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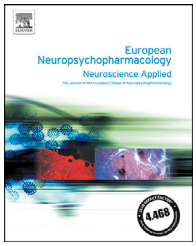

Serotonin 5-HT$_{2A}$ receptor expression and functionality in *postmortem* frontal cortex of subjects with schizophrenia: Selective biased agonism via G$_{\alpha i1}$-proteins

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Abstract
Serotonin 5-HT$_{2A}$ receptors (5-HT$_{2A}$Rs) have been implicated in schizophrenia. However, *postmortem* studies on 5-HT$_{2A}$Rs expression and functionality in schizophrenia are scarce. The 5-HT$_{2A}$R mRNA and immunoreactive protein expression were evaluated in *postmortem* tissue from dorsolateral prefrontal cortex (DLPFC) of antipsychotic-free ($n=18$) and antipsychotic-treated ($n=9$) subjects with schizophrenia, and matched controls ($n=27$). Functional coupling of 5-HT$_{2A}$R to G-proteins was tested by measuring the activation induced by the agonist (±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride ((±)DOI) in antibody-capture [^{35}S]GTPγS scintillation proximity assays (SPA). In antipsychotic-free schizophrenia subjects, 5-HT$_{2A}$R mRNA expression and protein immunoreactivity in total homogenates was similar to controls. In contrast, in antipsychotic-treated schizophrenia subjects, lower mRNA

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expression (60±9% *vs* controls) and a trend to reduced protein immunoreactivity (86±5% *vs* antipsychotic-free subjects) just in membrane-enriched fractions was observed. [^{35}S]GTPγS SPA revealed a significant ~6% higher stimulation of $G_{\alpha i1}$-protein by (±)DOI in schizophrenia, whereas activation of the canonical $G_{\alpha q/11}$-protein pathway by (±)DOI remained unchanged. Expression of $G_{\alpha i1}$- and $G_{\alpha q/11}$-proteins did not differ between groups. Accordingly, in rats chronically treated with clozapine, but not with haloperidol, a 30–40% reduction was observed in 5-HT_{2A}R mRNA expression, 5-HT_{2A}R protein immunoreactivity and [^{3}H]ketanserin binding in brain cortical membranes. Overall, the data suggest a supersensitive 5-HT_{2A}R signaling through inhibitory $G_{\alpha i1}$-proteins in schizophrenia. Together with previous results, a dysfunctional pro-hallucinogenic agonist-sensitive 5-HT_{2A}R conformation in *postmortem* DLPFC of subjects with schizophrenia is proposed. Atypical antipsychotic treatment would contribute to counterbalance this 5-HT_{2A}R supersensitivity by reducing receptor expression.

1. Introduction

Several findings support the involvement of the serotonin 5-HT_{2A} receptors (5-HT_{2A}Rs) in schizophrenia. Hallucinogenic drugs, such as psilocybin or lysergic acid diethylamide (LSD), trigger mental states resembling schizophrenia in healthy humans through the activation of 5-HT_{2A}Rs (Carhart-Harris et al., 2016; Geyer and Vollenweider, 2008; Nichols, 2016; Preller et al., 2018; Quednow et al., 2012; Schmid et al., 2015), and worsen psychosis in schizophrenia patients (Hoch et al., 1952). Most of the atypical antipsychotic drugs are characterized by high potency as 5-HT_{2A}R antagonists (Meltzer and Massey, 2011; Miyamoto et al., 2012). 5-HT_{2A}R knockout (KO) mice are insensitive to the behavioral effects of serotonergic psychedelics (González-Maeso et al., 2003, 2007), and genetic evidence links polymorphisms in the 5-HT_{2A}R (*HTR2A*) gene with sensorimotor gating deficits in schizophrenia (Geyer and Vollenweider, 2008). Moreover, a differential epigenetic methylation of the *HTR2A* gene in the brain of subjects with schizophrenia has been described (Cheah et al., 2017; Abdolmaleky et al., 2011). In addition, studies in immunological, pharmacological, congenital and genetic rodent models of schizophrenia have demonstrated increased 5-HT_{2A}R expression (Dean et al., 2008b; Fomsgaard et al., 2018; Holloway et al., 2013; Malkova et al., al.,2014; Moreno et al., 2011) and/or functionality (Holloway et al., 2013; Malkova et al., 2014; Moreno et al., 2011; Santini et al., 2013). Together, these findings suggest that up-regulation of cortical 5-HT_{2A}Rs could predispose to psychosis in schizophrenia.

The status of brain 5-HT_{2A}Rs in schizophrenia has been mainly evaluated with radiotracers, both by *in vivo* neuroimaging and *in vitro* radioligand binding techniques, raising different outcomes. Thus, while some *postmortem* studies support increased 5-HT_{2A}R density, others observed no changes and even decreases (see for reviews, Dean, 2003; González-Maeso and Sealfon, 2009; Selvaraj et al., 2014). These apparent discrepancies may depend on several confounding issues, such as the pharmacological properties of the radioligands used to bind 5-HT_{2A}Rs, tissue preparation (total homogenates, membranes, cytosol, tissue sections), discrimination between antipsychotic-free and antipsychotic-treated subjects, the brain area selected, and other demographic and methodological variables (age, *postmortem* delay, gender, *etc.*). Immunodetection is a feasible method for *in vitro postmortem* studies of neurotransmitter receptors. The use of antibodies to quantify receptor protein expression minimizes concerns of pharmacological selectivity associated with radioligands and avoids the influence of residual antipsychotic presence in samples competing for the receptor. However, protein immunodetection fails to keep intact the ternary structure and does not discriminate between molecular states of the receptor. In this context, only one study of 5-HT_{2A}R immunoreactivity in schizophrenia restricted to cerebellum evaluation has been reported (Eastwood et al., 2001). Similarly, studies with positron emission tomography (PET) in living humans have shown controversial results although the potential binding seems to be reduced when selective 5-HT_{2A}R radiotracers as [^{18}F]altanserin are used (see review in Selvaraj et al., 2014; Erritzoe et al., 2008; Rasmussen et al., 2010, 2016).

