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Dysregulated 5-HT$_{2A}$ receptor binding in postmortem frontal cortex of schizophrenic subjects

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Abstract

Previous postmortem and neuroimaging studies have repeatedly suggested alterations in serotonin 5-HT$_{2A}$ receptor (5-HT$_{2A}$R) binding associated with the pathophysiology of schizophrenia. These studies were performed with ligands, such as ketanserin, altanserin and LSD, that may bind with high-affinity to different structural or functional conformations of the 5-HT$_{2A}$R. Interpretation of results may also be confounded by chronic antipsychotic treatment and suicidal behavior in the schizophrenia group. We quantified 5-HT$_{2A}$R density by radioligand binding assays in postmortem prefrontal cortex of antipsychotic-free (n=29) and antipsychotic-treated (n=16) schizophrenics, suicide victims with other psychiatric diagnoses (n=13), and individually matched controls. [^{3}H]Ketanserin binding, and its displacement by altanserin or the LSD-like agonist DOI, was assayed. Results indicate that the number of [^{3}H]ketanserin binding sites to the 5-HT$_{2A}$R was increased in antipsychotic-free (128±11%), but not in antipsychotic-treated (92±12%), schizophrenic subjects. In suicide victims, [^{3}H]ketanserin binding did not differ as compared to controls. Aging correlated negatively with [^{3}H]ketanserin binding in schizophrenia, suicide victims and controls. The fraction of high-affinity sites of DOI displacing [^{3}H]ketanserin binding to the 5-HT$_{2A}$R was increased in antipsychotic-free schizophrenic subjects. Functional uncoupling of heterotrimeric G proteins led to increased fraction of high-affinity sites of altanserin displacing [^{3}H]ketanserin binding to the 5-HT$_{2A}$R in schizophrenic subjects, but not in controls. Together, these results suggest that the active conformation of the 5-HT$_{2A}$R is up-regulated in prefrontal cortex of antipsychotic-free schizophrenic subjects, and may provide a pharmacological explanation for discordant findings previously obtained.

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1. Introduction

5-Hydroxytryptamine (serotonin, 5-HT) receptors constitute a large family of evolutionary conserved seven transmembrane G protein-coupled receptors (GPCRs) (Millan et al., 2008). One of the serotonin receptor subtypes, the 5-HT_{2A}R, has been involved in the symptoms of schizophrenia, and proposed to contribute to the molecular mechanisms by which atypical antipsychotic drugs, such as clozapine, olanzapine and risperidone, induce their clinical effects (Gonzalez-Maeso and Sealfon, 2009; Fribourg et al., 2011; Kurita et al., 2012). Second generation, or atypical, antipsychotic drugs all have in common a high-affinity for the 5-HT_{2A}R, and a lower affinity for the dopamine D_2 receptor (Miyamoto et al., 2005). Hallucinogenic 5-HT_{2A}R agonists, such as mescaline, psilocybin and lysergic acid diethylamide (LSD), produce in normal subjects symptom profiles comparable to those seen in acutely ill unmedicated or first-episode schizophrenics (Young, 1974; Hermle et al., 1992; Vollenweider et al., 1998; Gouzoulis-Mayfrank et al., 2005; Quednow et al., 2011). Hallucinogenic drugs aggravate psychosis in schizophrenia patients (Hoch et al., 1952). Of particular interest is also the finding of hallucinogenic (LSD)-induced psychotic symptoms in relatives of schizophrenic patients (Anastasopoulos and Photiades, 1962), suggesting that individuals with greater predisposition to schizophrenia are more susceptible to the psychotic responses that require activation of the 5-HT_{2A}R. In murine models, we and others have demonstrated that 5-HT_{2A}R-regulated pathways on cortical pyramidal neurons are necessary to mediate the signaling pattern and behavioral responses to hallucinogenic drugs (see [Gonzalez-Maeso and Sealfon, 2009] for review).

