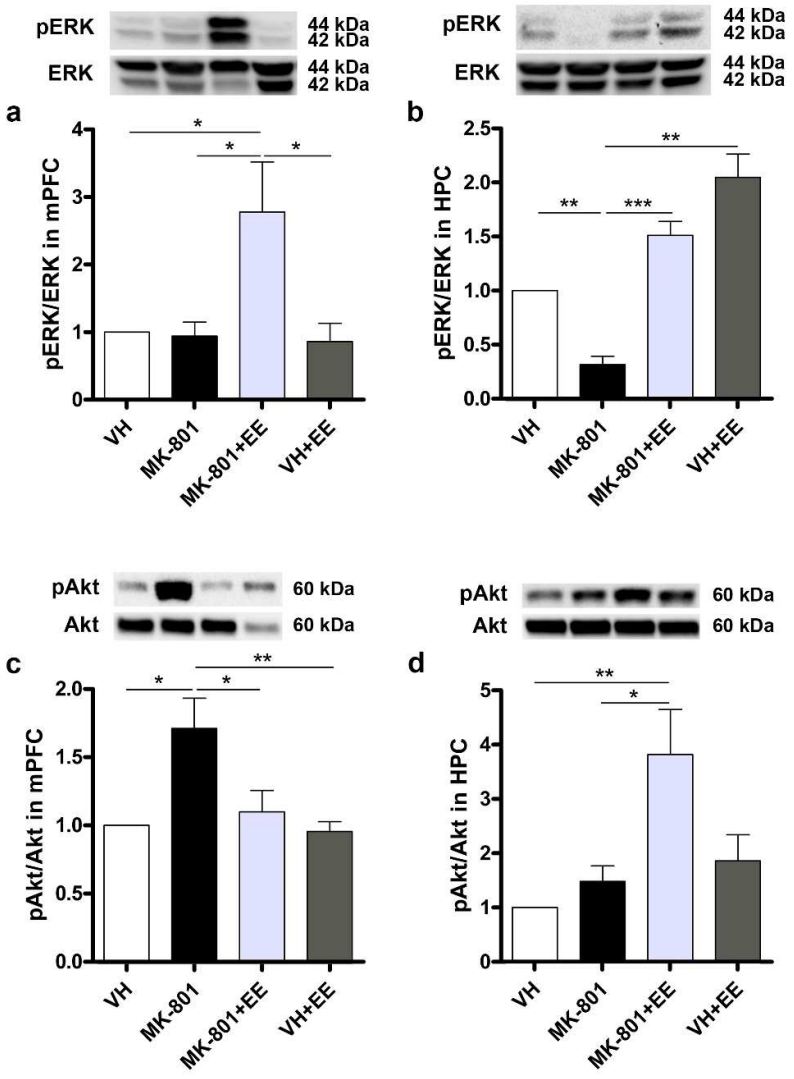


Figure 43. Activation of ERK1/2 and Akt pathways in animal housed in standard conditions and after adult enriched environment (EE) housing in (a,c) medial prefrontal cortex (mPFC) and (b,d) hippocampus (HPC). Western blots (upper panels) and quantification (histograms) of band densities for (a,b) relative phospho-ERK1/2 (pERK) to total ERK protein levels and for (c,d) relative phospho-Akt (pAkt) to total Akt protein expression. Data are represented as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 (one-way ANOVA).

As shown in Figure 43, the phosphorylation levels of ERK were reduced in MK-801-treated rats in hippocampus (VH vs. MK-801, p=0.002), but not in mPFC. Conversely, MK-801 caused a significant change in the phosphorylation levels of the Akt in the prefrontal cortex ($F(3,20)=6.288$, p=0.004; MK-801 vs. VH, p=0.011, MK-801 vs. MK-801+EE, p=0.035, MK-801 vs. VH+EE, p=0.007), but not in hippocampus. Exposing animals to enrichment significantly increased the relative expression levels of pERK and pAkt in hippocampus. In fact, EE reversed the MK-801-induced attenuation of phosphorylation levels of ERK ($p<0.0001$), and MK-801+EE group showed the highest levels of pAkt/Akt ratio in hippocampus (VH vs. MK-801+EE, p=0.006; MK-801 vs. MK-801+EE, p=0.027). Similarly, in mPFC pERK/ERK ratio was significantly increased in MK-801+EE animals ($F(3,16)=5.189$, p=0.011; MK-801+EE vs. VH, p=0.041; MK-801+EE vs. MK-801, p=0.033; MK-801+EE vs. VH+EE, p=0.025), and EE reversed MK-801-induced increase in Akt phosphorylation in mPFC.

Thus, intraperitoneal injections of MK-801 on early postnatal period induced the upregulation of Akt on mPFC with parallel downregulation of ERK pathway activation in dorsal hippocampus, and all these effects were counteracted by adult exposure to EE.

5.14. EE reverts MK-801-induced downregulation of NR1 in hippocampus and increases NR2A, NR2B and PSD-95 expression

In order to confirm whether adult short-exposure to EE was able to increase NMDAR subunit expression in mPFC and hippocampus, we performed Western Blot analysis against NR1, NR2A and NR2B subunits, and its scaffolding protein Postsynaptic Density-95 (PSD-95).

NR1 is the constitutive subunit of NMDA receptor. We found significant changes in both explored regions [mPFC ($F(3,20)=26.081$, $p<0.0001$) and hippocampus ($F(3,18)=48.336$, $p<0.0001$); Figure 44a and d]. This subunit was decreased in mPFC and hippocampus of MK-801 rats (VH vs. MK-801, p=0.04 in mPFC and $p<0.0001$ in hippocampus). MK-801+EE animals presented normal expression levels in hippocampus (VH vs. MK-801+EE, p=0.136; MK-801 vs. MK-801+EE, $p<0.0001$), but we only found a partial increase in mPFC (MK-801+EE vs. VH, p=0.136; MK-801+EE vs. MK-801,

p=0.199). Enrichment promoted NR1 expression in VH+EE animals in mPFC (VH vs. VH+EE, p=0.026; MK-801 vs. VH+EE, p<0.001; MK-801+EE vs. VH+EE, p=0.002).

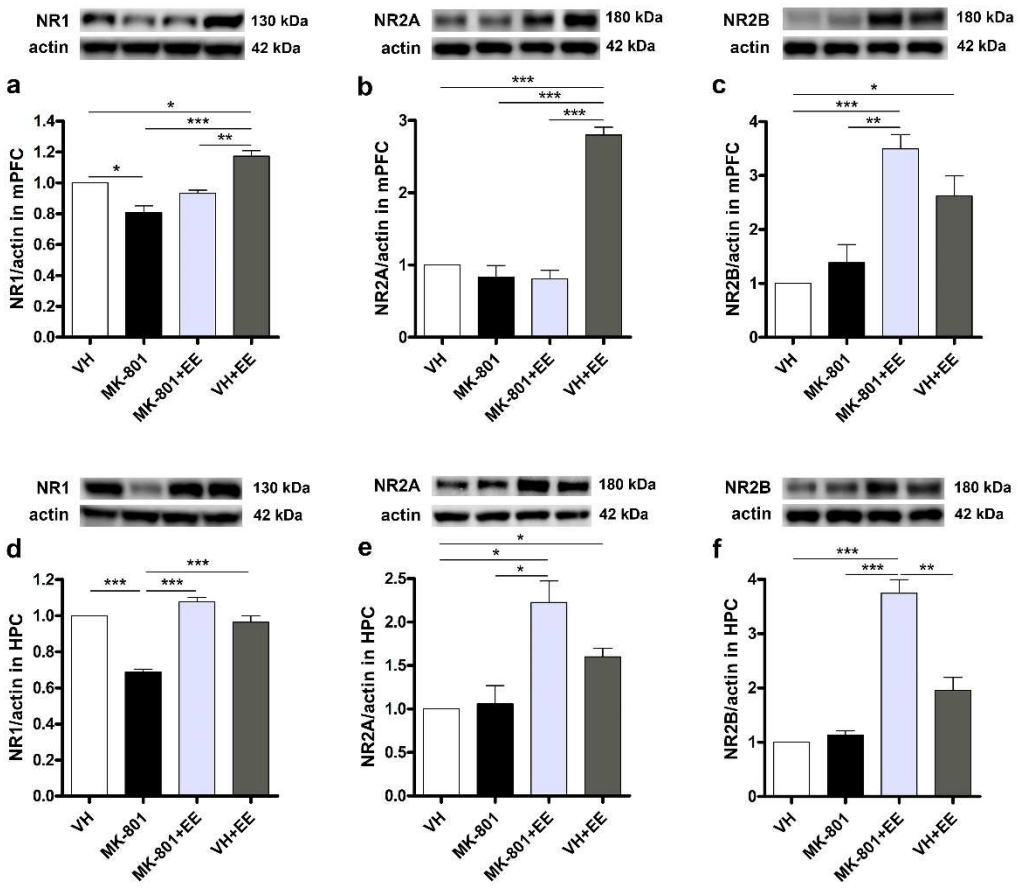


Figure 44. Effects of adult life exposure to EE after neonatal MK-801 treatment on NMDAR subunit expression. (a) The NMDAR vital subunit NR1 was significantly decreased in mPFC by MK-801 and EE had little effect on its expression in MK-801+EE animals. (b) Prefrontal NR2A subunit was significantly increased in VH+EE rats and (c) NR2B subunit increase was notorious in EE groups. (d) MK-801-induced attenuation of hippocampal NR1 subunit was increased up to normal values after EE. In addition, EE exposure increased (e) NR2A and (f) NR2B subunits in hippocampus. Histograms represent optical densities obtained for each primary antibody compared to actin, expressed as the ratio relative to vehicle-injected animals. Data are represented as mean ± SEM (n=6). *p < 0.05, **p < 0.01, ***p < 0.001; One-way ANOVA with Tamhane's T2 Post Hoc test was used to obtain significances.

In mPFC, there were significant changes in NR2A subunit ($F(3,18)=67.159$, $p<0.0001$; Figure 44b) accounted for a 3-fold increase in VH+EE group (VH+EE vs. the other 3 groups, $p<0.0001$). EE did not increase protein levels of NR2A in mPFC of MK-801+EE group (MK-801+EE vs. VH, $p=0.689$). However, we found a 3-fold increase of NR2B in mPFC of MK-801+EE animals ($F(3,20)=16.205$, $p<0.0001$), and was statistically different from standard condition groups (MK-801+EE vs. VH, $p=0.001$; MK-801+EE vs. MK-801, $p=0.004$). NR2B subunit expression was doubled in VH+EE compared to VH animals ($p=0.04$) (Figure 44c).

One-way ANOVA revealed a significant increase of NR2A subunit in HPC after EE ($F(3,19)=11.051$, $p<0.0001$; VH vs. MK-801+EE, $p=0.025$; VH vs. VH+EE, $p=0.02$; MK-801 vs. MK-801+EE, $p=0.03$) and the average increase was greater in MK-801+EE group compared to VH+EE group (Figure 44e). Similarly, regarding NR2B subunit in hippocampus, there were significant changes between groups ($F(3,19)=56.485$, $p<0.0001$). Similar to NR2A findings, NR2B increase was most notorious in MK-801+EE group (MK-801+EE vs. VH, $p<0.001$; MK-801+EE vs. MK-801, $p<0.0001$; MK-801+EE vs. VH+EE, $p=0.004$). Although expression levels of NR2B subunits in VH+EE group were also increased (Figure 44f), we failed to find statistically significant differences from standard condition animals.

