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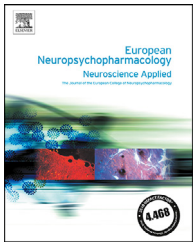

Pimavanserin exhibits serotonin 5-HT_{2A} receptor inverse agonism for $G_{\alpha i1}$- and neutral antagonism for $G_{\alpha q/11}$-proteins in human brain cortex

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Abstract
Pimavanserin is claimed as the first antipsychotic drug that shows selectivity for serotonin 5-HT_2 receptors (5-HT_2Rs) and lacks of affinity for dopamine D_2 receptors (D_2Rs). Cell-based functional assays suggest that pimavanserin and antipsychotics with D_2R/5-HT_2R affinity could act as inverse agonists of 5-HT_{2A}Rs. However, there is not evidence of such pharmacological profile in native brain tissue. 5-HT_{2A}Rs are able to engage both canonical $G_{\alpha q/11}$- and non-canonical $G_{\alpha i1}$-proteins. In the present study, the response to pimavanserin of the 5-HT_{2A}R coupling to $G_{\alpha q/11}$- and $G_{\alpha i1}$-proteins was measured in membranes of *postmortem* human prefrontal cortex by antibody-capture [^{35}S]GTPγS binding scintillation proximity assays. Pimavanserin promoted a concentration-dependant inhibition of the 5-HT_{2A}R coupling to $G_{\alpha i1}$-proteins whereas the response of $G_{\alpha q/11}$-proteins was unaltered, suggesting inverse agonism and neutral antagonism properties, respectively. The inhibition was abolished in the presence of the selective 5-HT_{2A}R antagonist MDL-11,939 and was absent in brain cortex of 5-HT_{2A}R *knock-out* mice when compared to respective 5-HT_{2A}R *wild-type* animals. In conclusion, the results demonstrate the existence of constitutive 5-HT_{2A}R activity in human brain for the signalling pathway mediated by $G_{\alpha i1}$-proteins. Pimavanserin demonstrates 5-HT_{2A}R functional selectivity and exhibits inverse agonist profile towards $G_{\alpha i1}$-proteins, which is considered the effector pathway promoting hallucinogenic responses. In contrast, pimavanserin behaves as neutral antagonist on the 5-HT_{2A}R

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coupling to the canonical $G_{\alpha q/11}$-protein pathway. The results strengthen the relevance of inverse agonism as potential mechanism of antipsychotic activity. Moreover, the existence of functional selectivity of 5-HT_{2A}Rs for different G_{α}-proteins could contribute to better design of 5-HT_{2A}R-related antipsychotic drugs.

1. Introduction

The development of new antipsychotic drugs with improved effectiveness, better impact on functional impairment, and less side effects represents a challenge for the current treatment of schizophrenia and related disorders. Traditionally, antipsychotic efficacy is considered to be mediated by antagonism of the dopamine D_2 receptor (D_2R). Most of second-generation or "atypical" antipsychotic drugs have in common a higher affinity for the serotonin 5-HT_{2A} receptor (5-HT_{2A}R) than D_2Rs (Meltzer, 1999). Nonetheless, the lack of efficacy of monotherapy with selective 5-HT_{2A}R antagonists (volinanserin or MDL-100,907) led to thought that 5-HT_{2A}R actions are incremental whereas affinity for D_2Rs represents the essential component of antipsychotic activity (Miyamoto et al., 2005). Recently, a potent and selective 5-HT_{2A}R drug, pimavanserin (ACP-103), was developed as a new alternative for the treatment of psychosis (Meltzer and Roth, 2013). Pimavanserin displays nanomolar potency as 5-HT_{2A}R inverse agonist, selectivity for 5-HT_{2A}Rs over 5-HT_{2C} receptors (5-HT_{2C}Rs), and no significant activity at any other G-protein coupled receptor (GPCR) (Vanover et al., 2006). Until now, pimavanserin has been studied as adjunctive therapy for schizophrenia (Meltzer et al., 2012) and has gained FDA approval to reduce delusions and hallucinations in Parkinson's disease (Cummings et al., 2014). In these patients, atypical antipsychotics administration could worsen efficacy of standard anti-parkinsonian drugs due to their antagonism on D_2R family. Besides, a pilot study demonstrated marked response to pimavanserin of refractory positive symptoms in clozapine-nonresponsive patients (Nasrallah et al., 2019). Therefore, pimavanserin is claimed as the first 5-HT_{2A}R drug that lacks of D_2R affinity but shows antipsychotic activity (Hacksell et al., 2014). The evaluation of pimavanserin pharmacological profile suggests that could act as inverse agonist of 5-HT_{2A}Rs (Vanover et al., 2006). However, the limited information on its inverse agonism properties have raised important concerns and prompted to the search of better evidence in human brain before to conclude that pimavanserin behaves as inverse agonist of 5-HT_{2A}Rs (Nutt et al., 2017).