The canonical 5-HT_{2A}R signaling pathway involves activation of $G_{\alpha q/11}$-proteins that, subsequently, activate phospholipase C, leading to inositol 1,4,5-triphosphate and 1,2-diacylglycerol accumulation, releasing Ca^{+2} from intracellular stores and activating protein kinase C. In heterologous expression systems and rodents, non-hallucinogenic LSD-analogous 5-HT_{2A}R agonists, such as lisuride, exclusively activate $G_{\alpha q/11}$-proteins, whereas hallucinogenic 5-HT_{2A}R agonists, such as LSD and (±)DOI, engage signaling through $G_{\alpha q/11}$ – but also through inhibitory $G_{\alpha i/o}$-proteins (González-Maeso et al., 2007; Ibarra-Lecue et al., 2018; Karaki et al., 2014). This differential activation is compatible with the presence of a "functional selectivity" (also known as "agonist-directed trafficking of receptor signaling" or "biased agonism") phenomenon in 5-HT_{2A}R activation (Urban et al., 2007). Despite the extensive evaluation of brain 5-HT_{2A}Rs density in schizophrenia, the status of their functional coupling to G-proteins remains unknown. Moreover, the hypothesis that a dysregulation of 5-HT_{2A}Rs in schizophrenia could selectively affect the receptor coupling to the hallucinogenic-mediated $G_{\alpha i/o}$-protein signaling has not been investigated.

The present study evaluated 5-HT_{2A}R mRNA and protein expression in *postmortem* dorsolateral prefrontal cortex (DLPFC) of subjects with schizophrenia and controls. A strict treatment-controlled design was used to verify the

influence of antipsychotic drug treatment. Moreover, the existence of dysfunctions in the coupling of 5-HT$_{2A}$R to different G-protein-mediated pathways between schizophrenia and control subjects was also tested. The DLPFC was selected as region of interest based on the described morphological alterations associated to schizophrenia (Jarskog et al., 2007; Lewis and González-Burgos, 2008) and previous findings in PET studies (Rasmussen et al., 2010, 2016).

2. Experimental procedures

2.1. Human brain samples

Human brains were obtained at autopsies in the Basque Institute of Legal Medicine, Bilbao, Spain, in compliance with policies of research and ethical boards for *postmortem* studies. Deaths were subjected to retrospective searching for diagnosis and treatment using examinerś information and medical records. Twenty-seven subjects with *antemortem* diagnosis of schizophrenia according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV, DSM-IV-TR) were selected and matched to 27 control subjects without neuropsychiatric or drug abuse disorders. Individual matching was performed for age, gender and, as much as possible, *postmortem* delay (PMD). A toxicological screening performed at the National Institute of Toxicology, Madrid, Spain, allowed the classification of subjects with schizophrenia as "antipsychotic-free" (AP-free, $n=18$) or "antipsychotic-treated" (AP-treated, $n=9$) according to the absence or presence of antipsychotic drugs in blood at the moment of death. It is noteworthy to remark that all the current AP-free subjects had received antipsychotic prescription and should not be considered never-treated naïve subjects. Specimens of DLPFC were dissected following standard procedures (Rajkowska and Goldman-Rakic, 1995) and stored at −80 °C. A full description of the AP-free, AP-treated subjects, and respective controls with tissue integrity information (PMD, storage time, RNA integrity number, brain pH) is shown in Supplementary Table S1.

2.2. Animals and treatments

Male Sprague-Dawley rats (220–320 g) were housed on a 12 h light/dark cycle at room temperature (22 °C) and 60% humidity with food and water available *ad libitum*. Animals were obtained and kept at the Animal Facility SGIker of the University of the Basque Country. Animal care and experimental protocols were in accordance with European Union regulations and were approved by the UPV/EHU institutional Ethics Committee for animal welfare (CEEA).

Rats were injected i.p., every 12 h, during 21 days, with saline (1 ml/kg, $n=10$), haloperidol (0.5 mg/kg, $n=10$) (Sigma Aldrich, MO, USA) or clozapine (5 mg/kg, $n=10$) (Tocris, Bristol, UK). Haloperidol and clozapine were selected as representative drugs with typical (selective affinity for dopamine D_2 receptor *vs* 5-HT$_{2A}$R) or atypical (higher affinity for 5-HT$_{2A}$R than dopamine D_2 receptor family) antipsychotic profile, respectively. Animals were sacrificed 48 h after the last injection of clozapine and 72 h after the last injection of haloperidol or saline. Brains were removed and the cortex was dissected and stored at −80 °C.

2.3. Quantitative real-time reverse transcription polymerase chain reaction

Sample preparation and procedures for determination of mRNA expression of genes encoding 5-HT$_{2A}$ (*HTR2A, Htr2a)* and 5-HT$_{2C}$ (*HTR2C, Htr2c)* receptors by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) were conducted by using published procedures (Muguruza et al., 2014). mRNA expression was normalized using the endogenous housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), beta-actin (*ACTB*) and ribosomal protein S13 (*RPS13*) for human samples, and *Gapdh, Actb* and ribosomal protein S29 (*Rps29*) for rat samples. A negative internal control and a reference sample (pool of controls) for each assay were included in all PCR plates. The assays identification numbers and conditions of the qRT-PCR are described in Supplementary Tables S2 and S3, respectively. Human samples were also assayed for RNA integrity number (RIN) using the Agilent 2100 Bioanalyzer (Agilent Technologies) (Stan et al., 2006).