Postsynaptic density-95 (PSD-95) is a scaffolding protein located in neural postsynaptic densities. It is associated with NR2 subunits of NMDA receptor, and it is required for synaptic plasticity associated with NMDA receptor signaling. In mPFC ($F(3,16)=16.953$, $p<0.0001$), PSD-95 levels tended to be downregulated in MK-801 group, but no statistical differences were found when compared to VH group. Animals housed in EE showed more PSD-95 than standard housed animals (VH vs. VH+EE, $p=0.012$; MK-801 vs. MK-801+EE, $p=0.031$; MK-801 vs. VH+EE, $p<0.001$; Figure 45a). PSD-95 levels in hippocampus were statistically different among groups ($F(3,18)=9.4$, $p<0.001$; Figure 45b). Increased protein expression was found in MK-801+EE group compared to VH group ($p=0.047$). Differences were near significance between MK-801 and MK-801+EE groups ($p=0.06$) and VH and VH+EE groups ($p=0.066$).

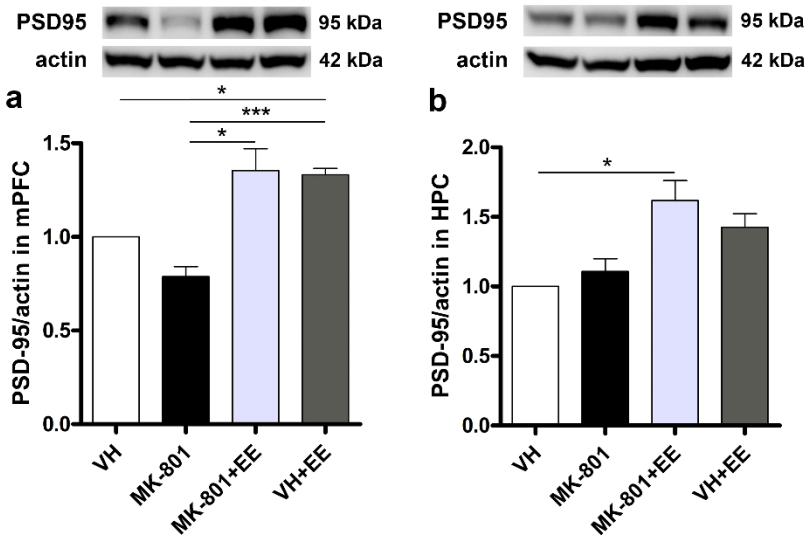


Figure 45. Protein levels of the scaffolding protein postsynaptic density 95 (PSD95) in (a) mPFC and (b) dorsal hippocampus. Histograms represent optical densities normalized to actin levels and are expressed as the ratio relative to vehicle-injected animals. Data are represented as mean ± SEM (n=6). *p < 0.05; **p < 0.01; ***p < 0.001 (one-way ANOVA).

Taken together, adult environmental intervention triggered a set of modifications on the components of glutamatergic neurotransmission, including the upregulation of the deficient NR1 subunit and NR2B-containing NMDARs with a parallel increase of the scaffolding protein PSD-95 and hippocampal NR2A subunit expression.

5.15. Recovery of $GABA_AR$ subunit ß2/3 expression following EE

The ionotropic receptor $GABA_A$ is necessary for the fast inhibition carried out by GABA neurotransmitter. We found significant differences of $GABA_AR$ subunit ß2/3 in mPFC ($F(3,19)=40.088$, $p<0.0001$) and hippocampus ($F(3,19)=4.623$, $p=0.014$; Figure 46). Post Hoc analysis showed that MK-801 treatment significantly reduced its expression in mPFC compared to control group ($p=0.044$).

After EE, this subunit was upregulated up to normal values in MK-801+EE group (MK-801 vs. MK-801+EE, $p=0.039$), and the average increase was more pronounced in VH+EE group (VH vs. VH+EE, $p=0.002$; MK-801 vs. VH+EE, $p<0.0001$; MK-801+EE

vs. VH+EE, p=0.044). On the other hand, the decrease in hippocampal expression of $GABA_AR$ subunit ß2/3 in MK-801 group was not statistically significant compared to VH group (p=0.081), although a significant increase was found in MK-801+EE group compared to MK-801 group (p=0.012).

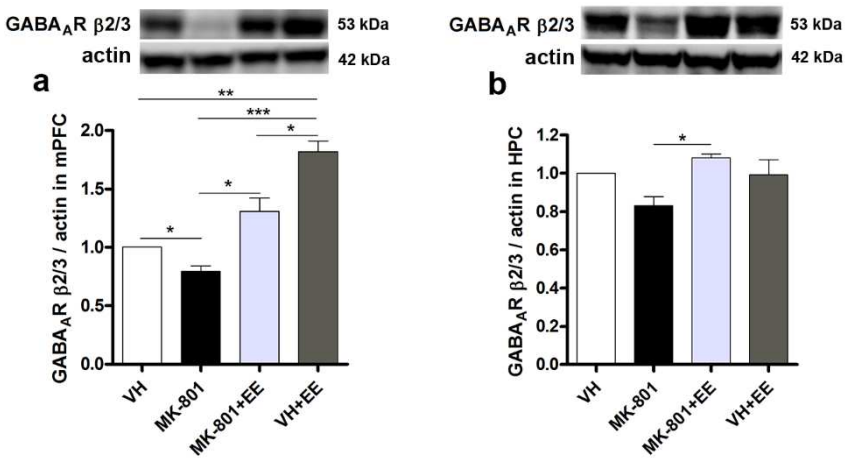


Figure 46. Protein levels of $GABA_AR$ subunit ß2/3 in (a) mPFC and (b) dorsal hippocampus. Histograms represent optical densities normalized to actin levels and are expressed as the ratio relative to vehicle-injected animals. Data are represented as mean ± SEM (n=6). *p < 0.05; **p < 0.01; ***p < 0.001 (one-way ANOVA).

6. Discussion

There is growing body of evidence suggesting that EE is remarkably beneficial in improving cognitive functions in health and disease. However, the impact of EE after neonatal MK-801 chronic administration in rats has not been profoundly studied, although it might be an effective strategy to manage cognitive symptoms and pathological findings related to schizophrenia. In this study, we administered Long Evans rats the NMDAR antagonist MK-801 chronically during neurodevelopment, and exposed rats to EE in adulthood in an attempt to reverse cognitive and neurochemical alterations that could be relevant for schizophrenia. Even though schizophrenia is considered a neurodevelopmental disorder, symptoms usually emerge at late adolescence/early adulthood. We tried to mimic the time course of the disease and exposed the animals to EE at a similar neurodevelopmental stage as for the diagnosis of schizophrenia in humans is done. This period usually coincides with prefrontal cortex maturation, myelization of frontal and temporal lobes, and with the rewiring of cortical circuits.

In our current study, several important findings are shown. First, we found that MK-801 impaired spatial learning, recognition memory, and associative memory. Secondly, MK-801 treatment reduced cortical and hippocampal volumes, as well as the number of PV, SST and CR-expressing interneurons in mPFC and hippocampus. MK-801 also diminished GAD67 immunoreactivity without overall cell loss, suggesting an impairment of excitatory-inhibitory balance. According to our results, a short-term exposure to EE in early adulthood improved cognitive functions and long-lasting GABAergic marker deficits. In addition, cell proliferation and hippocampal vasculature were altered in response to MK-801 and EE. At a molecular level, MK-801 impaired BDNF-TrkB signaling in mPFC, and EE upregulated this pathway in mPFC and hippocampus. Moreover, the obligatory NMDAR subunit NR1 was decreased by chronic MK-801 administration similar to $GABA_AR$ ß2/3 subunit. Adult EE exposure promoted the upregulation of NMDAR subunits, particularly in hippocampus. Alternatively, EE promoted the expression of $GABA_AR$ ß2/3 more notably in mPFC. Finally, MK-801 influenced the downstream signal transduction pathways that are common to the NMDA and TrkB receptors, namely ERK and Akt, in region specific manner.

The present study reveals new insights into the beneficial effects of EE exposure in adulthood of animals treated postnatally with MK-801, and renders EE a useful strategy to improve cognitive impairments, network disturbances and neurochemical alterations relevant to schizophrenia. Our findings propose that EE-mediated upregulation of BDNF-

TrkB signaling, and the regulation of NMDARs and related proteins, might be crucial neural mechanisms that mediate synaptic plasticity and improvement of cognitive alterations emerging upon NMDAR blockade.

6.1. Locomotor activity along neurodevelopment

The OFT was used to measure locomotor activity at different time points (P21, P24, P55) in response to repeated administration of MK-801 during postnatal period. Quantifying the total distance traveled and the relative distance in center, we found that the locomotor alterations produced by MK-801 did not persist long beyond treatment. The hypoactivity seen 24-hours after treatment cessation was absent at 96 hours and normal locomotor activity was conserved until P55. Our results coincide with those of Latysheva & Rayevsky *(Latysheva & Rayevsky, 2003)*, who found a transient decrease in spontaneous locomotor activity 23-hours after last injection, but failed to find any difference after 6 days or 4 months. Nevertheless, studies in the literature have mentioned either a robust increase of locomotion or decreased locomotor activity shortly after treatment *(Lim et al., 2012)*.

Long-term behavioral effects of MK-801 are also conflicting. For instance, some studies have found decreased locomotion in adulthood (P60) *(Baier et al., 2009)*, but others failed to observe any abnormalities *(Zhao et al., 2013; Su et al., 2014)*. The variability in results might be attributed to the diversity of dosing regimen. In line with other studies using similar doses *(Zhao et al., 2013; Su et al., 2014)*, we confirmed that MK-801 treatment had not long-term effects in locomotor activity. A number of authors have shown EE to reduce locomotor activity in OFT and habituate more rapidly than controls. This appears to be a relatively consistent finding independently of the employed protocol *(Zimmermann et al., 2001; Brenes et al., 2009)*. Our results are in line with earlier findings regarding decreased traveled distance in OFT after EE *(Zimmermann et al., 2001; Brenes et al., 2009)*. Moreover, after EE rodents also displayed reduced anxiety-like behavior in OFT indicated by the distance spent in center relative to periphery. Unfortunately, we only observed anxiolytic effects in saline controls housed in EE, and not in neonatally MK-801-injected rats with EE.

6.2. EE improves cognitive functions

EE is a widely used paradigm to stimulate cognitive processes, as mice and rats generally show improved performance in learning and memory tasks after EE than standard housed control animals *(van Praag et al., 2000)*. Many studies initiate EE at relatively early stages. Environmentally stimulating conditions from birth or weaning prevent cognitive and behavioral deficits secondary to chronic MK-801 treatment *(Nozari et al., 2014; Rahati et al., 2016)*. However, no single study to date has assessed the potential benefits of EE in adulthood after neonatal NMDAR blockade, although studies in which EE has started at adult stages also exhibited beneficial effects in other diseases, like diabetes *(Beauquis et al., 2010)*. In this work, we demonstrated that an intervention with EE in early adulthood enhanced cognitive functions of postnatally MK-801-treated animals.