Theory and experimental evidence suggest that GPCRs adopt multiple structural conformations when bound to different ligands. Drugs that increase or decrease the function of GPCRs are thought to modulate the proportion of receptors that are in the structurally active conformations relative to those in inactive, non-signaling conformations, respectively. Agonists bind with higher affinity to the active conformations of the receptor, whereas inverse agonists are ligands that preferentially bind and stabilize inactive conformational states. Interestingly, recent observations suggest that most clinically effective antipsychotic drugs are, in fact, 5-HT_{2A}R inverse agonists rather than simply neutral antagonists—ligands that compete for the same orthosteric binding site and prevent the cellular responses induced by agonists and inverse agonists (Egan et al., 1998; Fribourg et al., 2011). Since activation and decrease in the basal activity of the receptor represent a common feature of all hallucinogenic and atypical antipsychotic drugs, respectively, these findings suggest that the 5-HT_{2A}R may be involved in the mechanisms responsible for psychotic symptoms in schizophrenic patients.

Frontal cortex plays an important role in cognition and perception, and has been implicated more recently in schizophrenia and other psychotic disorders (Gonzalez-Maeso et al., 2008; Gonzalez-Maeso and Sealfon, 2009; Kurita et al., 2012). Many laboratories, including ours, have investigated the level of expression of 5-HT_{2A}R protein in frontal cortex of schizophrenic subjects. Most of this work has been performed in postmortem tissue samples with the use of radioligands, including [^{3}H]ketanserin (Reynolds et al., 1983; Mita et al., 1986; Laruelle et al., 1993; Burnet et al., 1996; Dean and Hayes, 1996; Dean et al., 1996; Dean et al., 1998; Dean et al., 1999; Marazziti et al., 2003; Matsumoto et al., 2005; Dean et al., 2008; Gonzalez-Maeso et al., 2008; Kang et al., 2009) and [^{3}H]LSD (Bennett et al., 1979; Whitaker et al., 1981; Joyce et al., 1993; Gurevich and Joyce, 1997), as well as in untreated first-episode schizophrenic patients by positron emission tomography (PET) with [^{18}F]altanserin (Rasmussen et al., 2010). Remarkably, there are striking differences in the results obtained: some studies suggested up-regulation of 5-HT_{2A}R binding sites, whereas others pointed toward absence of alterations or down-regulation in the number of binding sites. Recent reviews discuss the possible role of demographic and clinical measures, such as

age, treatment with antipsychotic or other psychotropic drugs, and suicide as cause of death, in these discrepant results between studies (Dean, 2003; Dean et al., 2008; Gonzalez-Maeso and Sealfon, 2009). Here, we extend our previous observations with evidence that [^{3}H]ketanserin binding sites are up-regulated in postmortem frontal cortex of antipsychotic-free schizophrenic subjects, and demonstrate that the density of [^{3}H]ketanserin binding sites is affected by antipsychotic drug treatment and aging. Our findings also suggest that this up-regulation may not be related to suicidal behavior, since the number of [^{3}H]ketanserin binding sites was unchanged in suicide victims with other neuropsychiatric disorders. With the use of ligands that preferentially bind different structural and/or functional receptor conformations, our findings could also provide a pharmacological explanation that may unify previous efforts on the quantification of 5-HT$_{2A}$R density in frontal cortex of schizophrenic subjects and controls.

2. Experimental procedures

2.1. Postmortem human brain samples

Human brains were obtained at autopsies performed in the Basque Institute of Legal Medicine, Bilbao, Spain, in compliance with policies of research and ethical boards for postmortem brain studies between 1992 and 2008. Deaths were subjected to retrospective searching for previous medical diagnosis and treatment using examiner's information and records of hospitals and mental health centers. After searching of antemortem information was fulfilled, 45 subjects who had met inclusion criteria of schizophrenia according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-III-R, DSM-IV or DSM-IV-R) were selected. Diagnosis of schizophrenia had been established by clinicians in charge of patients before death (Supplementary Tables 1 and 2). In the schizophrenia group, 37 subjects were suicide victims and the other 8 (4 antipsychotic-free and 4 antipsychotic-treated subjects) died of natural causes or motor vehicle accident. In order to explore the relationship between suicide completion and 5-HT$_{2A}$R density, an independent group of 13 suicide victims with other antemortem psychiatric disorders was collected (Supplementary Table 3). Of note, some of the antemortem psychiatric diagnoses are not included in the Diagnostic and Statistical Manual of Mental Disorders (*e.g.*, reactive depression and neurotic depression).

A toxicological screening for antipsychotics, antidepressants, other drugs and ethanol was performed on blood, urine, liver and gastric contents samples. Subjects who gave negative results for antipsychotic drugs in the toxicological screening were considered antipsychotic-free at death. The toxicological assays were performed at the National Institute of Toxicology, Madrid, Spain, using a variety of standard procedures including radioimmunoassay, enzymatic immunoassay, high-performance liquid chromatography and gas chromatography-mass spectrometry.