Inverse agonism is considered the pharmacological property of a drug to antagonize the action of agonists and simultaneously lower the basal constitutive activity of receptor signalling. Different studies have hypothesized that clinical activity of atypical antipsychotics is due to their inverse agonist properties at 5-HT_{2A}Rs and 5-HT_{2C}Rs (Sullivan et al., 2015; Weiner et al., 2001). However, it is noteworthy to remark that the negative intrinsic activity of these drugs has been described in cell culture assays where increased constitutive activity is usually present. In contrast, experiments with native tissue are scarce and the existence of constitutive 5-HT_{2A}R activity in brain is still a matter of debate (Aloyo et al., 2009; De Deurwaerdère et al., 2020). In this context, it is uncertain if the clinical effect of pimavanserin is due to its inverse agonism properties on 5-HT_{2A}Rs (Nutt et al., 2017).

GPCRs, and amongst them the 5-HT_{2A}R, can be activated by chemically distinct drugs to preferentially engage different signalling pathways. This mechanism is termed biased agonism or functional selectivity (Berg and Clarke, 2018). For instance, in brain, the 5-HT_{2A}R is able to discriminate activation of $G_{\alpha q/11}$-, $G_{\alpha i/o}$-proteins and other signalling pathways in response to diverse agonists (González-Maeso et al., 2007; Schmid and Bohn, 2010). Thus, 5-HT_{2A}R agonist drugs with hallucinogenic properties such as lysergic acid diethylamide (LSD), psilocybin, mescaline and (±)DOI promote activation of the canonical $G_{\alpha q/11}$-protein cascade as well as $G_{\alpha i/o}$-protein-mediated signalling pathways. In contrast, chemically analogous 5-HT_{2A}R agonists lacking of hallucinogenic properties (lisuride, ergotamine) only stimulate $G_{\alpha q/11}$-protein-dependant pathways (González-Maeso et al., 2007; López-Giménez and González-Maeso, 2018). These findings suggest that the fingerprint of pro-hallucinogenic and, perhaps, anti-hallucinogenic properties of 5-HT_{2A}R drugs depend on modulation of $G_{\alpha i/o}$-proteins and their downstream signalling pathways (López-Giménez and González-Maeso, 2018). Recently, supersensitivity of the 5-HT_{2A}R coupling to $G_{\alpha i1}$-proteins but not to $G_{\alpha q/11}$-proteins in *postmortem* brain of subjects with schizophrenia has been described (Garcia-Bea et al., 2019). This result provides further support for the suggestion that selective 5-HT_{2A}R antagonists/inverse agonists with functional selectivity on $G_{\alpha i1}$-proteins might be useful tools as antipsychotic drugs.

In the present study, the pharmacological profile of pimavanserin was investigated to elucidate whether this antipsychotic drug behaves as antagonist or inverse agonist of 5-HT_{2A}R in native *postmortem* human brain tissue. The modulation of 5-HT_{2A}R coupling to $G_{\alpha q/11}$- and $G_{\alpha i1}$-proteins in response to pimavanserin was measured to clarify the existence of putative functional selectivity of this drug towards pro-hallucinogenic pathways.

2. Experimental procedures

2.1. Brain cortex membranes preparation

Human brain samples were obtained at autopsy in the Basque Institute of Legal Medicine, Bilbao, Spain, in compliance with poli-

cies of research and ethical boards for *postmortem* brain studies at the moment of sample obtaining. All the subjects were determined to be free of neurological and psychiatric disorders based on medical records and *postmortem* tissue examinations. Positive blood toxicology for drugs or ethanol was considered exclusion criteria. Samples from the dorsolateral prefrontal cortex were dissected at autopsy following established protocols (Muguruza et al., 2013) and immediately stored at −70 °C until assay. Samples of six different subjects (5 males and 1 female) with ages between 29–77 years were included. The *postmortem* delay between death and storage of the samples ranged from 7 to 29 h.