The results of the MWM showed that rats of MK-801 group spent more time to find the platform than vehicle animals, indicating that MK-801 impaired spatial learning. On the other hand, adult intervention with EE reversed the spatial learning impairment. Previous studies have shown that early life MK-801 administration results in disturbed spatial learning in adult rodents. In fact, altered water maze performance seems to be predominantly manifested after maturity. In line with our results, Gorter and de Bruin *(Gorter & de Bruin, 1992)* also reported a slower learning in adult rats (P120-P140) following chronic neonatal MK-801 administration (0.2mg/kg from P8 to P19), even though reference memory was preserved. Similarly, Németh et al., *(Németh et al., 2002)* noted poorer water maze learning in comparison to controls, which is consistent with the results of Enomoto et al., *(Enomoto et al., 2008)* who found that MK-801 (0.15 mg/kg i.p.) significantly increased escape latency and distance. Few studies have addressed the potential benefits of enriched environment (EE) on MK-801-induced spatial learning deficits. According to Nozari et al., *(Nozari et al., 2014)*, EE prevented spatial learning and memory deficits in Morris Water Maze in juvenile rats after chronic high doses of MK-801 (1mg/kg) from P6-P10.

Novel Object Recognition (NOR) is a widely used test to assess memory function in schizophrenic humans and animal models. Previous studies have shown that acute administration of MK-801 before NOR impairs acquisition and encoding of object recognition memory *(Nilsson et al., 2007)* and it therefore decreases discrimination index

(Karasawa et al., 2008; Wierońska et al., 2013; Vishnoi et al., 2015). Nevertheless, long-term effects have been somewhat controversial. Adopting a once daily 0.25mg/kg dose from P6 to P21, Baier et al., *(Baier et al., 2009)* concluded that MK-801 did not had long-term adverse consequences on NOR. Likewise, Lim et al., *(Lim et al., 2012)* failed to find any impairment in recognition memory in adult rats using a daily dose of 0.2mg/kg between P7 and P10. It is now known that drug administration schedule highly influences NOR performance in adulthood. Chronic daily doses of 0.5mg/kg or higher during neurodevelopment seem to be necessary for long-lasting recognition memory impairment in adult animals *(van der Staay et al., 2011)* – the threshold dose for apoptotic injury *(Ikonomidou et al., 1999)*. On this background, Li et al., *(Li et al., 2015)* administered 0.25mg/kg MK-801 twice daily from P5 to P14, and demonstrated an impaired object recognition memory in adult rats that was already present in juvenile animals. This is consistent with our results, in which a daily dose of 0.5mg/kg from P10 to P20 impaired recognition memory. The low performance of MK-801-treated rats in NOR was not attributable to malnutrition or anxiety, in view of normal body weight and locomotor activity in Open Field Test. Previous studies have consistently shown that EE improves discrimination ratio in NOR task in healthy animals *(Kazlauckas et al., 2011; Doulames et al., 2014)*, and also in animal models of Parkinson's Disease *(Campêlo et al., 2017)*, Alzheimer's disease *(Polito et al., 2014)* or after traumatic brain injury *(Schreiber et al., 2014)*. Nevertheless, effect of environmental stimulation in reversing memory deficits in NOR has not been studied in animal models of neuropsychiatric disorders, including schizophrenia.

On the other hand, MK-801 disrupted associative recognition memory typified by decreased discrimination index in Object-in-Place task, and adult intervention with EE enhanced associative memory impairment. Altered performance of rats in Object-in-Place task resembles the impaired performance in working memory binding task *(Burglen et al., 2004)* and pattern-location association *(Wood et al., 2002)* of human patients with schizophrenia. In fact, associative memory seems to be more impaired in schizophrenic patients than recognition memory, which is often used to measure cognitive abilities in animal models of schizophrenia *(Achim & Lepage, 2003; Lepage et al., 2006; Luck et al., 2009)*. This might be so, because in the object–place associative tasks mPFC is more selectively involved than in recognition tasks *(Lee & Solivan, 2008)*. Actually, Object-in-Place associative memory depends on an interaction between the hippocampus (HPC),

perirhinal (PRH), and medial prefrontal (mPFC) cortices, and on the excitatory neurotransmission in any pair of structures within this networks mediated by AMPA and NMDARs *(Barker & Warburton, 2015)*. Li and colleagues *(Li et al., 2015)* demonstrated that early life MK-801 administration also altered associative memory in object-in-context task. However, it should be noted that object-in-context task is more dependent on neural ensembles of ventral hippocampus than of dorsal hippocampus, which is primarily involved in object-location representations.

As far as we know, this is the first time that late exposure to EE demonstrates to be effective in reversing spatial learning impairment in Morris Water Maze after postnatal MK-801 exposure. Similarly, this is the first study that demonstrates a beneficial effect of adult intervention with EE in recognition and associative memories in a neurodevelopmental model of schizophrenia.

6.3. Structural brain changes

Regarding the volumetric changes, our results are in agreement with previous reports describing a decrease in mPFC and hippocampal volume in Lister Hooded rats after perinatal MK-801 administration *(Gilabert-Juan et al., 2013)*. In contrast, this study showed that the volume of anterior cingulate was unaltered and mPFC reduction was mainly to be due to decreased volume in prelimbic cortex. Reductions in the volume of the PFC and hippocampus have also been found in schizophrenic patients, and this anatomical abnormality is often considered a hallmark of the disease, at it is present at the onset of illness *(Levitt et al., 2010; van den Noort et al., 2010)*. Decreased neuronal and glial size, and reduced microvascular density in schizophrenia patients might also contribute to cortical volume decrease *(Rajkowska et al., 1998; Stark et al., 2004; Schmitt et al., 2009; Hercher et al., 2014)*. One interesting result of the present study is that the environmental enrichment increased cortical and hippocampal volumes, thereby reversing MK-801-induced structural changes.

Another aspect of structural plasticity might be related to decline in microvascular structure. Altered capacity of cerebrovascular angiogenesis may result in a failure of vascular homeostasis, which in turn will affect the adjustment of dynamic changes in oxygen supply and demand. As increased neural activity induces changes in blood flow

and microvascular density, any abnormalities in the vasculature will lead to functional impairments and will alter neuronal integrity. In fact, several micro- and macrovascular abnormalities have been recently identified as having direct implications on the pathophysiology of schizophrenia. However, studies of microvascular brain changes in schizophrenia are scarce and sometimes contradictory. A *post mortem* stereological assessment of capillary diameters in anterior cingulate cortex in long-lasting schizophrenic patients, found no differences compared to controls, but smaller cortical thicknesses (layers III and V) were identified *(Sinka et al., 2012)*. In another study, an atypically simplified angioarchitecture and failure of normal arborization of brain vessels was verified *(Senitz & Winkelmann, 1991)*, supporting the existence of an abnormal vascular organization. We found by stereological means that MK-801-treated animals displayed reduced microvascular length in hippocampus, but not in mPFC. Interestingly, environmental intervention in adult life increased microvascular structure in MK-801-treated animals up to normal values.

6.4. Early life MK-801 administration reduces the number of PV and CR-expressing interneurons

Deficit in PV immunoreactive interneurons is the most consistent finding in animal models of schizophrenia *(Rujescu et al., 2006; Abekawa et al., 2007; Braun et al., 2007; Coleman et al., 2009; Li et al., 2015)* and human patients *(Volk et al., 2000; Zhang & Reynolds, 2002; Hashimoto et al., 2003)*. Our results showing a reduced number of PV+ interneurons in PL and AC cortices in mPFC, and CA1 and DG of hippocampus are in agreement with previous works. Studies using repeated injections of NMDAR antagonists have demonstrated a reduction in PV density in mPFC *(Nakatani-Pawlak et al., 2009; Redrobe et al., 2012)* and hippocampus *(Abekawa et al., 2007; Powell et al., 2012)*, and neurodevelopmental models further support this finding *(Abekawa et al., 2007; Coleman et al., 2009; Li et al., 2015)*. MK-801 is a non-competitive NMDAR antagonist that selectively disrupts GABAergic cells. A number of hypotheses have been proposed to explain the mechanisms by which PV+ can be selectively susceptible to damage after MK-801 administration. The open-probability of NMDAR of PV cells is higher than in other types of interneurons, as most of PV-expressing interneurons are fast-spiking (FS) cells. Thus, the probability of MK-801 for blocking NMDAR of FS-PV+ cells increases.

Moreover, Wang & Gao *(Wang & Gao, 2012)* showed that NMDAR in presynaptic glutamatergic terminals targeting pyramidal cells and FS interneurons were distinctly affected after subchronic MK-801 exposure. Presynaptic NMDAR are critical to modulate and facilitate neurotransmitter release. The authors demonstrated that MK-801 completely blocked presynaptic NMDARs of terminals that targeted FS-PV interneurons, whereas NMDARs of glutamatergic terminals that synapsed with pyramidal neurons were upregulated, shifting the excitation inhibition balance towards excitation.

Another hypothesis that supports a presumable selective disruption of PV+ interneurons is that our MK-801 administration schedule coincides with the developmental expression of PV *(Lema Tomé et al., 2007; Mukhopadhyay et al., 2009)*. PV expression is low to absent at P7 and gradually increases until P21 *(Lema Tomé et al., 2007; Mukhopadhyay et al., 2009)*. It has been suggested that calcium-binding proteins, especially PV, play a neuroprotective role when facing dysregulation of calcium homeostasis *(Lema Tomé et al., 2008)*. On the other hand, PV is the only calcium-binding protein that has been linked to specific brain functions, like attention *(Kim et al., 2016)* or cognitive flexibility *(Murray et al., 2015)*. Interneurons that express PV are also involved in feed-back loops that create gamma oscillation – the physiological correlate of higher cognitive functions *(Somogyi et al., 2005)*. It was therefore expected that our finding of persistent PV deficiency would result in cognitive impairment. In fact, we found a remarkable correlation between the number of PV+ interneurons and recognition memory. However, Bygrave et al., *(Bygrave et al., 2016)* claim that an exclusive NMDAR hypofunction of PV interneurons might not be the starting point of schizophrenia, but rather support the idea of NMDA hypofunction in several cells types.

Unlike what has been observed in the present study, CR-expressing interneurons are not altered in schizophrenic brains *(Daviss & Lewis, 1995; Reynolds et al., 2001a; Reynolds et al., 2001b; Abekawa et al., 2007)*, neither in animal models of MK-801 treatment *(Turner et al., 2010; Li et al., 2015)*. One of the reasons might be that usually cell densities are reported instead of absolute cell numbers, although reductions in cortical and hippocampal volume due to MK-801 have been repeatedly documented in the scientific literature *(Gilabert-Juan et al., 2013)*. This means that small changes in the number of CR+ interneurons could be masked by paralleled changes in volume. In fact, Gilabert-Juan et al., *(Gilabert-Juan et al., 2013)* found that CR gene expression was reduced in mPFC caused by MK-801 treatment. A plausible explanation could be that CR

is downregulated as a compensatory mechanism for brain hyperactivity. Hippocampal hyperactivity is a core feature of schizophrenia that has been replicated in MK-801 animal models *(Heckers & Konradi, 2015)*. CR+ interneurons are specialized in innervating other interneurons in mPFC and hippocampus *(Gulyás et al., 1996; DeFelipe J, 1997)*. In mPFC, they usually target somatostatin (SST) and PV-expressing interneurons *(Pi et al., 2013)*, whereas in hippocampus they predominantly contact with CB+ interneurons and other CR-expressing interneurons *(Gulyás et al., 1996)*, presumably those that co-express somatostatin *(Gulyás et al., 1996; Somogyi et al., 2005)*. Hence, CR+ interneurons are part of the dishinhibitory circuit, and they are in the position to govern the inhibition carried out by other interneurons *(Pi et al., 2013)*. CR could be downregulated in an attempt to compensate for network overactivation triggered by MK-801, but this hypothesis needs to be further confirmed.