Controls for the present study were chosen among the collected brains on the basis, whenever possible, of the following cumulative inclusion criteria: (1) negative medical information on the presence of neuropsychiatric disorders or drug abuse; (2) appropriate gender, age (mean of differences 2 years, range 0–9 years), and postmortem delay defined as the time between death and tissue dissection/freezing (mean of differences 12 h, range 0–51 h), to match each subject in the schizophrenia or suicide group; (3) sudden and unexpected death (motor vehicle accidents); and (4) toxicological screening for psychotropic drugs with negative results except for ethanol.

Specimens of prefrontal cortex (Brodmann's area 9) were dissected at autopsy (0.5–1 g tissue) on an ice-cooled surface following standard procedures (Rajkowska and Goldman-Rakic, 1995), and immediately stored at −80°C until use. Tissue pH values were as follow:

control subjects, 6.5±0.08; schizophrenic subjects, 6.4±0.09; suicide victims, 6.3±0.03. Brain samples were also assayed for RNA integrity number (RIN) using the Agilent 2100 Bioanalyzer (Agilent Technologies) (control subjects: 7.8±0.1; schizophrenic subjects: 7.3±0.3; suicide victims, 7.3±0.2). The definitive pairs of antipsychotic-free schizophrenics, antipsychotic-treated schizophrenics, suicide victims with other psychiatric disorders, and individually matched controls are shown in Supplementary Tables 1, 2 and 3, respectively. Younger and older control subjects were included to investigate the effect of aging on 5-HT_{2A}R binding (Supplementary Table 4).

2.2. Mouse brain samples

Experiments were performed on adult (8–12 weeks old) 5-HT_{2A}R knockout (KO) and wild-type male 129S6/Sv mice (Gonzalez-Maeso et al. 2008). Animals were housed at 12 h light/dark cycle at 23°C with food and water *ad libitum*. The day of the experiment, mice were sacrificed by cervical dislocation, and bilateral frontal cortex was dissected and frozen at −80°C, or immediately processed for radioligand binding assays. This brain region (bregma 1.90 to 1.40 mm) includes motor cortex and somatosensory cortex. The coordinates were taken according to a published atlas of the 129S6/Sv mouse strain (Hof et al., 2000). The Institutional Animal Use and Care Committee at Mount Sinai School of Medicine approved all experimental procedures.

2.3. Materials and drugs

1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), guanosine 5′-[β,γ-imido]triphosphate trisodium salt hydrate [Gpp(NH)p], GTPγS, lysergic acid diethylamide (LSD), and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich. [^{3}H]Ketanserin was obtained from PerkinElmer Life and Analytical Sciences, Inc. Altanserin and methysergide were purchased from Tocris Cookson Inc. All other chemicals were obtained from standard sources.

2.4. Radioligand binding

Tissue samples of frontal cortex were homogenized using an Ultra-Turrax T8 homogenizer in 1 ml of homogeneization buffer (5 mM Tris-HCl; 0.25 M sucrose, pH 7.4). The crude homogenate was centrifuged at 1,000μg for 5 min at 4°C, and the supernatant was re-centrifuged at 40,000μg for 15 min at 4°C. The resultant pellet (P_2 fraction) was washed twice in incubation buffer (50 mM Tris-HCl; pH 7.4) and re-centrifuged in similar conditions. Aliquots were stored at −80°C until assay.

[^{3}H]Ketanserin binding assays were performed as previously reported with minor modifications (Gonzalez-Maeso et al., 2008). Briefly, [^{3}H]ketanserin binding (10 nM) was measured at equilibrium in 250 μl aliquots (incubation buffer) of membrane preparations (20–40 μg protein per well) which were incubated in 96-well plates at 37°C for 60 min. We selected this concentration of [^{3}H]ketanserin because our previous findings in mouse and postmortem human frontal cortex membrane preparations demonstrate that at 10 nM the number of binding sites obtained is similar to the maximum density of receptors (B_{max}) estimated by non-linear regression analysis of [^{3}H]ketanserin binding saturation curves (Gonzalez-Maeso et al., 2008). Competition curves were carried out by incubating DOI (10^{-12}-10^{-4} M; eighteen concentrations), altanserin (10^{-16}-10^{-5} M; thirteen concentrations) or LSD (10^{-10}-10^{-4} M; fourteen concentrations) in binding buffer containing 2.0 nM [^{3}H]ketanserin. This concentration of [^{3}H]ketanserin is similar to its affinity (K_D) value (see [Gonzalez-Maeso et al., 2008]). In order to evaluate the affinity of competitive ligands for inactive states of the 5-HT_{2A}R, in postmortem human brain, some of the competition binding experiments were performed in incubation buffer supplemented with Gpp(NH)p (100 μM), EDTA (1 mM), and NaCl (140 mM). Likewise, in mouse brain, some of the