The 5-HT$_{2A}$R *knock-out* (5-HT$_{2A}$R$^{(-/-)}$) and *wild-type* (5-HT$_{2A}$R$^{(+/+)}$) mice were generously donated by Prof. R. Maldonado (Barcelona, Spain). Animals were originally generated on a 129S6/SvEv background and subsequently backcrossed onto the inbred C57BL/6J line following standard procedures (González-Maeso et al., 2007; Viñals et al., 2015). After donation, supplementary backcrosses were performed. Animals were genotyped by polymerase chain reaction (PCR). Absence of 5-HT$_{2A}$R expression in 5-HT$_{2A}$R$^{(-/-)}$ mice was confirmed by the lack of [^{3}H]ketanserin binding (Muguruza et al., 2013) and G$_{q/11}$-/G$_{i1}$-protein activation by (±)DOI (García-Bea et al., 2019). Adult mice were sacrificed by cervical dislocation, brains removed, cortex dissected and samples stored at −70 °C until assay. Animal care and experimental protocols were done in agreement with European Union regulations and approved by the UPV/EHU institutional Ethics Committee for animal welfare (CEEA).

Membrane enriched fractions (P$_2$) from human and mice samples were prepared as previously described (Diez-Alarcia et al., 2016).

2.2. Antibody-capture [^{35}S]GTPγS binding scintillation proximity assay (SPA)

Assays were performed as described (Diez-Alarcia et al., 2016) following experimental conditions for determination of inverse agonism properties of different drugs in *postmortem* human brain (Diez-Alarcia et al., 2019; Erdozain et al., 2012). Concentration-effect response (10^{-10}-10^{-4} M) curves for specific coupling/uncoupling of G$_{\alpha q/11}$- and G$_{\alpha i1}$-proteins by 1-(4-fluorobenzyl)−3-(4-isobutoxybenzyl)−1-(1-methylpiperidin-4-yl)urea (pimavanserin; ACP-103) and α-phenyl-1-(2-phenylethyl)−4-piperidinemethanol (MDL-11,939) were performed. MDL-11,939 was chosen as selective 5-HT$_{2A}$R vs 5-HT$_{2C}$R antagonist (Knight et al., 2004; Pehek et al., 2006). Pergolide and (±)−2,5-dimethoxy-4-iodoamphetamine (DOI) activity on G$_{\alpha q/11}$- and G$_{\alpha i1}$-proteins was used to validate the experimental conditions for detecting the functional selectivity on 5-HT$_{2A}$Rs. These compounds were chosen as representative non-hallucinogenic and hallucinogenic 5-HT$_{2A}$R agonists, respectively, and were tested at maximal concentration (10 μM) with and without the antagonist MDL-11,939 (1 μM). Additionally, specific coupling/uncoupling responses of inhibitory G$_{\alpha i2}$-, G$_{\alpha i3}$- and G$_{\alpha o}$-proteins at maximal pimavanserin concentration (10 μM) were performed. On the other hand, in order to confirm the 5-HT$_{2A}$R involvement in the effects of pimavanserin, one-point concentration experiments (10 μM) in the absence and presence of the antagonist MDL-11,939 (1 μM) were carried out both in human and in 5-HT$_{2A}$R$^{(+/+)}$ and 5-HT$_{2A}$R$^{(-/-)}$ mice brain samples. Briefly, [^{35}S]GTPγS binding was performed in buffer containing 0.4 nM [^{35}S]GTPγS, 11 μg of protein/well and 50–100 μM GDP. After [^{35}S]GTPγS binding, immunoprecipitation with specific antibodies for each G$_\alpha$-protein subunit and protein A polyvinyltoluene SPA beads was carried out. Plates were then centrifuged and bound radioactivity detected on a MicroBeta TriLux scintillation counter. Non-specific binding was defined as the remaining [^{35}S]GTPγS binding in the presence of 100 μM unlabelled GTPγS.