Regarding the number of CB+ cells, conflicting results are found in humans and animal models. Some studies have found increased CB expression in human schizophrenic patients *(Woo et al., 2008; Fung et al., 2010)*, others have found no changes *(Tooney & Chahl, 2004)*, and some have reported reduced CB immunoreactivity *(Beasley et al., 2002)*. Results from animal studies are equally variable. In this study, we excluded from stereological estimates those layers in which CB expression could be confounded by CB-expressing excitatory cells, like pyramidal layer and granular layer of hippocampus and LII/III of mPFC, and found no differences between groups. In an attempt to solve this question, Li et al., *(Li et al., 2015)* used double immunohistochemistry with CB and SST, and concluded that CB+ interneurons were decreased in both superficial and deep layer of mPFC as a consequence of MK-801 administration. However, it should be noted that not all CB+ cells express SST, neither all SST interneurons are CB+. Contrarily, Gilabert-Juan et al., *(Gilabert-Juan et al., 2013)* found increased CB mRNA in mPFC that was paralleled by an increase in the number of CB-expressing cells. More studies should be conducted to solve this discrepancy.

6.5. Alterations in somatostatin-expressing GABAergic interneurons

Post mortem mRNA studies of patients with schizophrenia have shown that SST decreased more significantly than any other biomarker in hippocampus and medial

prefrontal cortex *(Hashimoto et al., 2008; Morris et al., 2008; Konradi et al., 2011)*, and it seems that these differences are already noticeable during neurodevelopment *(Fung et al., 2010)*. Other studies have also examined the number and density of SST-expressing interneurons, showing a loss of this subpopulation *(Konradi et al., 2011)*. Reduced SST level is not pathognomonic of schizophrenia, as this observation is common to other psychiatric and neurological disorders such as bipolar disorder, Alzheimer's disease or Parkinson's disease *(Lin & Sibille, 2013)*. Nevertheless, a pattern of SST reduction in various areas implicate SST+ interneurons in the pathophysiology of schizophrenia *(Alherz et al., 2017)*.

We found that postnatal MK-801 treatment reduced the number of SST+ interneurons in mPFC and hippocampus, similar to what we observed in PV+ interneurons. Previous studies have also reported decreased SST-immunoreactivity after MK-801 administration *(Arif et al., 2006)*, as well as structural remodeling of spines and axonal boutons of hippocampal SST+ interneurons *(Perez-Rando et al., 2017)*. Although there are limited studies about the effect of NMDAR antagonists on this type of interneurons, MK-801 could presumably block NR2C and NR2D-containing NMDARs more selectively, due to their low affinity for magnesium *(Kotermanski et al., 2009)*. NMDAR subunit NR2C is preferentially expressed in PV+ and SST+ interneurons, and NR2D in PV+ fast-spiking cells *(von Engelhardt et al., 2015)*, making this two subtypes of interneurons especially vulnerable to NMDAR blockade. As far as we know, this is the first study that quantifies the number of SST+ interneurons in MK-801-treated animals.

Somatostatin is closely related to cognitive functions, as the deletion of somatostatin receptor 2 leads to cognitive impairment *(Dutar et al., 2002)*, and reductions in SST levels have been attributed to the cognitive decline seeing in healthy aging *(Dournaud et al., 1995)*. In this work, we observed that the number of SST+ interneurons was highly correlated with the performance in Novel Object Recognition and Object-in-Place tasks, and successfully predicted the discrimination index of the latter, further suggesting their contribution to learning and memory processes. However, the precise role of SST-expressing interneurons in cognition is still poorly understood. Synapse distribution between SST+ interneurons and pyramidal cells are crucial for determining the effects of local circuits and network inhibition *(Urban-Ciecko & Barth, 2016)*. Likewise, GABA release from axon terminals of SST+ interneurons activates fast synaptic $GABA_AR$, whereas somatostatin release slowly but persistently activates

metabotropic $GABA_BRs$ *(Urban-Ciecko & Barth, 2016)*. Therefore, distinct subpopulation of SST+ interneurons inhibit pyramidal cells at multiple levels and timescales that might lead to different functional outcomes *(Scheyltjens & Arckens, 2016)*.

Taken together, SST-interneurons are likely to be important mediators of learning and memory processes, as they are ideally situated to integrate, process, and modulate afferent inputs, but the precise mechanism are still to be determined.

6.6. EE promotes the expression of GABAergic markers

Calcium-binding proteins

EE intervention increased GABAergic cell immunoreactivity without changing overall cell quantity. This indicates that MK-801 reduced the expression of calcium-binding proteins in interneurons, and consequently their activity, instead of promoting programmed cell death. Nevertheless, the rate of apoptosis that previous studies have reported after MK-801 administration is relatively small compared to the total number of cells *(Ikonomidou et al., 1999)*. Thus, MK-801 probably augmented programmed cell death postnatally, but this loss could not be detected by stereological estimations in adulthood. In fact, our dose of 0.5mg/kg has been documented to be the threshold dose for apoptotic damage *(Ikonomidou et al., 1999)*.

It has been shown that hippocampal PV+ cells are especially sensitive to NMDAR blockade. This could partly explain why PV immunoreactivity in CA1 region was only partially increased. EE completely restored the number of PV+ and CR+ interneurons in anterior cingulate and PV+ interneurons in DG. However, only a partial restoration was found in PV+ and CR+ cells in prelimbic region of mPFC, and in CR+ in CA1. The partial recovery demonstrates the beneficial effects of EE, but it remains to be determined if longer periods in EE could further increase calcium-binding protein markers up to normal values, or the lack of complete recovery is caused by the postnatal MK-801-induced cell death.

Somatostatin

Somatostatin expression is CREB-dependent, and thus regulated by neuronal activity *(Urban-Ciecko & Barth, 2016)*. Therefore, the observed decrease in the number of SST+ interneurons after MK-801 would suggest a decrease in SST content resulting from NMDAR blockade rather than a loss of this type of interneurons. Based on this fact, we propose that the increase in the number of SST+ interneurons after EE exposure in adulthood was attributable to enhanced activity of these interneurons that would in turn promote the synthesis and expression of SST. However, we found that this effect was region-dependent, as the number of SST+ interneurons was completely rescued in CA3 region of hippocampus, but minimally in CA1 region. The heterogeneity within SST-expressing interneurons in hippocampus could explain these regional differences. For instance, even though cholinergic and noradrenergic inputs enhance the SST+ interneuron activity, cholinergic receptors are restricted to *oriens-lacunosum moleculare* (OLM) interneurons in CA1 *(Leão et al., 2011)*. Moreover, some OLM engage in gamma oscillations, but those that expression 5HT3A receptors do not *(Chittajallu et al., 2013)*. In addition, neocortical SST+ interneurons also display anatomical, molecular and electrophysiological differences and their functional roles are featured by their specific cortical wiring patterns that will invariably determine cognitive performance.

Glutamic-acid decarboxylase 67

In the mammalian brain, the primary inhibitory neurotransmitter GABA is mainly synthesized by GAD67. This isoform of glutamic acid decarboxylase is the responsible for over 90% of GABA production *(Asada et al., 1997)*. *Post mortem* studies of schizophrenic individuals have revealed reduced levels of GAD67 in the PFC *(Akbarian et al., 1995; Guidotti et al., 2000; Hashimoto et al., 2008)*. Despite the scarcity of investigations, the available findings thus far suggest that GAD67 expression is also decreased in hippocampus *(Thompson Ray et al., 2011)*. The present results are partially in accordance with human findings, showing reduction in GAD67 expression in hippocampus, albeit the expression in mPFC was maintained. It is noteworthy that GAD67 deficiency is primarily present in a subset of GABAergic cells, namely PV interneurons *(Hashimoto et al., 2003; Lewis et al., 2005)*. Given that we considered the overall GAD67 expression in mPFC, we were not able to detect significant differences.

Interestingly, EE in vehicle rats led to increased number of GAD67-positive cells. EE enhances glutamatergic neurotransmission through BDNF and its receptor TrkB *(Tyler et al., 2001; Tyler et al., 2006)*, and genetic approaches have demonstrated that GAD67 levels directly contribute to the strength of synaptic inhibition *(Lazarus et al., 2015)*. We speculate that the mild increase in GAD67 cells in VH+EE animals might occur in an attempt to counterbalance increased excitatory input.

6.7. EE reverses deficits on PV and GAD67 co-expression in CA1 and increases the relative ratio of co-expression in mPFC

Several lines of evidence point to alterations in inhibitory circuits as one of the main factors to explain the neurobiological basis of schizophrenia. Deficiencies in parvalbumin (PV) and glutamic-acid decarboxylase (GAD67) expression in post mortem brain studies of schizophrenics and in animal models of schizophrenia have been well established in the literature *(Turner et al., 2010; Gilabert-Juan et al., 2013; Fujihara et al., 2015)*. Moreover, GAD67 deficit in PV+ interneurons is thought to contribute to the disinhibition of cortical networks that result in cognitive impairment. Previous studies have shown that EE can prevent or reverse the loss of PV+ interneurons secondary to a variety of causes, including ischemia, maternal separation or stress. Here we show that adult EE exposure increases the number of PV+ interneurons co-expressing GAD67 in mPFC and hippocampus. However, this might be biased, in part, by decreased number of PV+ interneurons after MK-801 administration

Perinatal NMDAR antagonism instills disturbances in the GABAergic system like reduced PV and GAD67 expression that are consistently reported in *post mortem* brain of schizophrenic patients *(Lim et al., 2012)*. On the other hand, GAD67 seems to be selectively reduced in prefrontal basket and chandelier cells, two major subtypes of PV-expressing interneurons *(Hashimoto et al., 2003; Lazarus et al., 2015)*. We failed to find any differences in the relative number of interneurons co-expressing PV and GAD67 in mPFC between vehicle and MK-801-treated animals. Nevertheless, the reduced number of PV+ interneurons in this region, as previously reported *(van der Staay et al., 2011; Murueta-Goyena et al., 2017)*, could have biased the results. In dorsal hippocampus, we found that MK-801 reduced the expression of GAD67 in PV+ interneurons in CA1 and

dentate gyrus, similar to what Belforte et al., *(Belforte et al., 2010)* found in PV cells deficient for NMDARs. The connection between GAD67 reduction and altered inhibitory transmission have been difficult to predict, but a recent work by Lazarus et al., *(Lazarus et al., 2015)* has demonstrated that the deletion of one allele of GAD67 gene from PV+ interneurons contributed to reduced synaptic inhibition that could explain the increased activity of pyramidal neurons found in schizophrenia *(Heckers & Konradi, 2015)*. Interestingly, in the present work we found that EE increased the co-expression of PV and GAD67, probably in basket cells, as experience specifically modulates PV and GAD67 content in this subtype of interneurons *(Donato et al., 2013)*. These results might have important implications in cortical activity because GAD67-mediated GABA synthesis regulates the axon branching and perisomatic synapse formation of PV+ interneurons *(Chattopadhyaya et al., 2007)*.