experiments were performed in incubation buffer supplemented with GDP (50 μM), GTPγS (0.5 nM) EGTA (1 mM), $MgCl_2$ (3 mM) and NaCl (100 mM). Non-specific binding was determined in the presence of 10 μM methysergide. Reactions were incubated for 60 min at 37°C. Free ligand was separated from bound ligand by rapid filtration under vacuum (1450 FilterMate Harvester, PerkinElmer) through GF/C glass fiber filters. The filters were then rinsed three times with 300 μl binding buffer, dried (90°C, 10 min), and counted for radioactivity by liquid scintillation spectrometry using a MicroBeta TriLux counter (PerkinElmer).

2.5. Statistical analyses

Subject pairing was selected as the best design to control for experimental variance by the parallel processing of tissue samples from each subject pair (case and matched control). Therefore, analyses of antipsychotic-free and antipsychotic-treated schizophrenic subjects were conducted in separate along the study. [^{3}H]Ketanserin binding data at 10 nM followed a Gaussian distribution analyzed by the D'Agostino and Pearson omnibus normality test when expressed both as binding density in fmol/mg protein and as percentage of specific binding relative to individually matched controls. Importantly, when determining the number of [^{3}H]ketanserin binding sites in schizophrenic subjects (see Figs. 1 and 2 below), values are analyzed as percentage of specific binding relative to individually matched controls in order to avoid intra-group differences mostly due to aging (see below for discussion).

Radioligand binding displacement curves were analyzed using a nonlinear curve fitting (GraphPad Prism and InVivoStat softwares). The selection between models of competition curves by DOI, LSD, or altanserin against [^{3}H]ketanserin binding was made by the extra-sum-of-squares (F test). Following the nonlinear curve fitting, K_i values for DOI, LSD, and altanserin were calculated from the corresponding IC_{50} values. Differences in the binding profiles were assessed by unpaired Student's *t*-test of normalized parameters. Pearson's coefficient for simple correlation was calculated to test for possible association among variables. The influence of age as potential confounding variable on [^{3}H]ketanserin binding was assessed with ANCOVA. A further two-way ANOVA was used to evaluate the potential interaction between suicide and schizophrenia. The level of significance was chosen at p=0.05. All data are presented as mean±SEM.

3. Results

3.1. [^{3}H]Ketanserin Binding is Increased in Postmortem Frontal Cortex of Antipsychotic-Free Schizophrenic Subjects

[^{3}H]Ketanserin binds with high-affinity to both 5-HT_{2A} and 5-HT_{2C} receptors. Using the 5-HT_2 receptor ligand methysergide (10 μM) to define non-specific binding, we found that [^{3}H]ketanserin binding sites are abolished in frontal cortex of 5-HT_{2A} knockout (KO) mice as compared to wild type littermate controls (Supplementary Fig. 1). These findings validate the use of specific [^{3}H]ketanserin biding as a tool to measure 5-HT_{2A}R density.

We first investigated the number of [^{3}H]ketanserin binding sites in postmortem frontal cortex of schizophrenic subjects and controls. We compared two different groups of subjects: those either antipsychotic-free or antipsychotic-treated at time of death. Each of these two groups included individually matched controls (see Supplementary Tables 1 and 2 for demographic and toxicological information). Similar to our previous observations (Gonzalez-Maeso et al., 2008), [^{3}H]ketanserin binding to the 5-HT_{2A}R was higher (128±11%) in antipsychotic-free schizophrenic subjects as compared to individually matched controls (Fig. 1A). However, no differences (92±12%) were observed between schizophrenic subjects treated with antipsychotic drugs and individually matched controls