2.4. Immunodetection

Western blot experiments were performed in total homogenates (García-Fuster et al., 2008), and in membrane-enriched (P_2) fractions (Muguruza et al., 2013) as previously described. The selected primary antibodies were rabbit polyclonal anti-5-HT$_{2A}$R (ab16028; 1:2000) from Abcam (Cambridge, UK), mouse monoclonal anti-5-HT$_{2A}$R (sc-166775; 1:20,000), mouse monoclonal anti-$G_{\alpha i1}$- (sc-56536; 1:100) and rabbit polyclonal anti-$G_{\alpha q/11}$-proteins (sc-392; 1:300) from Santa Cruz (CA, USA), and mouse monoclonal anti-β-actin (A1978; 1:200,000) from Sigma Aldrich, as loading control. The fluorescent conjugated secondary antibodies Alexa Fluor™ 680 goat anti-rabbit (A21076; 1:2500–1:8000) and Alexa Fluor™ 680 goat anti-mouse (A21057; 1:8000) from Invitrogen (OR, USA), and IRDye™ 800 donkey anti-mouse (610-732-002; 1:10,000) and IRDye™ 800 goat anti-rabbit (611-132-002; 1:5000) from Rockland Immunochemicals (PA, USA), were used to detect and quantify the immunoreactive signal by using the Odyssey Infrared Imaging System (LI-COR Biosciences, NE, USA). All samples were analyzed at least two times in different gels, obtaining a minimum of four measurements for each sample.

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

Specific activation of the different G_α-proteins was determined by using a [^{35}S]GTPγS binding assay followed by incubation with specific antibodies (see above) and SPA beads, as described (Diez-Alarcia et al., 2016; Erdozain et al., 2012). The 5-HT$_{2A/C}$R agonist (±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride ((±)DOI) (Sigma Aldrich) was used to stimulate [^{35}S]GTPγS (1250 Ci/mmol) (Perkin Elmer Life Sciences, Maanstraat, Germany) binding at two concentrations close to those that elicit the 50% ($EC_{50}\approx$300 nM) and the maximal (10 μM) effects, estimated from concentration-effect curves (data not shown). The 5-HT$_{2A}$R antagonist ketanserin (10 μM) (Tocris, UK) was used to determine the 5-HT$_{2A}$R specific component of the stimulation induced by (±)DOI. Preliminary results demonstrated that $G_{\alpha i1}$ displays, among the inhibitory $G_{\alpha i/o}$-proteins, the most efficient response of [^{35}S]GTPγS binding stimulation by (±)DOI (Miranda-Azpiazu et al., 2013).

2.6. Radioligand binding

Saturation binding assays (0.03–9 nM, ten concentrations) with [^{3}H]ketanserin (specific activity 67 Ci/mmol; Perkin Elmer) in rat brain cortex membrane fractions were performed as described (Muguruza et al., 2013, 2014). At this concentration range, [^{3}H]ketanserin exclusively binds 5-HT$_{2A}$Rs in rodent brain cortex (Muguruza et al., 2013).

2.7. Data analysis and statistical procedures

All studies were performed under a paired design where each schizophrenia case and the respective matched control were always processed simultaneously. The qRT-PCR data analysis was performed by StepOne Software v2.1 (Applied Biosystems™). The relative mRNA amount was calculated as $2^{-\Delta\Delta Ct}\pm$SEM - normalized by both housekeeping genes and reference sample expression - and data were standardized to control samples. In Western blot assays, the immunoreactivity value of the target proteins was corrected by the corresponding value of β-actin; and then, calculated as percentage of a standard sample loaded in every single gel to control for inter-assay variability. Finally, data were standardized considering the mean value of the control samples as 100%. In radioligand binding experiments, the apparent equilibrium dissociation constant (K_D) and the maximum density of specific binding sites (B_{max}) were obtained. Specific binding data obtained from antibody-capture [^{35}S]GTPγS SPA were transformed to percentage of basal binding value (binding in the absence of any exogenous drug) for each G_α-protein subtype.

Statistical comparisons were made by Student's *t*-test and one-way analysis of variance (ANOVA) followed by Dunnett's *post-hoc* test. Potential association of experimental results with age, PMD, RIN, storage time and pH were tested. If significant correlation existed, the interaction was explored by analysis of covariance (ANCOVA) where the group (schizophrenia or control) was the independent variable, age, PMD, RIN, storage time or pH were the covariates, and the experimental result was the dependent variable. Statistical comparisons of non-linear analyses in radioligand binding assays were conducted by *F*-test.

The analyses were carried out by using GraphPad Prism 5™ and InVivo Stat™ softwares. Values are shown as mean±SEM. The level of significance was chosen as $p < 0.05$.

3. Results

3.1. *HTR2A* mRNA expression and protein immunoreactivity in *postmortem* brain of subjects with schizophrenia and matched controls

The mRNA expression of *HTR2A* showed no difference between subjects with schizophrenia and controls (Fig. 1(a)). However, when subjects were grouped depending on the absence/presence of antipsychotics in blood, lower *HTR2A* mRNA expression was observed in AP-treated group (60±9% % *vs* matched controls, $p < 0.05$), but not in AP-free group (110±9% *vs* matched controls) (Fig. 1(a)). As *HTR2A* mRNA expression showed significant correlation with the RIN value ($r = 0.43$, $p < 0.01$), results were reanalyzed by ANCOVA with RIN as covariate. This analysis further confirmed the significant decrease of *HTR2A* mRNA expression in AP-treated subjects ($F[1,15] = =18.72$, $p < 0.05$). The mRNA expression of *HTR2C* was determined in parallel as negative control. When compared to respective controls, *HTR2C* mRNA expression was unaltered in both AP-free and AP-treated subjects (Fig. 1(b)).

In order to quantify the protein expression of 5-HT$_{2A}$R, immunodetection with a specific antibody was performed. Western blot assays showed a single band at ~55 kDa (Supplementary Fig. S1) whose specificity has previously been demonstrated in 5-HT$_{2A}$R KO mice (Fribourg et al., 2011). The 5-HT$_{2A}$R protein immunoreactivity in total brain homogenates showed no significant alteration in schizophrenia (Fig. 1(c)), even when subjects with schizophrenia were separated in AP-free and AP-treated groups (Fig. 1(c)). This lack of modulation contrasts with the elevated 5-HT$_{2A}$R density previously observed by radioligand binding assays in brain membrane fractions of AP-free subjects (Muguruza et al., 2013). In order to check whether methodological issues could underlie this inconsistency (radioligand binding *vs* immunodetection, and use of total homogenates *vs* membrane-enriched fractions), new Western blot experiments were performed in membrane preparations from DLPFC of the same subjects. No clear differences were observed in membrane 5-HT$_{2A}$R immunoreactivity between schizophrenia and corresponding matched controls neither in the AP-free group nor in the AP-treated group. However, although not in statistically significant way, immunoreactive signal of 5-HT$_{2A}$Rs in P$_2$-membrane preparations was 17±6% lower in AP-treated than in AP-free subjects (Fig. 1(d)).