6.8. MK-801 increases the number of proliferative cells is subgranular layer and EE increases immature granule cells phenotype in hippocampus

Cell division markers like BrdU are incorporated into DNA when DNA is synthesized during cell division. This marker is often used to assess cell proliferation in subgranular zone of dentate gyrus. It has been previously shown that the administration of both competitive and non-competitive NMDAR antagonists increases proliferation in this hippocampal region *(Nacher et al., 2001; Nacher et al., 2003)*, suggesting that adult neurogenesis may be regulated by NMDARs present in precursor cells and in differentiating granule neurons, and control the development of adult-born neurons at most stages of their differentiation *(Nácher et al., 2007)*. This is in accordance with our results, in which we found increased and long-lasting cell proliferation after postnatal MK-801 treatment. EE-induced cell proliferation in VH+EE rats was not as high as cell proliferation secondary to MK-801 treatment. This finding lead us to question which proportion of this cells will differentiate into granule cells that could potentially integrate into hippocampal circuits.

Mature granule cells of dentate gyrus express CB and NeuN. Heretofore post-mitotic neuronal cells transiently express CR, before being fully integrated in the

hippocampal circuitry. Former studies have speculated about the immature dentate gyrus as being a potential endophenotype of neuropsychiatric disorders, including schizophrenia *(Walton et al., 2012)*. *Post mortem* immunohistochemical analysis of schizophrenic brain revealed that they displayed significantly increased CR expression in dentate gyrus compared to normal controls *(Walton et al., 2012; Shin et al., 2013)*. Considering that glutamate inhibits cell proliferation and NMDAR blockade increases it in the subgranular zone of DG *(Cameron et al., 1995)*, it seemed possible that MK-801 administration could increase CR+ cells in granular cell layer. Nevertheless, we failed to find any difference in CR-expressing cells in granular cell layer, which indicates that chronic MK-801 administration during neurodevelopment does not promote immature phenotype of DG, at least, on a long-term basis. Other studies have demonstrated long-lasting increased cell proliferation and neurogenesis after MK-801 administration, but in adult rats *(Nacher et al., 2001; Nacher et al., 2003; Petrus et al., 2009)*. Contrarily, we observed significantly more CR+ cells in VH+EE animals compared to all other 3 groups. Several studies confirm that EE increases adult neurogenesis, and this is often paralleled with improvements in learning and memory tasks *(Kempermann et al., 2002; Nilsson et al., 2007)*. It has been discussed that EE promotes survival of newborn neurons rather than increasing cell proliferation *(van Praag et al., 2000)*. Independently of the mechanism, any of these options will result in more CR+ expressing immature neurons in granule cell layer, which coincides with our results.

6.9. Molecular modifications underlying EE-induced cognitive enhancement

The mechanisms by which EE is able to improve cognition and restore GABAergic cell immunoreactivity are not fully uncovered. Previous studies have reported that exercise alone has also beneficial effects at anatomical and behavioral levels *(van Praag, 2009)*, and exposure to novelty improves spatial memory *(Yang & Tang, 2011)*. EE promotes structural changes in the brain like increased spine density and dendritic branching, induces changes in neurotransmitters, and enhances the gene expression and protein levels of different growth factors *(van Praag et al., 2000; Mohammed et al., 2002)*, such as nerve growth factor (NGF) *(Mohammed et al., 1990; Pham et al., 1999)*, brain-derived neurotrophic factor (BDNF) *(Falkenberg et al., 1992)*, glial-derived

neurotrophic factor (GDNF) *(Young et al., 1999)* or vascular endothelial growth factor (VEGF) *(Bengoetxea et al., 2008)*. Accumulating evidence has yielded to the notion that NMDAR hypofunction underlies cognitive dysfunction and anatomical alterations of schizophrenia. Albeit the scarcity of investigations about the pathways that regulate NMDAR trafficking and mobility related to EE, it is known that EE increases neurotransmitter receptors, including NR1, NR2A and NR2B subunits of NMDARs in several regions of the brain, including hippocampus, forebrain and amygdala *(Tang et al., 2001)*. Based on this evidences, we focused on studying the expression levels of NMDAR subunits and the signaling pathway BDNF-TrkB that might have relevant implications in schizophrenia, and could be the underlying molecular cascades involved in cognitive and cellular improvements.

6.9.1. NMDAR subunits

NMDAR antagonists, including MK-801, lead to a decrease in GABAergic interneurons and behaviors reminiscent to schizophrenia in animals *(Olney & Farber, 1995; Abekawa et al., 2007; Murueta-Goyena et al., 2017)*. In this study, we demonstrate that neonatal MK-801 administration in rats downregulates the obligatory NR1 subunit of NMDAR in hippocampus and mPFC that further supports NMDAR hypofunction and its relation to the pathophysiology of schizophrenia. Weickert et al., *(Weickert et al., 2013)* provided evidences of reduced mRNA and protein levels of NR1 subunit in dorsolateral prefrontal cortex of schizophrenic patients, and more recently, Catts et al., *(Catts et al., 2015)* have corroborated these results. After adult environmental exposure, NR1 subunit increased in dorsal hippocampus of both saline and MK-801-treated rats with a parallel increase in NR2A and NR2B subunits that was more evident in MK-801+EE group. These data indicate that EE-promoted synthesis and expression of NMDAR subunits is particularly notorious in rats neonatally treated with MK-801, perhaps as a compensatory mechanism for the induced NMDAR blockade. However, in mPFC EE-mediated NR1 and NR2A protein level enhancement was minimal, but we found a major increase in NR2B levels. NR2B-containing NMDARs reveal long currents, carry more charge for a single synaptic event than NR2A-containg NMDARs *(Zhu et al., 2002; Erreger et al., 2005)* and couple to intracellular signaling cascades, like Ras-GRF1, RasGAP and CaMKII *(Barria & Malinow, 2005)* that might favor LTP induction, and

consequently could enhance memory functions as seen in MK-801+EE rats, but this viewpoint remains highly speculative.

Moreover, NMDA channel gating and surface expression is regulated by postsynaptic density protein 95 (PSD95) *(Lin et al., 2004)* – a scaffolding protein that is required for activity-driven synapse stabilization *(Ehrlich et al., 2007)*. Most molecular studies of PSD-95 protein levels in prefrontal cortex find no change in individuals with schizophrenia *(Funk et al., 2009; Kristiansen et al., 2010)*, though studies of the anterior cingulate cortex have observed a decrease in PSD-95 protein levels *(Funk et al., 2009; Kristiansen et al., 2010; Funk et al., 2012)*. Nonetheless, Catts et al., *(Catts et al., 2015)* found a 20% decrease in NR1 protein and a 30% decrease in PSD-95 in postsynaptic density-enriched fractions from individuals with schizophrenia relative to unaffected controls in prefrontal cortex. Although the decrease in PSD-95 in mPFC of our animal model was not statistically significant, environmental intervention increased PSD-95 expression levels, suggesting increased NMDAR-mediated neurotransmission after EE intervention. Nithianantharajah et al., *(Nithianantharajah et al., 2004)* also reported increased PSD-95 after 30 days of EE, which is consistent with the results of Rampon et al., *(Rampon et al., 2000)* who also found increases in PSD-95 gene expression or protein levels following EE.

Furthermore, both PV and GAD67 are expressed upon the activation of NMDARs containing the NR2A subunit *(Kinney et al., 2006)*. According to our results, perinatal NMDAR antagonism instills disturbances in the GABAergic system like reduced PV and GAD67 expression that are consistently reported in *post mortem* brain of schizophrenic patients *(Lim et al., 2012)*. In animal studies, genetic ablations of NMDARs have been found to reduce PV and GAD67 in cortex and hippocampus *(Belforte et al., 2010)*. These results, in combination with studies in cultured neurons support the notion that NMDARs are necessary for maintaining PV and GAD67 expression in cortical interneurons, especially NR2A-containing NMDARs *(Kinney et al., 2006)*.

These results suggest that EE increases the expression of different NMDAR subunits and upregulates PSD-95. This can be due to increased number of synaptic boutons after EE or merely the insertion of new receptor in preexisting synapses. In MK-801+EE animals the insertion of new NMDARs would indicate enhanced NMDAR-mediated neurotransmission necessary for the synthesis of learning-related proteins, but

also for the expression of interneurons markers that are essential for the proper functioning of the brain.

6.9.2. BDNF-TrkB signaling

BDNF is a neurotrophic factor that has been related to cognitive functions, mainly learning and memory *(Yamada & Nabeshima, 2003; Bekinschtein et al., 2014)*. In human patients, the mRNA levels of BDNF and its receptor TrKB are decreased in prefrontal cortex *(Weickert et al., 2003; Weickert et al., 2005)*, hippocampus *(Durany et al., 2001)*, as well as plasma levels of BDNF *(Toyooka et al., 2002)*. Hippocampal reduction of BDNF, TrkB receptor and TrKB receptor phosphorylation in hippocampus of rats treated with MK-801 during development have also been documented *(Yang et al., 2014)*. In addition, decreased GABA synthesis in PV interneurons has been attributed to a decreased signaling of BDNF through TrkB receptor *(Lewis et al., 2005)*. In fact, mice hypomorphic for TrkB demonstrate a reduction in GAD67 and parvalbumin expression *(Hashimoto et al., 2005)*. Taken together, these findings indicate the relevance of BDNF-TrkB signaling in schizophrenia, as has been suggested previously *(Hashimoto et al., 2005)*.

It has been reported that hippocampal-specific deletion of the BDNF gene impairs novel object recognition as well as spatial learning in the water maze *(Heldt et al., 2007)*. Object-place association task is also impaired in BDNF lacking mice *(Aarse et al., 2016)*. Rats treated with MK-801 during development show hippocampal reduction of BDNF, TrkB receptor and TrKB receptor phosphorylation *(Yang et al., 2014)*. However, reduced expression of BDNF in hippocampus after MK-801 seems to take place over a short period of time *(Fumagalli et al., 2003)*. Hill et al., *(Hill et al., 2015)* showed a decrease in the BDNF levels in hippocampus 24h post-injection (0.05mg/kg MK-801) that was recovered at 48h in male Wistar rats. On the other hand, Guo et al., *(Guo et al., 2010)* administered MK-801 (0.5mg/kg twice daily) to Sprague-Dawley rats from postnatal day 5 to 14. BDNF protein levels in hippocampus and prefrontal cortex were analyzed on P15, P42 and P77. According to their results, different patterns of BDNF increase were found in prefrontal cortex and hippocampus: they found an early and transient increase in BNDF levels in prefrontal cortex after MK-801 injection, whereas a late increase was

observed in hippocampus. We failed to find any differences in BDNF levels in either region between vehicle and MK-801-treated rats, but the expression and activation of TrkB receptor was significantly decreased in mPFC, suggesting impaired BDNF-TrkB signaling in this region that could account for cognitive deficits. EE-related cognitive enhancement is elicited by increased availability of mature BDNF, thus prompting the hypothesis that upregulated BDNF-TrkB signaling might be a crucial neural mechanism that mediates the molecular, cellular and cognitive improvements that we observed after EE. In line with our results, EE promoted the upregulation of BDNF and subsequent phosphorylation of TrkB receptor.