(Fig. 1B). Of note, 25 (13 antipsychotic-free and 12 antipsychotic-treated) out of these 45 schizophrenic subjects (29 antipsychotic-free and 16 antipsychotic-treated) and control pairs were included in our previous study (Gonzalez-Maeso et al., 2008). In a effort to control for confounding influence of age, we performed an ANCOVA with control, antipsychotic-free and antipsychotic-treated as the grouping effect, [^{3}H]ketanserin binding as the dependent variable and age as covariate. Consistent with the previous finding, [^{3}H]ketanserin binding to the 5-HT$_{2A}$R was higher ($F[2,86]=3.71$, $p<0.05$) in antipsychotic-free subjects as compared to controls ($p<0.05$, Fisher's post-hoc test), but did not differ between antipsychotic-treated subjects and controls ($p>0.05$, Fisher's post-hoc test).

Postmortem brain studies of suicide victims have suggested a relationship between 5-HT$_{2A}$R density in frontal cortex and suicidal behavior (Oquendo et al., 2006). We found that up-regulation of [^{3}H]ketanserin binding to the 5-HT$_{2A}$R remained statistically significant in non-suicide antipsychotic-free schizophrenic subjects (Fig. 2A), without changes in non-suicide antipsychotic-treated schizophrenic subjects (Fig. 2B). However, the low number of cases in these groups of non-suicide schizophrenic subjects (n=4 per group) prompted us to investigate the number of [^{3}H]ketanserin binding sites in frontal cortex of suicide victims with other psychiatric disorders and individually matched controls (see Supplementary Table 3 for demographic information). We found no significant differences in [^{3}H]ketanserin binding to the 5-HT$_{2A}$R between suicide subjects and controls (Fig. 2C). Among antipsychotic-free subjects, two-way ANOVA demonstrated the absence of interaction between schizophrenia and suicide ($F[1,83]=1.99$, $p>0.05$) with significant effect of schizophrenia condition ($F[1,83]=6.78$, $p<0.01$) but not of suicide ($F[1,83]=2.11$, $p>0.05$). In contrast, antipsychotic-treated subjects did not show influence of schizophrenia condition ($F[1,68]=1.47$, $p>0.05$), suicide ($F[1,68]=1.22$, $p>0.05$) or interaction between both factors ($F[1,68]=1.10$, $p>0.05$).

3.2. Effect of age on [^{3}H]ketanserin binding

The [^{3}H]ketanserin binding to postmortem frontal cortex membrane preparations displayed a negative correlation with the age at death ($R=-0.39$; $p<0.0001$) (Fig. 3, see also Supplementary Table 4 for additional control subjects not included in Supplementary Tables 1–3). Previous findings suggest that a second order polynomial equation (parabola) provides the best fit to the data describing the effect of aging on [^{3}H]ketanserin binding in postmortem human frontal cortex (Gross-Isseroff et al., 1990b). We found that a first order polynomial equation (straight line) provided the best fit to the data (first order polynomial versus second order polynomial: $F[1,106]=0.15$, $p>0.05$, all subjects in Supplementary Tables 1–4). Similar findings were obtained in antipsychotic-free schizophrenics (Supplementary Table 1), antipsychotic-treated schizophrenics (Supplementary Table 2), suicide victims with other psychiatric disorders (Supplementary Table 3), and controls (Supplementary Tables 1–4), (data not shown). Based on this, the first order polynomial equation was chosen as the best-fit model. According to this linear model, the average decrease per decade for [^{3}H]ketanserin binding was 6.67% relative to the estimated value in a 10-year-old subject. Further analysis showed that the differences between the slopes of controls and antipsychotic-treated schizophrenics are significant ($F[1,65]=6.02$, $p<0.01$). No significant differences were observed between the slopes of controls and antipsychotic-free schizophrenics ($F[1,76]=0.27$, $p>0.05$). Slopes of linear regressions were as follows: -2.25 ± 0.79 (controls in Supplementary Tables 1–4); -3.55 ± 2.55 (antipsychotic-free schizophrenics); -8.05 ± 2.84 (antipsychotic-treated schizophrenics). No significant correlation was observed between [^{3}H]ketanserin binding and postmortem delay (time between death and tissue dissection/freezing; $R=0.02$; $p>0.05$), freezing storage time at $-80°C$ ($R=-0,02$; $p>0.05$), RIN values ($R=0,06$; $p>0.05$), or tissue pH ($R=-0.05$; $p>0.05$).