2.3. Data analysis and statistical procedures

Data were analysed with GraphPad Prism™ 5.01. Specific [^{35}S]GTPγS binding values obtained from SPA assays were transformed to percentage of basal [^{35}S]GTPγS binding (binding values in the absence of any exogenous drug) obtained for each G$_\alpha$-protein and considered as 100%. Concentration that promotes half-maximal effect (IC$_{50}$) and maximal inhibitory effect (I$_{max}$) values were calculated, when possible, by nonlinear curve fitting of concentration-response experiments. Results from single-point maximal concentration experiments were analysed by one-, and two-sample Student's *t*-test vs basal values (expressed as 0%) or between experimental groups, respectively. Data are described as mean±S.E.M. values. Graphical representations of results are displayed as concentration-response curves or as box and whiskers plots.

2.4. Antibodies, drugs and reagents

Monoclonal antibodies against G$_{\alpha q/11}$- (sc-515689), G$_{\alpha i1}$- (sc-56536), G$_{\alpha i2}$- (sc-13534), G$_{\alpha i3}$- (sc-365422) and G$_{\alpha o}$- (sc-393874) proteins were obtained from Santa Cruz Biotechnologies (USA). Specificity demonstration of G$_{\alpha q/11}$- and G$_{\alpha i1}$-protein antibodies vs the different recombinant and brain tissue G-proteins is shown in Supplementary Figure S1. Pimavanserin and pergolide were supplied by MedChem Tronica Ab (Sweden) and MDL-11,939 (glemanserin) by Tocris Bioscience (UK). (±)DOI was obtained from Sigma-Aldrich (USA). [^{35}S]GTPγS was purchased from Perkin Elmer Life Sciences (Germany). Other reagents for SPA were obtained from Sigma-Aldrich and Perkin Elmer Life Sciences.

3. Results

3.1. Effects of pimavanserin and MDL-11,939 on G$_{\alpha q/11}$-, and G$_{\alpha i/o}$-proteins in human brain

The [^{35}S]GTPγS binding to G$_{\alpha q/11}$-proteins in response to increasing concentrations of pimavanserin and MDL-11,939 was unaltered (Fig. 1A), suggesting that these drugs act as neutral antagonists on the canonical pathway of 5-HT$_{2A}$Rs. In contrast, pimavanserin displayed a concentration-dependant inhibition of the [^{35}S]GTPγS binding to G$_{\alpha i1}$-proteins with IC$_{50}$=3.5 nM (-logIC$_{50}$=8.46±0.2) and I$_{max}$=25±2% (Fig. 1B), which denotes an inverse agonist profile on this signalling pathway in human prefrontal cortex. MDL-11,939 showed a null effect on [^{35}S]GTPγS binding to G$_{\alpha i1}$-proteins, indicating a pharmacological profile of neutral antagonist (Fig. 1B).

In a new set of experiments, responses to pimavanserin (10 μM) were tested on other G$_{\alpha i/o}$-proteins (G$_{\alpha i2}$, G$_{\alpha i3}$ and G$_{\alpha o}$). At this maximal concentration, pimavanserin did not display significant activity on [^{35}S]GTPγS binding to any of these inhibitory G$_\alpha$-proteins, which could reflect a pure antagonist profile or, alternatively, the absence of constitutive activity of these G$_{\alpha i/o}$-proteins in human brain cortex (Fig. 2).

3.2. Involvement of 5-HT$_{2A}$Rs in the effects of pimavanserin in human brain

In order to demonstrate that pimavanserin-induced [^{35}S]GTPγS binding inhibition to G$_{\alpha i1}$-proteins represents in-