The increase in interneurons markers after EE might be not only the consequence of enhanced NMDA-mediated neurotransmission, but also of upregulated BDNF-TrkB signaling. Primary neocortical cultures have shown increased NR1 subunits peptide levels in response to BDNF, and BDNF-mediated NR1 transcription depends upon induction of ERK pathway through activation of the TrKB receptor *(Kim et al., 2012)*. However, other neurotrophic factors could also be contributing to these modifications, such as NGF, a neurotrophic factor involved in the upregulation of NR1 promoter *(Liu et al., 2001)*. Vascular endothelial growth factor (VEGF) not only has effects on the vasculature, but also acts as a neurotrophic factor. Its role in synaptic function is poorly understood, but VEGF is known to be involved in synaptic plasticity. According to Licht et al., *(Licht et al., 2011)*, increases in VEGF expression improve memory, primarily by affecting synaptic plasticity, rather than promoting neurogenesis or vasculogenesis. In fact, silencing VEGF-R2 in neural cells impair hippocampal-dependent synaptic plasticity *(De Rossi et al., 2016)*.

6.9.3. ERK and Akt pathways

Several lines of evidence converge in implicating abnormal ERK and Akt activity in the pathogenesis of schizophrenia. *(Funk et al., 2012; Hino et al., 2016)*. ERK1/2 pathway is involved in the mechanisms of synaptic plasticity, learning and memory. ERK activation is required for the full expression of long-term potentiation (LTP), the principal cellular mechanism thought to underlie neuronal plasticity *(Giovannini, 2006)***.** ERK pathway is known to be a downstream signal transduction pathway that is common to the

NMDA and TrkB signaling pathways, and mediates short- and long-term effects of intracellular signaling in neurons *(Ishii et al., 2010)*. Abnormal activity of ERK-associated pathways has been described in frontal cortical areas of schizophrenic patients *(Funk et al., 2012)*, and the uncoupling of NMDAR-ERK pathway has been proposed as one of the mechanisms MK-801-induced damage. Moreover, NMDAR-mediated ERK signaling is involved in memory processing, including associative memories *(Al Rahim et al., 2009)*, and its lack of activation in MK-801-treated animals in dorsal hippocampus could account for the deficiencies in Object-in-Place task. Previous studies have also reported that MK-801 induces a decrease in the level of ERK phosphorylation *(Al Rahim et al., 2009)* that seems to be exclusive of hippocampus, because according to Ishii et al., 1.0 mg/kg treatment with MK-801 did not affect pERK/ERK ratio in frontal cortex of mice *(Ishii et al., 2010)***.** In line with this, Goeldner et al., *(Goeldner et al., 2008)* showed that MK-801 concomitantly suppressed ERK activation in hippocampus and disrupted recognition memory.

A variety of studies have addressed the phosphorylation levels of Akt pathways in MK-801 animal model of schizophrenia. Results are diverse and highly depend on the dosing and timing of the treatment. According to Ahn et al., *(Ahn et al., 2005)* phosphorylation of Ser473-Akt was increased after injection of 1 mg/kg of MK-801 in the rat frontal cortex but not in hippocampus. This increase peaked at 30 min and was maintained until 90 min after injection. The phosphorylation showed a dose-dependent increase up to 1 mg/kg MK-801, followed by a decrease at higher dosage. Moreover, same authors later found that administration of MK-801 from 0.25 to 1 mg/kg caused ERK phosphorylation to decrease, but Akt-GSK-3 phosphorylation to increase in frontal cortex of rats *(Ahn et al., 2006)*, which is consistent with our results. Similarly, Seo et al., *(Seo et al., 2007)* observed that repeated administration of 0.5, 1, and 2 mg/kg of MK-801 increased the phosphorylation levels of Akt-GSK-3beta pathway in the rat frontal cortex. Although it is still unclear why, it might be a compensatory mechanism to counteract the prefrontal hyperactivity, as Akt-mediated phosphorylation increases the number of $GABA_AR$ on the plasma membrane surface, thereby increasing fast inhibitory neurotransmission. In this respect, it is worth noting that adult exposure to EE normalized phosphorylation levels of Akt and ERK in previously MK-801-treated rats.

6.9.4. $GABA_A$ receptor subunit β2/3

The inhibitory neurotransmitter GABA can act through $GABA_A$ ionotropic receptors or $GABA_B$ metabotropic receptors. $GABA_A$ receptors are ligand-gated chloride channels, which usually reduce the membrane excitability by reducing the membrane potential to chloride potential, and thus inhibiting neurotransmitter release. The β subunit of the $GABA_AR$ is vital for the regulation of ion selectivity and general properties of the chloride channel *(Chang et al., 1996)*. Beneyto et al., *(Beneyto et al., 2011)* observed a reduction in mRNA for the β2-subunit in DLPFC of subjects with schizophrenia. However, there have been conflicting results regarding mRNA levels of β3-subunit. According to Hashimoto et al., *(Hashimoto et al., 2008)* this protein was reduced in DLPFC, but a separate study found no change *(Beneyto et al., 2011)*. Similarly, Bullock et al., *(Bullock et al., 2008)* did not found differences in $GABA_AR$ β3-subunit mRNA expression in lateral cerebella of subject with schizophrenia, but Fatemi et al., *(Fatemi et al., 2013)* found an increase.

On the other hand, Kim et al., *(Kim et al., 2000)* demonstrated that chronic blockade of calcium influx though NMDAR with MK-801 reduced β3-subunit and increased β2-subunit mRNA in hippocampus, but they failed to find differences in protein expression. In this study, we found downregulated expression of β2/3, which is consistent with previous studies of rats chronically injected with MK-801 *(Matthews et al., 2000)*, although our current data supports larger changes in medial prefrontal cortex than in hippocampus. GABRβ3 KO mice display deficits in learning and memory *(DeLorey et al., 2008)*, suggesting that reduced expression of this subunit is relevant to the cognitive symptomatology of schizophrenia.

Electrophysiological studies have shown that NMDAR activation is essential for $GABA_AR$-mediated inhibition in the developing neocortex *(Chan & Yeh, 2013)*, suggesting that alterations in $GABA_AR$ subunits could be in part accounted for changes in NMDARs, although this viewpoint is speculative. Previous studies have reported that environmental stimulation increases $GABA_ARs$ *(He et al., 2010)*, including subunit β3 *(Cai et al., 2010)*, which is consistent with our results. However, those changes have not been previously described in the literature in MK-801 neurodevelopmental model of schizophrenia.

6.10. General discussion and future perspectives

The present study reveals new insights into the beneficial effects of adult life exposure to EE of neonatally MK-801-treated animals as a strategy to improve cognitive impairments and neurochemical alterations relevant to schizophrenia. NMDAR hypofunction is one of the leading hypothesis in the field of schizophrenia to explain the neurochemical, metabolic, functional and behavioral alterations of the disease *(Snyder & Gao, 2013; Weickert et al., 2013)*. NMDAR antagonists, including MK-801, lead to a decrease in GABAergic interneurons and behaviors reminiscent to schizophrenia in animals *(Olney & Farber, 1995; Abekawa et al., 2007; Murueta-Goyena et al., 2017)*. In this study, we demonstrate that adult short-exposure to EE is able to improve most of the pathophysiological findings related to MK-801 administration.

NMDAR blockade in perinatal period confers long-lasting brain and behavioral changes that recapitulate the human schizophrenia phenotype *(Bubeníková-Valesová et al., 2008; Lim et al., 2012)*. In early postnatal development, GABAergic cells express more NMDARs than in late neurodevelopment, especially PV+ interneurons, and they are thought to be particularly sensitive to NMDAR blockade *(Grunze et al., 1996)*. Even though the precise mechanism for this selectivity is unknown, it could be accounted for the higher open probability of fast-spiking cells (PV+ interneurons) and the subunit composition of NMDARs that might make them especially vulnerable *(Kotermanski et al., 2009; Wang & Gao, 2009)*. Reduction on inhibitory tone during development would impair cortical maturation process and lead to increased excitability of cortical circuits, as seen in schizophrenic patients. Indeed, studies in human patients show abnormal hippocampal activity *(Heckers & Konradi, 2015)*. This is consistent with the presence of glutamate metabolites in schizophrenic patients before the onset of the disease *(Merritt et al., 2016)*. Accordingly, the administration of NMDAR antagonists perturbs the maturation of GABAergic circuits, including disrupted synaptic integration in early brain development and modified network activity and plasticity in adulthood. NMDAR blockade also generates a dyssynchrony in gamma oscillations, thus disturbs attention maintenance, executive functions and overall cognition in humans and rodents. According to the current literature, there is unifying hypothesis of NMDAR hypofunction in schizophrenia, and this might take place at two dissociable time points *(Nakazawa et al., 2017)*. The first NMDAR hypofunction might occur in GABAergic cells in early

development, as previously described, and a second period of NMDAR hypofunction will result during adolescence by internalizing NMDARs, in an attempt to counterbalance the overly activated cortical and hippocampal circuits. This second NMDAR hypofunction is probably no longer selective of GABAergic cells, and presumably takes place at pyramidal cells. This could explain why NMDAR deletion from PV interneurons does not completely recapitulate the disease *(Bygrave et al., 2016)* (Figure 47).

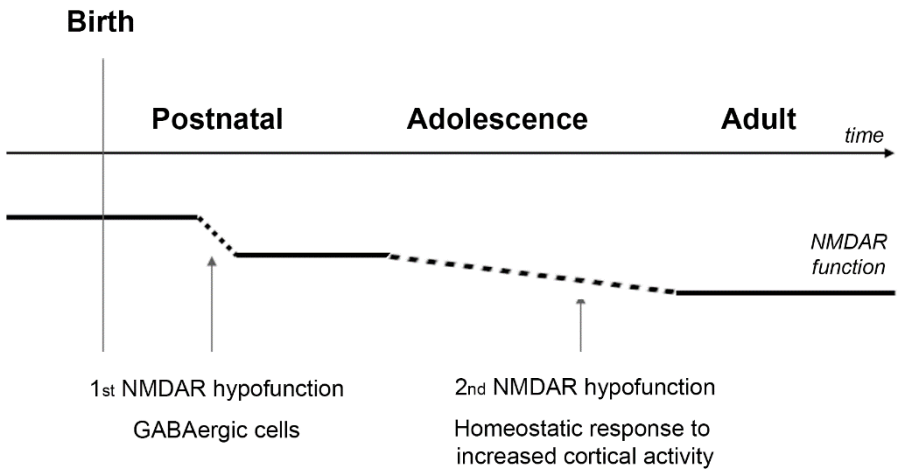


Figure 47. Dual NMDAR hypofunction hypothesis. During early development NMDAR hypofunction in GABAergic cells could cause disinhibition of pyramidal cells that in turn would lead to a generalized homeostatic downregulation of NMDARs. Extracted and adapted from: Nakazawa et al. NPJ Schizophr. 2017.