3.3. Fraction of high-affinity sites of DOI displacing [^{3}H]ketanserin binding is increased in antipsychotic-free schizophrenic subjects

We first examined the pharmacological parameters of the 5-HT_{2A}R agonist DOI displacing [^{3}H]ketanserin binding in postmortem frontal cortex. As shown in Fig. 4, we found that DOI displaced [^{3}H]ketanserin binding in a triphasic manner (monophasic versus biphasic: $F[2,286]=64.15$, $p<0.0001$; biphasic versus triphasic: $F[2,284]=15.75$, $p<0.0001$).

Next, we compared the bindings sites for DOI displacing [^{3}H]ketanserin binding in schizophrenic subjects and controls. Interestingly, the fraction of high-affinity sites of DOI displacing [^{3}H]ketanserin binding was significantly increased in antipsychotic-free as well as in antipsychotic-treated schizophrenic subjects compared to individually matched controls (Fig. 5A, Fig. 5B, Fig. 5C, and Table 1). Notably, this fraction of high-affinity binding sites was increased in antipsychotic-free as compared to antipsychotic-treated schizophrenic subjects (Fig. 5C, and Table 1). No statistically significant differences were found between $K_{i\text{-}high}$, $K_{i\text{-}medium}$, $K_{i\text{-}low}$, or fractions of medium and low affinity sites of DOI displacing [^{3}H]ketanserin binding in untreated schizophrenics, treated schizophrenics, and individually matched controls (Figs. 5A–5D, and Table 1). No differences were found between the two groups of control subjects (Fig. 5C, and Table 1).

3.4. Functional uncoupling of G proteins increases the fraction of high-affinity sites of altanserin displacing [^{3}H]ketanserin binding in schizophrenic subjects, but not in controls

The highly specific alterations of both [^{3}H]ketanserin and DOI binding to the 5-HT_{2A}R in postmortem frontal cortex of antipsychotic-free schizophrenic subjects (see Fig. 1 and Fig. 5 above), together with previous findings by PET scan showing decrease in [^{18}F]altanserin binding in antipsychotic-naïve patients with first-episode schizophrenia (Rasmussen et al., 2010), led us to examine the pharmacological profile of altanserin displacing [^{3}H]ketanserin binding in antipsychotic-free and antipsychotic-treated schizophrenic subjects, and individually matched controls. In order to provide additional information regarding the affinity of altanserin for active and inactive states of the 5-HT_{2A}R, we also investigated the effect of functional uncoupling of heterotrimeric G proteins by the non-hydrolyzable GTP analog Gpp(NH)p on the affinity of altanserin displacing [^{3}H]ketanserin binding.

Non-hydrolyzable GTP analogs are characterized for their ability to uncouple heterotrimeric G proteins from their respective GPCRs, shifting agonist or inverse agonist competition curves to lower or higher overall affinities, respectively (see Supplementary Fig. 2). In control subjects, displacement of [^{3}H]ketanserin binding by altanserin was best described by a two-site model (Fig. 6A, Fig. 6B, and Table 2). We also found that the affinities of altanserin displacing [^{3}H]ketanserin binding were not affected in the presence of Gpp(NH)p. In schizophrenic subjects, altanserin also displaced [^{3}H]ketanserin binding in a biphasic manner, with no differences between antipsychotic-free or antipsychotic-treated schizophrenics, and individually matched controls (Fig. 6C, Fig. 6D, and Table 2). Notably, the presence Gpp(NH)p significantly increased the fraction of high-affinity sites of altanserin, without changes in $K_{i\text{-}high}$ and $K_{i\text{-}low}$ binding (Fig. 6C, Fig. 6D, and Table 2). This effect of Gpp(NH)p on altanserin binding was observed in both antipsychotic-free and antipsychotic-treated schizophrenics (Fig. 6E and Fig. 6F). No differences were found between the two groups of control subjects (Fig. 6 and Table 2).

Additional control experiments were performed with the 5-HT_{2A}R agonists DOI and LSD displacing [^{3}H]ketanserin binding in the presence or in the absence of a non-hydrolyzable GTP analog (Supplementary Fig. 3A and Fig. 3B). As expected, we found that uncoupling of heterotrimeric G proteins leads to lower affinities of DOI and LSD for the 5-HT_{2A}R. Together, these findings suggest the existence in schizophrenic subjects, but not in controls,

of a fraction of 5-HT_{2A}Rs that is sensitive to G protein uncoupling by non-hydrolyzable GTP analogs.