3.2. Functional coupling of 5-HT$_{2A}$Rs to $G_{\alpha q/11}$- and $G_{\alpha i1}$-proteins in *postmortem* brain of subjects with schizophrenia and matched controls

Experiments of 5-HT$_{2A}$R agonist-mediated stimulation of [^{35}S]GTPγS binding to different G_α-protein subtypes were performed in DLPFC membranes to test the different 5-HT$_{2A}$R signaling patterns. This approach informs about the functional status of 5-HT$_{2A}$Rs in *postmortem* brain and allows the evaluation of alterations in schizophrenia.

The 5-HT$_{2A/C}$R agonist (±)DOI (300 nM and 10 μM) produced a selective activation of $G_{\alpha q/11}$- and $G_{\alpha i1}$-proteins (Fig. 2(a) and (b)) but not of $G_{\alpha i2}$, $G_{\alpha o}$ and $G_{\alpha s}$ subtypes (Supplementary Fig. S2). The activation of these G_α-proteins was antagonized by the selective 5-HT$_{2A}$R antagonist ketanserin (10 μM) (Fig. 2(a) and (b)). Moreover, the stimulation of [^{35}S]GTPγS binding by (±)DOI was absent in cortex of 5-HT$_{2A}$R KO mice (Supplementary Fig. S3). (±)DOI stimulatory effects did not correlate with any of the potential confounding factors (age, PMD, RIN, storage time, pH and basal binding values).

In membrane fractions, basal binding of [^{35}S]GTPγS to $G_{\alpha q/11}$- and $G_{\alpha i1}$-proteins, before (±)DOI stimulation, was not different between schizophrenia subjects (45±4 fmol/μg protein for $G_{\alpha q/11}$; 41±4 fmol/μg protein for $G_{\alpha i1}$) and controls (37±4 fmol/μg protein for $G_{\alpha q/11}$; 38±3 fmol/μg protein for $G_{\alpha i1}$).

The stimulation of $G_{\alpha q/11}$-protein induced by (±)DOI showed no significant differences between schizophrenia and control groups (Fig. 2(a)), even when considering the presence of antipsychotic treatment. By contrast, $G_{\alpha i1}$-protein stimulation induced by (±)DOI at the two concentrations tested was significantly higher in the whole group of schizophrenia (~5-6% increase; $p < 0.05$) compared to controls (Fig. 2(b)). This pattern of higher $G_{\alpha i1}$-protein stimulation was slightly more robust in AP-free subjects (~6-7% increase *vs* matched controls) than in AP-treated subjects (~5% increase *vs* matched controls). Therefore, the canonical $G_{\alpha q/11}$-protein pathway of 5-HT$_{2A}$Rs was unaltered whereas the "pro-hallucinogenic" $G_{\alpha i1}$-protein pathway was functionally overactive in DLPFC of subjects with schizophrenia.