The complex interaction of genetic, epigenetic and environmental factors that contribute to the onset of schizophrenia and the lack of consistent molecular basis in human patients make difficult to find effective treatments. Environment affects the symptomatology of schizophrenia, as isolation usually worsen them. In addition, enriched environment increases neurotrophic factors, upregulates the expression of neurotransmitter receptors, increases the complexity of dendritic branching and enhances learning and memory processes *(van Praag et al., 2000)*, all of which are disturbed in schizophrenia. Based on those findings, we proposed that rearing adult animals in EE could be successful in managing schizophrenia-related alterations. The major challenge

of the present study was to modify enduring cellular and molecular alterations. Interestingly, our findings suggest that short-term EE exposure in adulthood may be a useful approach to manage enduring perturbations linked to schizophrenia and shed new light on the neurobiological modifications used by EE to reverse the actions of MK-801 (Figure 48). In this respect, increased BDNF-TrkB signaling and upregulated expression NMDARs after EE were associated with improved structural and cellular findings and better cognitive outcomes.

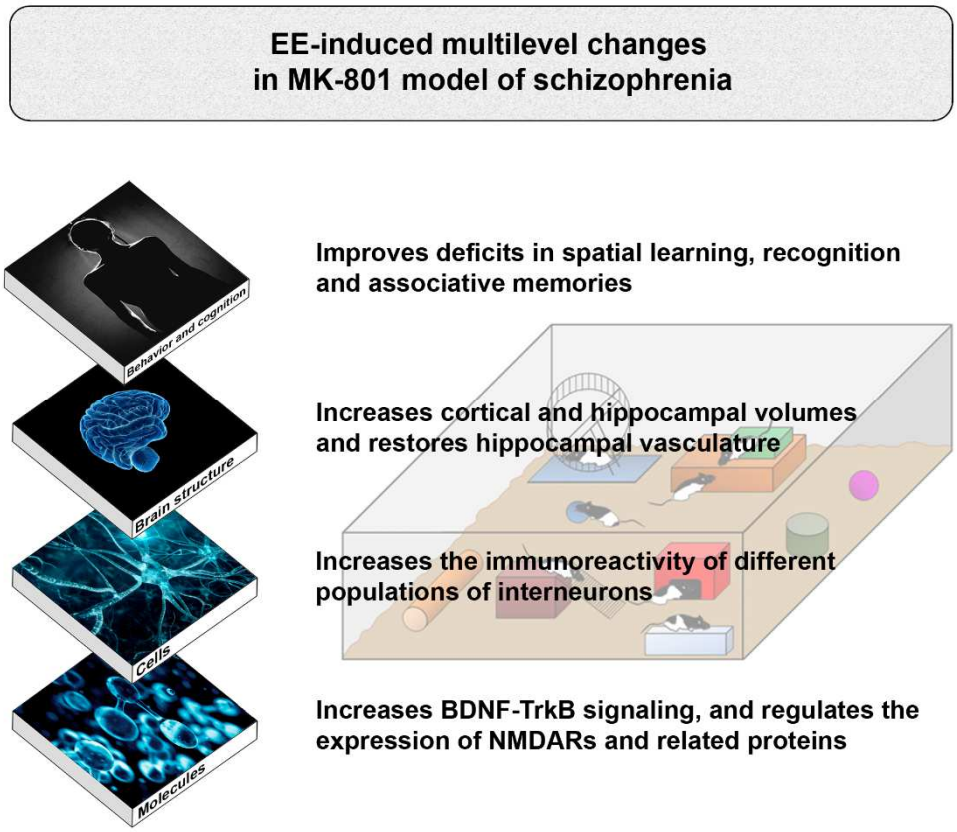


Figure 48. Major modifications following exposure to enriched environment (EE) in adulthood of postnatally MK-801-treated animals. EE-induced changes range from behavior and cognition to structural brain changes, cells and protein expression.

In spite of the evidences of this study linking adult intervention with EE to improved cognitive performance in a neurodevelopmental animal model of schizophrenia, several aspects need to be further investigated. Firstly, although we found concomitant increase in BDNF-TrkB signaling and neurochemical and behavioral modifications, we did not determine the mechanism of action and the cause-effect relationship between these factors. In addition, we focused this study in adult animals and we used short intervention with environmental enrichment. However, it would be important to explore the temporal boundaries of MK-801 and EE-induced changes, like when the functional downregulation of NR1 occurs or determine if earlier and/or longer environmental intervention would result in different functional outcomes. Moreover, monitoring the animals while housed in EE would help to better establish the relationship between activity and functional outcomes after EE. Finally, NMDAR hypofunction contributes to the manifestation of the disorder, but the etiopathology of schizophrenia itself remains unexplained. A complex combination of genetic, epigenetic and environmental factors could render NMDARs hypoactive, and a better understanding of the neurobiological changes is required to further support of EE as a possible intervention for schizophrenia. While future studies are required to address these issues, our results establish the foundation for future research in environmental interventions and open possibilities for novel therapeutic targets.

7. Conclusions

1. Administration of MK-801 (0.5mg/kg) during early postnatal development impaired spatial learning in Morris Water Maze, decreased discrimination index in Novel Object Recognition task and altered object-place associative memory assessed in Object-in-Place task.

2. Cortical and hippocampal volumes were decreased in MK-801 group. However, total microvascular length was only diminished in hippocampus.

3. Interneurons expressing parvalbumin (PV), calretinin (CR), somatostatin (SST) were decreased in mPFC and hippocampus secondary to NMDAR antagonism, measured by stereological means. Similarly, the total number of inhibitory cells expressing GAD67 was significantly decreased in hippocampus, although the overall number of neurons was unaltered after MK-801 treatment.

4. Western Blot analysis showed a decrease in the obligatory subunit NR1 of NMDAR in MK-801-treated animals without altering the expression levels of NR2A and NR2B subunits or PSD-95. Similarly, $GABA_AR$ subunit ß2/3 was decreased because of NMDAR blockade.

5. Exposure to Environmental Enrichment (EE) in adult life (P55-P73) improved the above-mentioned parameters. Environmental intervention restored spatial learning and recognition memory impairment. However, object-place associative memory was partially reversed. In a similar vein, it promoted the increase in cortical and hippocampal volumes, as well as in the length of the microvasculature. Similarly, the expression of interneurons markers was increased after EE.

6. Adult environmental intervention triggered a set of modifications on the components of glutamatergic neurotransmission, including the upregulation of the deficient NR1 subunit and NR2B-containing NMDARs with a parallel increase of the scaffolding protein PSD-95 and hippocampal NR2A subunit expression, as well as upregulation of inhibitory drive by increasing $GABA_AR$ subunit ß2/3 expression.

7. Intraperitoneal injections of MK-801 on early life induced long-lasting BDNF-TrkB signaling disruption and upregulated Akt activation on mPFC with parallel downregulation of ERK pathway activation in dorsal hippocampus, and all these effects were counteracted by short-term exposure to EE in adulthood.

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Appendix 1

SOLUTIONS

4% Paraformaldehyde

- Add 800 mL of PBS 0.1M to a glass beaker on a stir plate in a ventilated hood. Heat while stirring to approximately 60 °C.
- Add 40 g of paraformaldehyde powder (Ref: 141451.1211, Panreac, Spain) to the heated PBS solution.
- Add 7-8 tablets of NaOH (Sodium hydroxide, Ref: 30620, Sigma-Aldrich, Spain) to raise the pH.
- Once the paraformaldehyde is dissolved, cool the solution.
- Adjust the volume of the solution to 1 L with PBS 0.1M.
- Adjust the pH to 7.4 with 37% hydrochloric acid (Ref: 131020.1611, Panreac, Spain)
- Filter the solution.

Sodium Chloride 0.9%

- 9 g of Sodium Chloride (Ref: 31434, Sigma-Aldrich, Spain) to 1L of H_2O_d.
- Stir until the solution completely dissolves.

30% sucrose solution

- 150g of sucrose (Ref: 131621.1211, Panreac, Spain) in 350ml of PBS 0.1M
- Mix and adjust the volume to 500 ml.

PBS 1M

- Dissolve the following in 1L of H_2O_d:
    1. 3,45g Sodium phosphate monobasic (Ref: S9638, Sigma-Aldrich, Spain)

2. 10.65g Sodium phosphate dibasic (Ref: S9763, Sigma-Aldrich, spain)
3. 83g Sodium Chloride (Ref: 31434, Sigma-Aldrich, Spain)

- Mix to dissolve and adjust pH to 7.4
- Store this solution at room temperature

PBS 0.1M

- Dissolve 100 ml of PBS 1M in 900 ml of deionized water.
- Adjust pH to 7.4 if necessary.

PBS 0.1M + 0.5% Triton X-100

- Add 500 µL of Triton X-100 (Ref: T9284, Sigma-Aldrich, Spain) in 100 ml of PBS 0.1M.

PBS 0.1M + 4% sodium azide

- Add 2g of sodium azide (Ref: S-2002, Sigma-Aldrich, Spain) to 50 ml of distilled water.
- Use 1ml of previous solution and mix with 200 ml of PBS 0.1M.

TrisHCl 0.05M

- 0,605g Trizma Base (Ref: T-1503, Sigma-Aldrich, Spain)
- 100 ml of distilled water
- Adjust pH to 7.4 with 37% HCl (Ref: 131020.1611, Panreac, Spain)

Sodium Citrate Buffer

- Solution A: 21,10g of citric acid (Ref: 3655188, Sigma-Aldrich, Spain) in 1L of H_2O_d

- Solution B: 29,41g of sodium citrate (Ref: 6448, Merck, Germany) in 1L of H_2O_d
- Mix 9ml of solution A with 41ml of solution B in 450ml of H_2O_d

DAB (50mg/ml)

- Mix 50 mg of DAB (Ref: D-5637, Sigma-Aldrich, Spain) for every ml of H_2O_d
- Use laboratory hood.

Pretreatment solution for BrdU IHC

- Saline-Sodium Citrate (20x SSC):
  a) 175,3g NaCl (Ref: 31434, Sigma-Aldrich, Spain)
  b) 88,2g Tri-Sodium Citrate (Ref: S1804, Sigma-Aldrich, Spain)
  c) 800 ml H_2O_d.
  d) Adjust the pH to 7 and add 200ml of H_2O_d

- Use 50 ml of SSC 20x
- Add 250ml of formamide (Ref: 1.04008.1000, Merck, Germany).
- Add 200ml H_2O_d

Denaturing solution of DNA for BrdU IHC (2N HCl)

- 10N HCl → 365g HCl en 1L H_2O_d
- Use 20ml of 10N HCl and add 80ml of H_2O_d.

Preparation of gelatin-coated slides

- Prepare the gelatin-coating solution by dissolving 3g of gelatin (Ref: 4078.500, Merck, Germany) in 250 ml of heated, deionized H_2O (60-80ºC)
- After the gelatin has dissolved, add 0,125mg of chromium (III) potassium sulfate dodecahydrate (Ref: 1035, Merck, Germany). Chromium potassium sulfate

dodecahydrate will positively charge the slides allowing them to attract negatively charged tissue sections.

- Stir until solution is completely dissolved.
- Place the histological slides into racks made of glass.
- Dip the racks containing the slides (~10 seconds) into the gelatin-coating solution.
- Remove the racks containing the slides and let them drain. Blot excess solution from the racks onto filter paper (gently tap the racks against the filter paper for better drainage).
- Place the racks containing the slides on the oven at 37ºC for 24-48 hours.
- Put back dried slides into the original boxes, and store at room temperature until use.