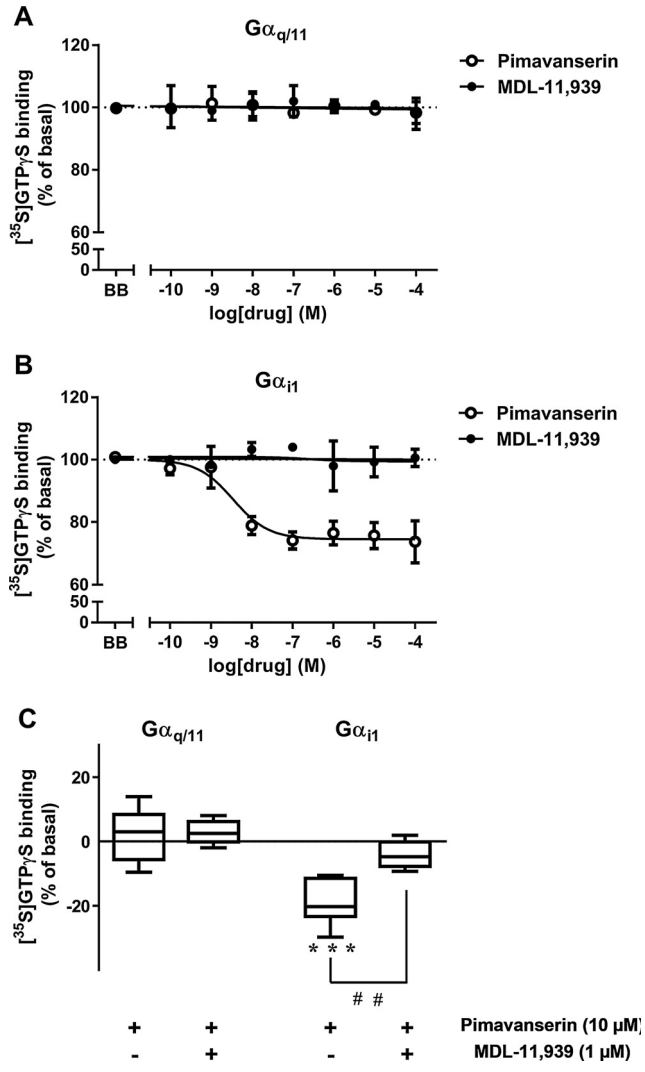

Fig. 1 Concentration-response curves of the specific [^{35}S]GTPγS binding to $G_{\alpha q/11}$-proteins (**A**) and $G_{\alpha i1}$-proteins (**B**) obtained for pimavanserin and MDL-11,939 in prefrontal human brain cortex membranes. The 100% line denotes the specific [^{35}S]GTPγS basal binding to respective G_{α}-proteins. Points and bars represent mean±S.E.M. response values for each drug concentration. **C.** Modulation of specific [^{35}S]GTPγS binding to $G_{\alpha q/11}$- and $G_{\alpha i1}$-proteins by 10 μM pimavanserin alone and co-incubated with the 5-HT$_{2A}$R antagonist MDL-11,939 (1 μM) in prefrontal human brain cortex. Basal values of specific [^{35}S]GTPγS binding to $G_{\alpha q/11}$- and $G_{\alpha i1}$-proteins are expressed as 0% and stimulatory/inhibitory effects on the respective basal are shown. Each box spans the 25–75 interquartile range and whiskers extend to the 5%–95% observations. The line inside the box indicates median value. Independent experiments were carried out by triplicate in samples of six subjects. $^{***}p<0.001$ vs basal values (one-sample *t*-test); $^{\#\#}p<0.01$ between groups (two-sample *t*-test).

verse agonism mediated by 5-HT$_{2A}$Rs, MDL-11,939 (1 μM) was employed to antagonize the effect. The inhibition induced by pimavanserin at 10 μM (20±2%) on [^{35}S]GTPγS binding to $G_{\alpha i1}$-proteins was abolished when co-incubated with MDL-11,939 (1 μM) (2 ± 2%) ($p<0.01$ between groups),

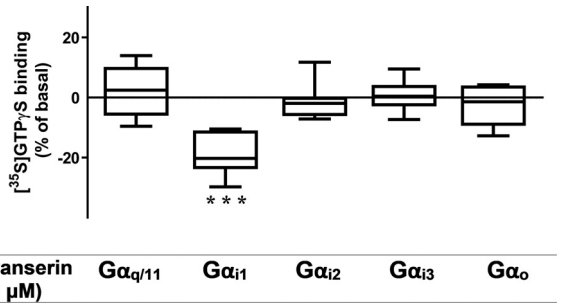

Fig. 2 Modulation of specific [^{35}S]GTPγS binding to $G_{\alpha q/11}$-, $G_{\alpha i1}$-, $G_{\alpha i2}$-, $G_{\alpha i3}$- and $G_{\alpha o}$-proteins by 10 μM pimavanserin in prefrontal human brain cortex. Basal values of specific [^{35}S]GTPγS binding to the different G-proteins are expressed as 0% and stimulatory/inhibitory effects on the respective basal are shown. Each box spans the 25–75 interquartile range and whiskers extend to the 5%–95% observations. The line inside the box indicates median value. Independent experiments were carried out by triplicate in samples of six subjects. $^{***}p<0.001$ vs basal values (one-sample *t*-test).