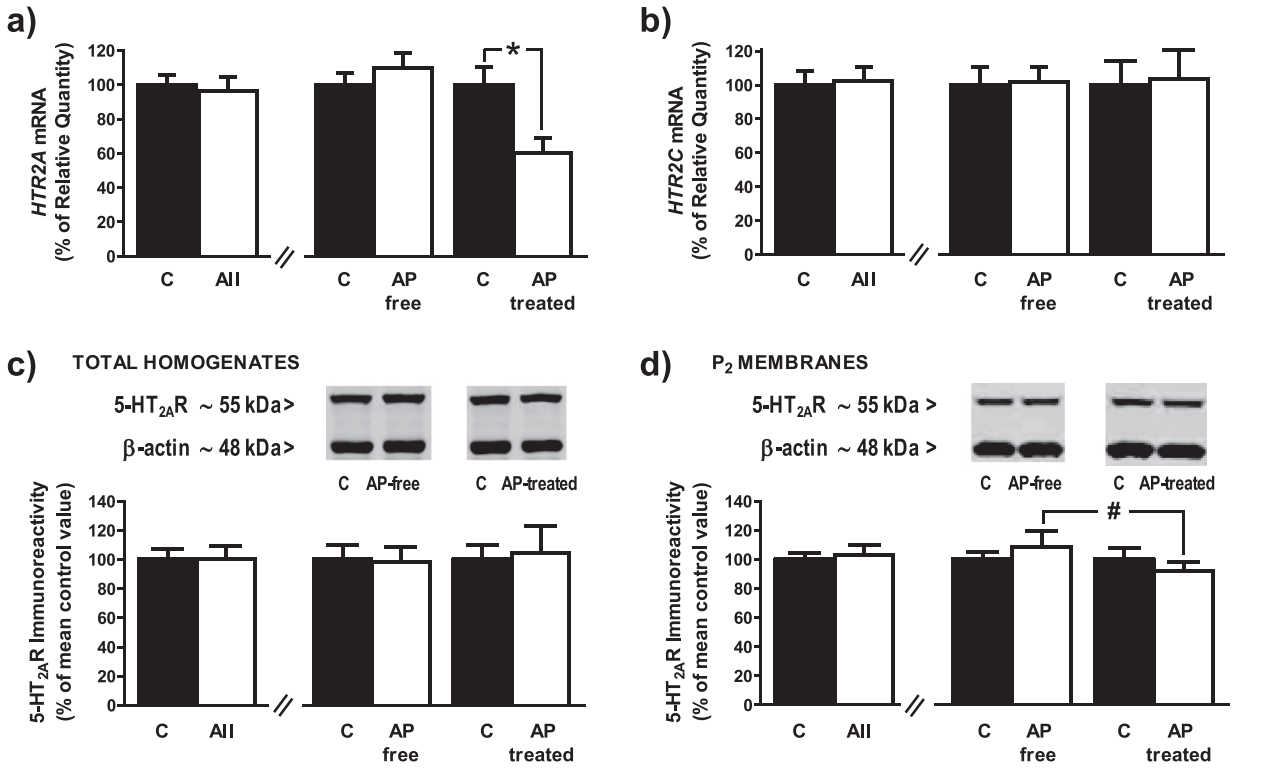

Fig. 1 Relative expression levels of (a) *HTR2A* mRNA, (b) *HTR2C mRNA,* (c) 5-HT$_{2A}$R protein in total homogenates and (d) 5-HT$_{2A}$R protein in P$_2$-membranes of DLPFC samples in the schizophrenia group (All), antipsychotic-free (AP-free) and antipsychotic-treated (AP-treated) subjects, and their respective matched controls (C). Inset (c) and (d): Representative images of 5-HT$_{2A}$R and β-actin (loading control) immunoreactivities in an AP-free, an AP-treated subjects, and in their respective controls. Bars represent mean±SEM values. $^{*}p < 0.05$ *vs* matched controls (Student's *t*-test), $^{\#}p = 0.08$ *vs* AP-free immunoreactive values (Student's *t*-test).

In order to test whether the supersensitivity of the (±)DOI stimulation of [^{35}S]GTPγS binding may lay directly on 5-HT$_{2A}$Rs or on enhanced G$_{\alpha i1}$-protein density, expression experiments of G$_{\alpha q/11}$- and G$_{\alpha i1}$-protein subtypes were performed in membrane fractions of DLPFC samples from the same subjects. No significant differences were observed between schizophrenia and control groups in the immunoreactivity neither of G$_{\alpha q/11}$- (100±4% *vs* matched controls, $p > 0.05$) nor of G$_{\alpha i1}$-proteins (89±5% *vs* matched controls, $p > 0.05$) (Fig. 2(c) and (d)), independently of the absence/presence of antipsychotic treatment (Fig. 2(c) and (d)).

3.3. Effects of chronic treatment with antipsychotics on *Htr2a* and *Htr2c* mRNA expression, 5-HT$_{2A}$R immunoreactivity and [^{3}H]ketanserin binding in rat brain cortex

Taking into account the results obtained in human DLPFC of subjects with schizophrenia, the effects of chronic (21 days) treatment with two different antipsychotic drugs (haloperidol and clozapine) on *Htr2a* and *Htr2c* mRNA expression and 5-HT$_{2A}$R protein immunoreactivity were tested in rat brain cortex.

Htr2a mRNA expression was lower in rats after chronic treatment with the atypical antipsychotic clozapine (70±5% *vs* saline, $p < 0.01$), but not with the typical antipsychotic haloperidol (Fig. 3(a)). In contrast, *Htr2c* mRNA expression did not vary among the different groups (Fig. 3(b)).

The 5-HT$_{2A}$R protein immunoreactivity in total homogenates from rat cortex revealed no changes in the different experimental groups (Fig. 3(c)). By contrast, in P$_2$-membrane preparations from the same animals, decreased 5-HT$_{2A}$R immunoreactivity was observed in clozapine-treated rats (63±6% *vs* saline, $p < 0.01$) with absence of changes in haloperidol-treated animals (Fig. 3(d)).

In order to confirm the down-regulation observed in rat brain cortical membranes by immunoblotting assays, saturation binding experiments with [^{3}H]ketanserin were performed in the same membrane preparations. In this case, a significant decrease of 5-HT$_{2A}$R density was also observed in clozapine-treated (B_{max} 64±4% *vs* saline; $p < 0.01$) but not in haloperidol-treated rats. No significant differences were observed in K_D values for any experimental group (Fig. 3(e); Table S4).

4. Discussion

The present study shows that density of 5-HT$_{2A}$Rs in DLPFC of subjects with schizophrenia is not altered when evaluated by protein immunodetection. This finding apparently contrasts with previous results in a similar population showing that [^{3}H]ketanserin binding to 5-HT$_{2A}$Rs was increased in AP-free and returned to control values