Tris Maleate Buffer

- 12,1g Trizma Base (Ref: T-1503, Sigma-Aldrich, Spain)
- 11,6g Maleic Acid (Ref: M-0375, Sigma-Aldrich, Spain)
- 1L of H_2O_d

Adjust pH to 6.0 with NaOH (Sodium hydroxide, Ref: 30620, Sigma-Aldrich, Spain) pills.

Inhibitory solution for butyrylcholinesterase histochemistry

- 50ml of Tris Maleate Buffer (pH 6.0)
- 1,416mg of BW284CS1 0,05M (1,5-bis (4-allyldimethylammoniumphenyl)-pentan-3-one dibromide) (Ref: A-9013, Sigma-Aldrich, Spain).

Incubation Solution

- 32,5ml Tris Maleate 0.1M (pH 6.0)
- 50mg Butyryl thiocholine iodide (1mg/ml)
- 2,5ml Sodium citrate 0.1M (Ref: 6448, Merck, Germany)
- 5ml Cooper sulphate 30mM – dropwise

- 5ml Potassium ferricyanide (5mM) – dropwise
- 5ml of H_2O_d

RIPA Lysis Buffer

- 50 mM Trizma Base (Ref: T-1503, Sigma-Aldrich, Spain) - 0,302g
- 150 mM NaCl (Ref: 31434, Sigma-Aldrich, Spain) - 0,438g
- 0.5% Sodium Deoxycholate (Ref: D6750, Sigma-Aldrich, Spain) - 0,25g
- 0.1% SDS (Dodecyl sulfate sodium, Ref:1.13760.0100, Merck, Germany) - 0,05g
- 1% Triton X-100 - 0,5ml

Add 15 µL of protease inhibitor (Ref: P3840, Sigma-Aldrich, Spain) every 1 ml lysis buffer

LAEMMLI Sample Buffer 4x

- 1,25ml Tris 2M pH: 6.8
- 4 ml glycerol 100%
- 2,67ml 30% SDS (Dodecyl sulfate sodium salt, Ref: 1.13760.0100, Merck, Germany)
- 83 µl 1% BFB (Bromophenol Blue, Ref: B-5525, Sigma-Aldrich, Spain)

Add 100 µl of 2-mercaptoethanol (Ref: 8.05740.0250, Merck, Germany) for every 400 µl of SB4x.

Running buffer (EB) 10x

- 15,5g Trizma Base (Ref: T1503, Sigma-Aldrich, Spain)
- 72g Glycine (Ref: G8818, Sigma-Aldrich, Spain)
- 500ml H_2O_d

Store at 4ºC

Running buffer (EB) 1x

- Dilute EB 10x (100ml EB10x + 900 ml H_2O_d).
- Add 10 ml of 10% SDS.

Transfer Buffer (for wet transfer)

- 900ml of EB 1x
- 100 ml of methanol (Ref: 131091.1211, Panreac, Spain).

TBS 5x

- 6,08g Trizma Base (Ref: T1503, Sigma-Aldrich, Spain)
- 20g NaCl (Ref: 31434, Sigma-Aldrich, Spain)
- Adjust the volume to 500ml with H_2O_d
- Adjust pH to 7,6

TBST

- 100 ml TBS 5x
- 400 ml H_2O_d
- 500 µl Tween-20 (Ref: P1379, Sigma-Aldrich, Spain)

Appendix 2

Number of interneurons, total neuronal cells and vessel length estimation in medial prefrontal cortex (PL: prelimbic region, AC: anterior cingulate cortex) and hippocampus (CA1: Cornus Ammonis 1 of hippocampus, DG: dentate gyrus) in the four experimental groups. Mean ± standard deviation (SD).

Parvalbumin (PV)

		PV PL	PV AC	PV CA1	PV DG
VH	Mean	11016	13601	5332	1357
	SD	3771	5395	1624	158
MK-801	Mean	4818	3711	1705	812
	SD	1564	1226	669	294
MK-801+EE	Mean	9933	14611	3235	1360
	SD	2609	4681	901	156
VH+EE	Mean	15990	19317	4573	1547
	SD	4397	6002	1402	450

Calretinin (CR)

		CR PL	CR AC	CR CA1	CR DG
VH	Mean	11747	18162	6745	2063
	SD	3499	3238	955	336
MK-801	Mean	7088	10052	5033	2465
	SD	1415	1855	685	137
MK-801+EE	Mean	9278	16069	6322	2095
	SD	1389	1954	1643	626
VH+EE	Mean	12478	20262	6825	2640
	SD	2847	3895	670	557

Calbindin (CB)

		CB PL	CB AC	CB CA1	CB DG
VH	Mean	10571	10213	4962	1739
	SD	4727	5295	752	364
MK801	Mean	11072	12793	3782	1615
	SD	3713	2435	1327	408
MK-801+EE	Mean	12711	12673	5213	1716
	SD	5666	2024	1607	293
VH+EE	Mean	14759	26197	4693	1603
	SD	5549	23122	1116	661

Somatostatin (SST)

		SST PL	SST AC	SST CA1	SST DG
VH	Mean	28762	40410	11273	9756
	SD	4363	7467	1022	1509
MK801	Mean	20399	31632	8529	8641
	SD	2791	7048	1255	1214
MK-801+EE	Mean	26058	38754	9595	8556
	SD	3645	4672	1173	1038
VH+EE	Mean	28839	41296	10557	9895
	SD	3328	3968	917	473

Total interneurons number (GAD67)

		GAD67 PL	GAD67 AC	GAD67 CA1	GAD67 DG
VH	Mean	44468	41808	25809	15338
	SD	12714	8523	4103	1895
MK801	Mean	33859	37605	16298	11223
	SD	6643	5730	3131	1999
MK-801+EE	Mean	35960	44964	24255	19188
	SD	7611	12305	6384	4100
VH+EE	Mean	54883	59622	26702	16060
	SD	13456	10817	5292	3428

Total neuronal number (NeuN)

		NeuN PL	NeuN AC	NeuN CA1	NeuN DG
VH	Mean	405206	549775	44764	36657
	SD	34813	67933	5718	4745
MK801	Mean	337989	537363	43251	31975
	SD	57216	56291	7543	6059
MK-801+EE	Mean	379244	541020	45313	38117
	SD	35628	70440	6795	4205
VH+EE	Mean	376755	570172	45609	34938
	SD	45755	31888	8210	5075

Vessel length (BCh histochemistry)

		BCh mPFC	BCh HPC
VH	Mean	26930870	27880545
	SD	1265949	1665099
MK-801	Mean	30062633	23425169
	SD	2986637	2159999
MK-801+EE	Mean	27436287	28213173
	SD	3168025	2655144
VH+EE	Mean	30136982	29073083
	SD	1654461	2595177

Number of proliferative cells in the subgranular zone of dentate gyrus of the hippocampus (BrdU+) and number of immature granule cells (CR+ cells) in granular cell layer of dentate gyrus in the four experimental groups. Mean ± standard deviation (SD).

BrdU+ and CR+ cells in granule cell layer

		BrdU+	CR+ granular layer
VH	Mean	116	587
	SD	20	118
MK-801	Mean	168	712
	SD	35	200
MK-801+EE	Mean	166	715
	SD	33	271
VH+EE	Mean	156	1063
	SD	20	274

Appendix 3

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Short-Term Exposure to Enriched Environment in Adult Rats Restores MK-801-Induced Cognitive Deficits and GABAergic Interneuron Immunoreactivity Loss

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Abstract Perinatal injections of *N*-methyl-D-aspartate (NMDA) receptor antagonist in rodents emulate some cognitive impairments and neurochemical alterations, such as decreased GABAergic (gamma aminobutyric acid) interneuron immunoreactivity, also found in schizophrenia. These features are pervasive, and developing neuroprotective or neurorestorative strategies is of special interest. In this work, we aimed to investigate if a short exposure to enriched environment (EE) in early adulthood (P55–P73) was an effective strategy to improve cognitive dysfunction and to restore interneuron expression in medial prefrontal cortex (mPFC) and hippocampus (HPC). For that purpose, we administered MK-801 intraperitoneally to Long Evans rats from postnatal days 10 to 20. Twenty-four hours after the last injection, MK-801 produced a transient decrease in spontaneous motor activity and exploration, but those abnormalities were absent at P24 and P55. The open field test on P73 manifested that EE reduced anxiety-like behavior. In addition, MK-801-treated rats showed cognitive impairment in novel object recognition test that was reversed by EE. We quantified different interneuron populations based on their calcium-binding protein expression (parvalbumin, calretinin, and calbindin), glutamic acid decarboxylase 67, and neuronal nuclei-positive cells by means of unbiased stereology and found that EE enhanced interneuron immunoreactivity up to normal values in MK-801-treated rats. Our results demonstrate that a timely intervention with EE is a powerful tool to reverse long-lasting changes in cognition and neurochemical markers of interneurons in an animal model of schizophrenia.

Keywords MK-801 · Interneurons · Cognitive dysfunction · Calcium-binding proteins · Enriched environment

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Introduction

Interneurons are the main source of inhibitory input in the central nervous system, and gamma aminobutyric acid (GABA) is their primary neurotransmitter. Although GABAergic interneurons only account for 10–25% of total cell number, depending on the brain region, they display extremely distinct chemical, morphological, and functional features, making their classification a challenging task. It has been postulated that the rich variety of interneurons is essential for providing constant matched inhibitory input to the remarkably diverse incoming stimuli [1]. In addition, the computational diversity they provide allows proper dynamics for higher cognitive functions. Nevertheless, GABAergic interneurons also play an important role during postnatal development [2]. GABA promotes the migration of glutamatergic and GABAergic neurons and dictates the final location of different subpopulations of interneurons. GABA also

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Neuropathological Background of MK-801 for Inducing Murine Model of Schizophrenia

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Abstract

Schizophrenia is a complex psychiatric disorder with a developmental component that compromises neural circuits. Understanding the neuropathological basis of schizophrenia remains a major challenge for establishing new therapeutic approaches. In this review, causal factors for abnormal brain development in schizophrenia are discussed, with particular focus on N-methyl-D-aspartate (NMDA) receptor hypofunction and GABAergic circuit-mediated neurotransmission. Changes in interneuron structure and function have been reported in schizophrenia, and current evidence points to a specific involvement of interneuronal NMDA receptor signaling. Furthermore, altered gamma-band oscillations in schizophrenic patients drew attention to a possible deficit in fast-spiking parvalbumin-expressing interneurons, which play an essential role in regulating complex interaction between pyramidal cells, and represent a key to the understanding of network operations. Here, we describe the major biochemical, neuropathological, and cognitive deficits present in schizophrenic human individuals, and the faithfulness of animal models for mimicking those impairments. In NMDA receptor antagonism-based animal models, repeated injections of MK-801 (dizocilpine) during early postnatal brain development, disrupt the excitation/inhibition balance.

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Murueta-Goyena A, Gallastegui M, Pulido L, Ortuzar N, Lafuente JV, Bengoetxea H.

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UNTANGLING HIPPOCAMPAL CHANGES IN A NEURODEVELOPMENTAL MODEL OF SCHIZOPHRENIA: THE ROLE OF ENRICHED ENVIRONMENT
Ane Murueta-Goyena, José Vicente Lafuente, Bengoetxea Harkaitz
Faculty of Medicine and Nursing. University of the Basque Country (UPV/EHU), Leioa (Bizkaia), Spain

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