supporting the involvement of 5-HT$_{2A}$Rs (Fig. 1C). As expected, pimavanserin (10 μM) neither alone nor in the presence of MDL-11,939 (1 μM) modified [^{35}S]GTPγS basal binding to $G_{\alpha q/11}$-proteins (Fig. 1C).

Given the absence of pimavanserin and MDL-11,939 effects on $G_{\alpha q/11}$-protein activation, it was necessary to demonstrate a measurable response of this signalling pathway to 5-HT$_{2A}$R activation by agonists. Pergolide was chosen as a non-hallucinogenic drug with high (although non-selective) affinity for 5-HT$_{2A}$Rs (Odagaki et al., 2019). On the other hand, (±)DOI was selected as representative hallucinogenic LSD-like drug (López-Giménez and González-Maeso, 2018) with high affinity for 5-HT$_{2A}$Rs and 5-HT$_{2C}$Rs (Knight et al., 2004). Consistent with the hypothesis, pergolide (10 μM) was able to stimulate [^{35}S]GTPγS basal binding to $G_{\alpha q/11}$- (31±3%; $p<0.001$ vs basal) but not to $G_{\alpha i1}$-proteins (1 ± 2%). In contrast, (±)DOI at 10 μM increased the [^{35}S]GTPγS binding to both $G_{\alpha q/11}$- (17±2%; $p<0.001$ vs basal) and $G_{\alpha i1}$-proteins (15±2%; $p<0.001$ vs basal) (Supplementary Figure S2). The selective 5-HT$_{2A}$R antagonist MDL-11,939 (1 μM) partially abolished the pergolide-mediated $G_{\alpha q/11}$-protein activation (13±2%) ($p<0.001$ between groups). In the case of (±)DOI, the presence of MDL-11,939 (1 μM) blocked the stimulation of both $G_{\alpha q/11}$- (−1 ± 1%; $p<0.0001$ between groups) and $G_{\alpha i1}$-proteins (0 ± 2%; $p<0.001$ between groups) (Supplementary Figure S2).

3.3. Effects of pimavanserin on $G_{\alpha q/11}$-, and $G_{\alpha i1}$-proteins in 5-HT$_{2A}$R$^{(-/-)}$ and 5-HT$_{2A}$R$^{(+/+)}$ mice

To further ensure that the pharmacological profile of pimavanserin obtained in human brain corresponds to activity on 5-HT$_{2A}$Rs, equivalent assays were performed in brain cortex of 5-HT$_{2A}$R$^{(+/+)}$ and 5-HT$_{2A}$R$^{(-/-)}$ mice. No effects of pimavanserin (10 μM) were observed for [^{35}S]GTPγS binding to $G_{\alpha q/11}$-proteins in any of the genotypes (Fig. 3). However, a significant inhibition (13±2%; $p<0.01$ vs basal) of