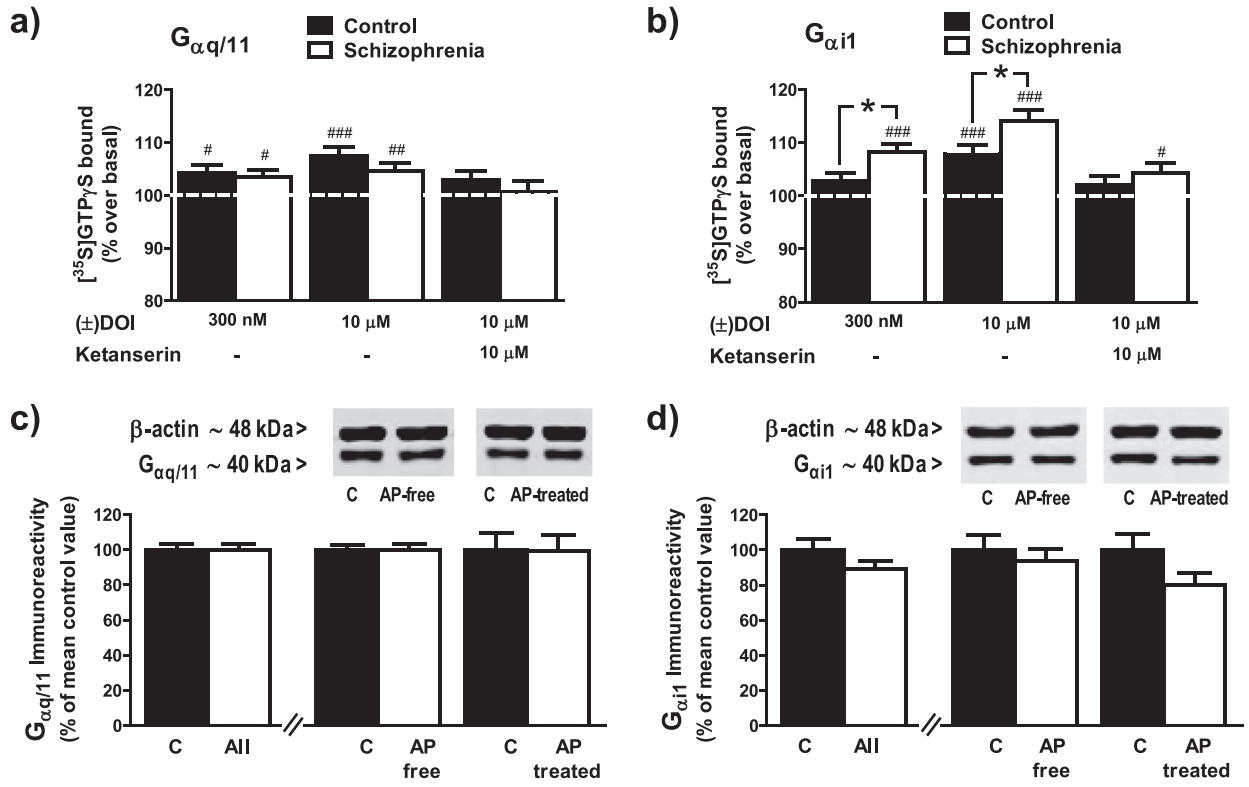

Fig. 2 (±)DOI (300 nM and 10 μM) induced stimulation of [^{35}S]GTPγS binding to (a) $G_{\alpha q/11}$- and (b) $G_{\alpha i1}$-proteins in DLPFC of schizophrenia (white bars) and control (black bars) subjects, and reversion by the selective antagonist ketanserin (10 μM). Data are shown as percentage of basal [^{35}S]GTPγS binding values for each G_α-protein subtype. Graphic representation and representative images of (c) $G_{\alpha q/11}$- and (d) $G_{\alpha i1}$-protein immunoreactivities in P_2-membranes of DLPFC in the schizophrenia group (All), antipsychotic-free (AP-free) and antipsychotic-treated (AP-treated) subjects, and their respective matched controls (C). Bars represent mean±SEM values. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ (one sample *t*-test *vs* 100%); $^{*}p < 0.05$ *vs* matched controls (Student's *t*-test).

in AP-treated subjects (Gonzalez-Maeso et al., 2008; Muguruza et al., 2013). Likewise, evidence is provided that mRNA expression of *HTR2A* is unaltered in DLPFC of AP-free subjects. On the other hand, the study reports, for the first time, that the hallucinogenic agonist (±)DOI displays a supersensitive coupling of 5-HT_{2A}R to the $G_{\alpha i1}$-protein pathway in schizophrenia, while no alterations in the canonical $G_{\alpha q/11}$-protein pathway are detected. Altogether, the findings in the present and previous studies (Muguruza et al., 2013) prompt the hypothesis of an enhanced proportion of agonist-sensitive 5-HT_{2A}Rs in DLPFC of AP-free schizophrenia subjects. This increased 5-HT_{2A}R sensitivity would not be the consequence of increased receptor synthesis and expression, but seems to represent an altered conformational exchange between G-protein-coupled (active) and G-protein-uncoupled receptor states (Muguruza et al., 2013). The equilibrium displacement towards the agonist-sensitive active conformation of the 5-HT_{2A}R constitutes the signal-driving stimulus that is experimentally observed as an increased coupling activity to G-proteins in schizophrenia. This 5-HT_{2A}R supersensitivity occurs without changes in basal [^{35}S]GTPγS binding values or G_α-protein immunoreactive levels, which discards a primary alteration of G-protein density. Whether this trend to 5-HT_{2A}Rs hyperactivity represents an intrinsic constitutive dysfunction of the receptor or is a consequence of abnormalities in associated proteins (Dean et al., 2008a; Roth, 2011) deserves further investigation.

Despite the multiple mRNA expression and radioligand binding studies performed in *postmortem* brain, a definitive understanding of 5-HT_{2A}R status in schizophrenia, especially in the absence of antipsychotic treatment, remains uncertain. Thus, *postmortem* radioligand binding assays have found decreased, unchanged and increased brain 5-HT_{2A}R density in schizophrenia (see for references, Dean, 2003; González-Maeso and Sealfon, 2009). *In vivo* PET neuroimaging studies have mainly proposed a reduction of the 5-HT_{2A}R binding potential in AP-free patients (see for references, Selvaraj et al., 2014; Rasmussen et al., 2010, 2016). As already mentioned, these apparent discrepancies might be related to the different pharmacological profile of the radiolabeled drugs used as radiotracers. Thus, it seems that the population of 5-HT_{2A}Rs with high-affinity for agonists, that represents the active receptor conformational state, would be increased in schizophrenia whereas the total 5-HT_{2A}R protein density would remain unchanged. Consequently, the population of 5-HT_{2A}Rs with high-affinity for inverse agonists, which represents the uncoupled receptor conformational state, should be decreased in AP-free subjects. Indirect data obtained in studies with *postmortem* DLPFC