4. Discussion

In the present study we characterized the number of [^{3}H]ketanserin binding sites in postmortem frontal cortex of antipsychotic-free schizophrenic subjects, schizophrenic subjects treated with antipsychotic drugs, and control subjects individually matched by gender, age, and postmortem delay. We found that [^{3}H]ketanserin binding was increased in frontal cortex of antipsychotic-free schizophrenic subjects, with absence of changes in antipsychotic-treated schizophrenics. Since specific binding was absent in frontal cortex of 5-HT_{2A}-KO mice, these data suggest that changes in [^{3}H]ketanserin binding represent alterations at the 5-HT_{2A}R in frontal cortex of antipsychotic-free schizophrenics. Most of the schizophrenic subjects included in this study had committed suicide. Our findings, suggest that these findings are not affected by suicidal behavior, as [^{3}H]ketanserin binding was unchanged in suicide victims with other psychiatric disorders. We also found that the fraction of high-affinity sites of the hallucinogenic 5-HT_{2A}R agonist DOI displacing [^{3}H]ketanserin binding was significantly increased in antipsychotic-free schizophrenic as compared to individually matched controls, and that this increase was partially reversed in antipsychotic-treated schizophrenic subjects. Additionally, functional uncoupling of receptors and heterotrimeric G proteins significantly increased the fraction of high-affinity sites of altanserin to the 5-HT_{2A}R in schizophrenic subjects, yet this effect was not observed in controls. These data support the hypothesis of a selective up-regulation of [^{3}H]ketanserin binding in frontal cortex of antipsychotic-free schizophrenic subjects, with an specific increase in the fraction of active states of the 5-HT_{2A}R. Other hypotheses, including down-regulation of radioligand binding, have been proposed to account for the potential role of the 5-HT_{2A}R in schizophrenia frontal cortex. In the course of this study, we have tested several of these alternative explanations for our results.

4.1. Effect of antipsychotic drugs on 5-HT_{2A}R binding

In this study, our findings suggest that [^{3}H]ketanserin binding to the 5-HT_{2A}R is increased in frontal cortex of antipsychotic-free schizophrenic subjects, and that treatment with antipsychotic drugs decreases this number of binding sites to control levels. Similar findings were previously reported in postmortem frontal cortex of antipsychotic-free and antipsychotic-treated schizophrenic subjects (Gonzalez-Maeso et al., 2008). This also correlates with our previous findings that chronic treatments with the atypical antipsychotics clozapine and risperidone down-regulate [^{3}H]ketanserin binding in mouse cortical neurons, an effect that is not observed after chronic treatment with the typical antipsychotic haloperidol (Gonzalez-Maeso et al., 2008; Kurita et al., 2012). Since only two of the schizophrenic subjects included in this study were treated with haloperidol (see Supplementary Table 2), these and our previous findings in postmortem human brain and mouse models support the hypothesis that chronic treatment with atypical antipsychotic drugs decreases the number of binding sites for [^{3}H]ketanserin in frontal cortex.

Some researchers have suggested that [^{3}H]ketanserin binding is decreased or unchanged in schizophrenia. Most (Reynolds et al., 1983; Laruelle et al., 1993; Burnet et al., 1996; Dean and Hayes, 1996; Dean et al., 1998; Dean et al., 1999; Matsumoto et al., 2005; Dean et al., 2008; Kang et al., 2009), but not all (Mita et al., 1986; Laruelle et al., 1993), of these studies were performed in frontal cortex of treated schizophrenic subjects. Our findings in rodent models (Gonzalez-Maeso et al., 2008) and postmortem human brain (Fig. 1) suggest that absence of changes and/or decreased [^{3}H]ketanserin binding in postmortem frontal cortex of treated schizophrenic subjects may be a consequence of the effect of chronic treatment with atypical antipsychotic drugs. These effects of chronic treatment with atypical antipsychotic