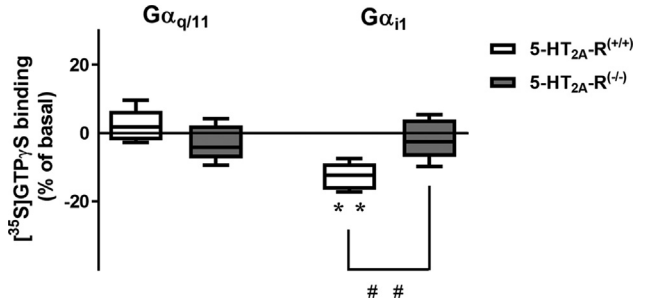

Fig. 3 Modulation of specific [^{35}S]GTPγS binding to G$_{\alpha q/11}$- and G$_{\alpha i1}$-proteins by 10 μM pimavanserin in brain cortex membranes of 5-HT$_{2A}$R$^{(+/+)}$ (white boxes) and 5-HT$_{2A}$R$^{(-/-)}$ (dark boxes) mice. Basal values of specific [^{35}S]GTPγS binding to G$_{\alpha q/11}$- and G$_{\alpha i1}$-proteins are expressed as 0% and stimulatory/inhibitory effects on the respective basal are shown. Absolute values of the specific [^{35}S]GTPγS basal binding to G$_{\alpha q/11}$-, and G$_{\alpha i1}$-proteins were not different between 5-HT$_{2A}$R$^{(+/+)}$ and 5-HT$_{2A}$R$^{(-/-)}$ mice (data not shown). Each box spans the 25–75 interquartile range and whiskers extend to the 5%—95% observations. The line inside the box indicates median value. Independent experiments were carried out by triplicate in samples of five to seven animals. $^{**}p<0.01$ vs basal values (one-sample *t*-test); $^{\#\#}p<0.01$ between groups (two-sample *t*-test).

the [^{35}S]GTPγS binding to G$_{\alpha i1}$-proteins was observed in 5-HT$_{2A}$R$^{(+/+)}$ mice. In contrast, the pimavanserin-induced inhibition of the G$_{\alpha i1}$-protein activity was not found in 5-HT$_{2A}$R$^{(-/-)}$ mice (2 $\pm$ 2%) ($p<0.01$ between genotypes), confirming the inverse agonist properties on this non-canonical signalling pathway of 5-HT$_{2A}$Rs. (Fig. 3).

4. Discussion

The present study provides evidence that pimavanserin displays inverse agonist properties on 5-HT$_{2A}$Rs in *postmortem* human frontal cortex. This pharmacological profile of pimavanserin is highly selective for the 5-HT$_{2A}$R coupling to G$_{\alpha i1}$-proteins whereas the drug operates as neutral antagonist on the signalling pathway mediated by G$_{\alpha q/11}$-proteins. Overall, the finding sheds light on consistent objections raised about the true pharmacological profile of pimavanserin in native tissue and, specifically, in human brain (Nutt et al., 2017). Moreover, since the hallucinogenic-like effects of 5-HT$_{2A}$R agonists such as LSD, psilocybin, mescaline, (±)DOI and analogues seem to be mediated through the G$_{\alpha i1}$-protein (González-Maeso et al., 2007; López-Giménez and González-Maeso, 2018), the ability of pimavanserin to preferentially uncouple 5-HT$_{2A}$Rs from this G$_{\alpha}$-protein should be regarded as critical to better understand the mechanisms of antipsychotic activity. Accordingly with this hypothesis, the present results in *postmortem* human brain of differential G$_{\alpha}$-protein recruitment by the agonists pergolide and (±)DOI confirm previous findings in animals and cell cultures that proposed activation of the G$_{\alpha i1}$-protein signalling pathway as a fingerprint of hallucinogenic-like properties for 5-HT$_{2A}$R agonist drugs (López-Gimenez and González-Maeso, 2018).