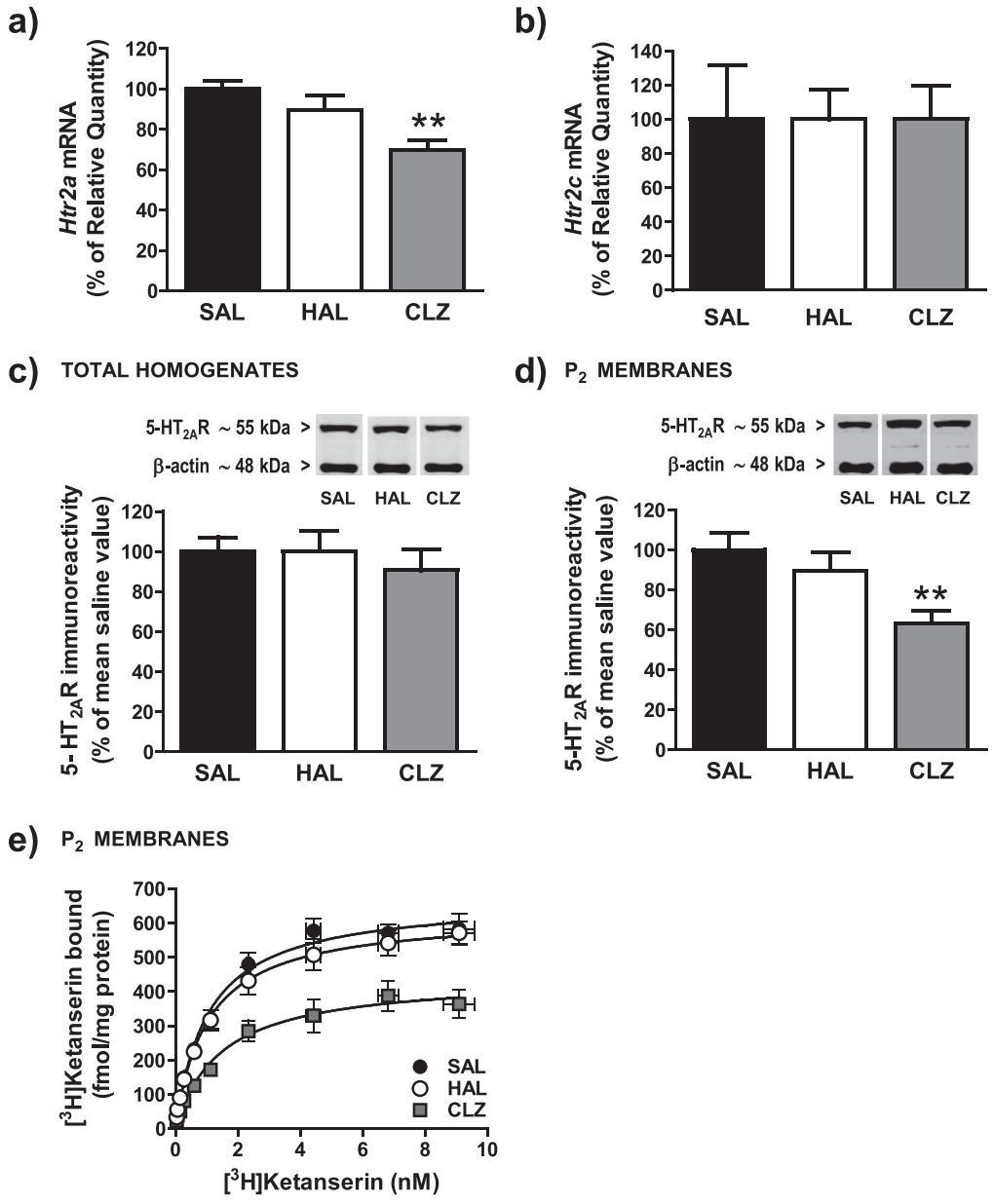

Fig. 3 Relative expression levels of (**a**) *Htr2a* mRNA, (b) *Htr2c* mRNA, (c) 5-HT$_{2A}$R protein in total homogenates, (d) 5-HT$_{2A}$R protein in P$_2$-membranes, and (e) specific [^{3}H]ketanserin binding saturation curves (0.03–9 nM) in P$_2$-membranes from brain cortex of rats chronically treated with saline (SAL), haloperidol (HAL), or clozapine (CLZ). Inset (c) and (d): Representative images of 5-HT$_{2A}$R and β-actin (loading control) immunoreactivities for each group. Bars and points represent mean±SEM values of 10 animals. $^{**}p < 0.01$ *vs* saline (one-way ANOVA followed by Dunnettś *post-hoc* test). In (e), Bmax differences between saline- and clozapine-treated rats were confirmed by the non-linear co-analysis of [^{3}H]ketanserin binding saturation curves (F[3,382]=7.43, $p < 0.001$).

tissue suggest that the PET radioligand most commonly used to identify 5-HT2ARs receptors, altanserin, acts as an inverse agonist and shows responses compatible with the documented reduction of [^{18}F]altanserin binding described in PET studies of schizophrenia (Muguruza et al., 2013). Indeed, the interest in the design and further use of 5-HT$_{2A}$Rs agonist radioligands for PET studies in schizophrenia has been recognized (L'Estrade et al., 2018).

Apart from selection of suitable immunological or radioligand tools for identification of 5-HT$_{2A}$Rs, confounding variables such as age, sex, PMD, and pharmacological treatment could also underlie the observed differences between *postmortem* evaluations of this receptor in schizophrenia. In the present work, subjects were individually matched in a way that significant differences for confounding factors between schizophrenia and control subjects were absent. It is worth mentioning that twenty-one of the 27 subjects with schizophrenia had died by suicide. Although suicide has been proposed as a confounding factor in evaluation of 5-HT$_{2A}$Rs (Underwood et al., 2018 and references therein), previous data in frontal cortex of suicide victims with

a variety of psychiatric disorders (see for references, Muguruza et al., 2013, 2014; Zhao et al., 2015) and a recent meta-analysis have argued that suicide unlikely represents a major confounder in 5-HT_{2A}R binding studies (Selvaraj et al., 2014).

The presence of antipsychotic treatment in schizophrenia is an essential confounding factor to be considered. Second generation antipsychotics induce changes in 5-HT_{2A}Rs detection through modulation of their expression as demonstrated in animal models (González-Maeso et al., 2008; Kurita et al., 2012; Yadav et al., 2011), or by blockade of the radioligand binding site due to residual presence of antipsychotics acting as 5-HT_{2A}R antagonists (Dean et al., 2008a). Interestingly, most of the studies suggesting decreased or unchanged [^{3}H]ketanserin binding in schizophrenia have been performed in brain of AP-treated subjects (see for references, Muguruza et al., 2013). Therefore, in order to discard eventual influences of antipsychotics in *postmortem* studies, independent and well-matched groups of AP-free and AP-treated subjects should be selected and independently analyzed. The influence of residual presence of antipsychotics competing for the radioligand binding pocket can also be overcome by using immunodetection of the 5-HT_{2A}R protein with selective antibodies as alternative methodology. In the present study, the immunoreactive expression of 5-HT_{2A}R protein is not modified in DLPFC homogenates of AP-treated subjects with schizophrenia. A similar pattern has been found in cerebellum cytosolic fractions of AP-treated schizophrenia subjects (Eastwood et al., 2001). Reevaluation of 5-HT_{2A}R protein expression in membrane-enriched fractions of the same subjects indicates a trend towards a reduction, associated to antipsychotic treatment, suggesting that this down-regulation mainly affect the 5-HT_{2A}R fraction expressed in plasma membranes. This membrane P_2 fraction represents the population able to interact with G-proteins and sensitive to agonist interaction. Moreover, the analysis of *HTR2A* gene expression clearly confirms a reduction of *HTR2A* mRNA in AP-treated that was not present in AP-free subjects. This finding is consistent with previous *postmortem* studies performed in AP-treated populations that proposed decreased *HTR2A* mRNA levels in schizophrenia (Burnet et al., 1996; Hernandez and Sokolov, 2000; López-Figueroa et al., 2004). In order to confirm these effects of antipsychotic treatment on mRNA and protein expression, long-term administration of haloperidol and clozapine, which display low and high affinity for 5-HT_{2A}R, respectively, was performed in rats. The results show a reduction in *Htr2a* gene expression and in 5-HT_{2A}R protein immunoreactivity in membrane-enriched preparations induced by clozapine but not by haloperidol treatment. In contrast, no changes in 5-HT_{2A}R protein immunodetection in cortical total homogenates are observed. Moreover, radioligand binding assays in membrane fractions of the same animals demonstrate that chronic clozapine decreases [^{3}H]ketanserin binding, which mimics previous results in DLPFC from AP-treated subjects (Muguruza et al., 2013). All these findings make the overactive $G_{\alpha i1}$-protein stimulation by (±)DOI an improbable consequence of long-term exposition of both AP-free and AP-treated subjects to antipsychotic drugs and point to an intrinsic alteration in schizophrenia. Globally considered, the present and previous data indicate that up-regulation of the agonist-sensitive functional 5-HT_{2A}R conformation in schizophrenia before treatment is counterbalanced through a decreased receptor synthesis and cell membrane protein expression induced by atypical antipsychotics. The expression of 5-HT_{2A}R in the cytosolic fraction of human brain homogenates is higher than in plasma membranes, a fact that induces greater experimental variability (Eastwood et al., 2001). However, although representing the minor population, 5-HT_{2A}R membrane fraction probably represents the population accessible to the neurotransmitter and, consequently, the most sensitive for functional coupling to G-proteins following interaction with agonists (Bhatnagar et al., 2001). The relevance that this reversion mechanism of the enhanced 5-HT_{2A}R function in schizophrenia plays on the therapeutic activity of atypical antipsychotics is unknown.

The present results provide evidence of a 5-HT_{2A}Rs coupling to different G_{α}-proteins in human DLPFC membranes when stimulated by the agonist (±)DOI, in concordance with the existence of functional selectivity or biased agonism through this receptor. In animals, activation of inhibitory $G_{\alpha i/o}$-proteins by (±)DOI and other LSD-like agonist drugs is considered a fingerprint of hallucinogenic properties mediated by 5-HT_{2A}Rs (González-Maeso et al., 2007). The enhanced (±)DOI-mediated 5-HT_{2A}R stimulation by (±)DOI in schizophrenia involves signaling through inhibitory $G_{\alpha i1}$- but not $G_{\alpha q/11}$-proteins, indicating that the functional hyperactivity of 5-HT_{2A}Rs is selectively exerted on hallucinogenic signal transduction pathways of this receptor. Whether other non-canonical 5-HT_{2A}R pathways involving G-protein dependent and independent pathways (Roth, 2011) are also altered in schizophrenia remains to be studied. The drug (±)DOI represents a 5-$HT_{2A/C}$R agonist (Knight et al., 2004) that promotes psychotic-like behaviors through 5-HT_{2A}Rs (González-Maeso et al., 2003; Halberstadt and Geyer, 2013; Moreno et al., 2013) and disorganizes electrophysiological network activity of prefrontal cortex (Celada et al., 2008). The blockade of (±)DOI stimulation by the selective 5-HT_{2A}R antagonist ketanserin (Knight et al., 2004) and the absence of this stimulation in 5-HT_{2A}R KO mice discard the implication of 5-HT_{2C}Rs in the observed effects of (±)DOI. The apparent scarce 5–7% increase of $G_{\alpha i1}$-protein activation by 5-HT_{2A}Rs in schizophrenia is not particularly surprising, since functional response in [^{35}S]GTPγS binding assays are known to be highly dependent on experimental conditions to detect GDP/GTP exchange. Along the same line, the possibility that this limited increase of $G_{\alpha i1}$-protein stimulation by (±)DOI could represent a selective dysfunction of some DLPFC neuron subtypes, generating a dilution effect, is an alternative explanation that deserves further investigation.

In conclusion, an overactive 5-HT_{2A}R signal trafficking through $G_{\alpha i1}$-proteins in DLPFC of subjects with schizophrenia that would predispose to psychotic symptoms has been found. In this context, schizophrenic patients and relatives have been described as more susceptible to psychotic responses induced by the hallucinogenic 5-HT_{2A}R agonist LSD (Anastasopoulos and Photiades, 1962; Hoch et al., 1952). Based on the present findings, it is tempting to speculate that drugs with 5-HT_{2A}R functional selective properties as antagonists or inverse agonists in $G_{\alpha i1}$-protein signaling pathways could represent better pharmacological tools for

the treatment of schizophrenia than non-selective 5-HT$_{2A}$R antagonists.

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Contributors

Aintzane García-Bea, Patricia Miranda-Azpiazu, Carolina Muguruza and Sara Marmolejo-Martinez-Artesero performed the experiments and undertook the statistical analysis. Rebeca Díez-Alarcia, Ane M Gabilondo, Luis F Callado and J Javier Meana contributed to literature searches, and supervised and interpreted the data. Benito Morentin and Luis F Callado provided access and conducted collection of human brain samples and toxicological information. Javier González-Maeso and J Javier Meana designed the study. Aintzane García-Bea, Ane M Gabilondo and J Javier Meana wrote 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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