Demonstration that a particular drug shows inverse agonist properties requires the existence of constitutive basal activity that should be reduced by the candidate compound. The decrease of constitutive activity must be sensitive to blockade with an antagonist and be absent in *knock-out* animals for the involved receptor (Aloyo et al., 2009). Up to now, the existence of constitutive activity of the 5-HT$_{2A}$R represented an unresolved issue (De Deurwaerdère et al., 2020). Attempts to demonstrate in vitro constitutive basal activity of this receptor have required either receptor mutagenesis (Egan et al., 1998) or overexpression (Weiner et al., 2001) in transfected cells. However, these approaches do not reproduce accurately the molecular signalling of 5-HT$_{2A}$Rs in native tissue, which is essential to identify the antipsychotic mechanisms of action. In the current in vitro study, the existence of constitutive 5-HT$_{2A}$R basal activity toward the G$_{\alpha i1}$-protein subtype is demonstrated in human and mice brain cortex. The inverse agonist activity of pimavanserin on this signalling pathway is supressed by a candidate antagonist drug, MDL-11,939, whose neutral antagonist properties are also proved. Finally, the inverse agonism profile of pimavanserin is absent in 5-HT$_{2A}$R$^{(-/-)}$ mice. A further condition to establish definitively the existence of constitutive receptor activity under in vitro conditions is the demonstration that signalling deactivation occurs in the absence of the endogenous neurotransmitter (Aloyo et al., 2009). In the present condition, the processing of brain tissue homogenates to obtain membrane-enriched fractions is based on strong centrifugations and washouts, which precludes the presence of significant trace amounts of serotonin in the medium. Even more, if that possibility were true, inverse agonism of pimavanserin would be expected to appear on all the G$_{\alpha}$-proteins, lacking of subtype selectivity, and MDL-11,939 would show similar profile to pimavanserin. Previously, indirect evidence of constitutive 5-HT$_{2A}$R activity in *postmortem* human frontal cortex was suggested by demonstrating that the non-hydrolyzable GTP analogue Gpp(NH)p, which uncouples receptor from G-proteins, enhances the 5-HT$_{2A}$R population identified by the inverse agonist altanserin (Muguruza et al., 2013). However, identification of the G$_{\alpha i1}$-protein as the specific effector pathway responsible for the constitutive activity of 5-HT$_{2A}$Rs in brain has not been conclusively determined until now.

The possibility that antipsychotic drugs with significant affinity for D$_2$R and 5-HT$_{2A}$Rs such as clozapine, risperidone and olanzapine could mediate some of their clinical effects through inverse agonism on 5-HT$_{2A}$Rs and other GPCRs has been speculated (Sullivan et al., 2015). The argument is supported on transient expression screening studies performed in cells and the assessment of phospholipase C and phospholipase A$_2$ signalling responses in these cell cultures (Egan et al., 1998; Weiner et al., 2001). However, caution should be taken in mind since no functional study has been designed yet in native tissue conditions to test the putative inverse agonist profile of antipsychotics and, particularly, their activity on the non-canonical G$_{\alpha i1}$-protein pathway.

Certainly, the present study does not directly demonstrate that recruitment of G$_{\alpha i1}$-protein signalling pathway represents the mechanism of antipsychotic activity of pimavanserin and, perhaps, other relevant drugs. However, the coupling preference of 5-HT$_{2A}$Rs to G$_{\alpha i1}$-protein instead of canonical G$_{\alpha q/11}$-proteins to induce hallucinogenic-like symptoms in animals (González-Maeso et al., 2007; López-Giménez and González-Maeso, 2018) and the over-

active 5-HT_{2A}R coupling to $G_{\alpha i1}$-, but not $G_{\alpha q/11}$-proteins, in schizophrenia (García-Bea et al., 2019) should be relevant outputs to consider in the analysis of the therapeutic role played by the 5-HT_{2A}R inverse agonism. Elucidation of the structure of 5-HT_{2A}Rs in complex with antipsychotic drugs and docking studies could help to the rational design of compounds displaying functional selectivity for the $G_{\alpha i1}$-protein pathway (Kimura et al., 2019). On the other hand, a crucial point is to ascertain whether an enhanced constitutive activity of 5-HT_{2A}R coupling to $G_{\alpha i1}$-proteins is present in brain of subjects with schizophrenia. This would strengthen the relevancy of inverse agonism vs neutral antagonism as mechanism to improve the efficacy and/or tolerability of 5-HT_{2A}R-related antipsychotic drugs. In this context, it should be kept in mind, when considering clinical evaluation of pimavanserin as adjunct treatment to antipsychotics, that the singular efficacy elicited by pimavanserin might be hidden by addition of another antipsychotic with neutral antagonist profile on 5-HT_{2A}Rs signalling towards $G_{\alpha i1}$-proteins.

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Contribution of authors

Itziar Muneta-Arrate, Rebeca Diez-Alarcia and Igor Horrillo performed the experiments and undertook the statistical analysis. Rebeca Diez-Alarcia and J Javier Meana designed the study, and supervised and interpreted the data. J Javier Meana provided access to human brain samples and toxicological information. Itziar Muneta-Arrate and J Javier Meana wrote the different versions of the manuscript. All authors revised the manuscript and approved the final version.

Conflict of interest

The authors declare no conflicts of interest.

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Supplementary materials

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