drugs are consistent with the fact that the slopes of linear regression between [^{3}H]ketanserin binding and age were significantly steeper (more negative) in antipsychotic-treated, but not in antipsychotic-free, schizophrenic subjects as compared to controls (see below for further discussion regarding the effect of aging on [^{3}H]ketanserin binding). Overall, our results suggest up-regulation of [^{3}H]ketanserin binding in frontal cortex of antipsychotic-free schizophrenics. Although further investigation is needed, it is tempting to speculate that this up-regulation of 5-HT$_{2A}$R in frontal cortex may be associated to psychotic symptoms in schizophrenia patients, hypothesis that is also supported by findings in postmortem temporal cortex of parkinsonian subjects with visual hallucinations treated with medications that were not antipsychotics (Huot et al., 2010). Previous findings suggest absence of effect of ethanol present in postmortem toxicological analyses (Gross-Isseroff et al., 1990b), and absence of differences in [^{3}H]ketanserin binding between alcoholics and controls (Underwood et al., 2008). However, further investigation is also needed to determine the effect, if any, of ethanol and psychoactive drugs such as amphetamine and tetrahydrocannabinol on 5-HT$_{2A}$R binding in postmortem human frontal cortex.

Together with the effects of treatment with antipsychotic drugs, potential explanations for these discrepancies in [^{3}H]ketanserin binding findings are also parameters intrinsic to the studies carried out in postmortem human brain tissue samples such as age, inclusion of control subjects that are not individually matched by demographic variables, and the reliability of postmortem psychiatric diagnosis mostly based on postmortem family interviews. In schizophrenia patients, hallucinations and delusions typically attenuate with aging (Davidson et al., 1995). This clinical measure is further supported by the negative correlation of [^{3}H]ketanserin binding and age at time of death that we and others have found in postmortem frontal cortex tissue samples (Fig. 3 and [Gross-Isseroff et al., 1990b; Gonzalez-Maeso et al., 2008]), and by the decline in [^{18}F]altanserin binding relative to age in healthy volunteers (Moses-Kolko et al., 2011). The significant effect of aging on [^{3}H]ketanserin binding highlights the importance of employing study designs that include schizophrenic subjects individually, and not collectively, matched to control subjects by demographic factors such as gender, age and postmortem delay (see Supplementary Tables 1–3). This profound effect of aging also provides the rationale for the statistical analysis of data presented as percentage of specific [^{3}H]ketanserin binding in schizophrenic subjects relative to individually matched controls, and not as binding density in fmol/mg protein, in order to avoid intra-group differences (*i.e.*, age-related differences in [^{3}H]ketanserin binding within the control group). The two most commonly used approaches for assessing psychiatric diagnoses in postmortem human brain research are clinical record reviews and postmortem interviews with family and peers. Recent publications suggest that psychiatric diagnoses are difficult to ascertain based on postmortem family interviews (Deep-Soboslay et al., 2005). Our findings were obtained in postmortem tissue samples of subjects with antemortem diagnosis of schizophrenia or other neuropsychiatric disorders based on the clinical information obtained at mental health centers (see Experimental procedures, above).

Previous findings demonstrate an age-dependent decrease in [^{3}H]ketanserin binding that reaches a minimum in the 5th decade followed by an age-dependent increase in older subjects (6th and 7th decade) (Gross-Isseroff et al., 1990b). Our findings suggest that linear regression provides the best fit to the data describing the effect of aging on [^{3}H]ketanserin binding in postmortem human frontal cortex (ages ranged from 4 to 86 years, see Supplementary Tables 1–4). These divergent results might be explicable by differences in experimental approaches (radioligand binding in plasma membrane preparations or tissue sections) as well as the effect of age at different anatomical regions in frontal cortex.

4.2. Different findings with ketanserin, altanserin and LSD

Agonists bind with greatest affinity to the active (G protein-coupled) state of the receptor. Our findings with the hallucinogenic 5-HT_{2A}R agonist DOI show a selective increase in the fraction of high-affinity sites displacing [^{3}H]ketanserin binding as an alteration in postmortem frontal cortex of antipsychotic-free as well as antipsychotic-treated schizophrenics when compared to control subjects. Taking into consideration that the density of [^{3}H]ketanserin binding sites was unchanged in antipsychotic-treated schizophrenics (Fig. 1), further investigation is needed to better understand the persistence of these changes in the fraction of high-affinity binding sites of DOI displacing [^{3}H]ketanserin binding after antipsychotic treatment. However, and importantly, these changes with the hallucinogen DOI were significantly more pronounced in antipsychotic-free as compared with antipsychotic-treated schizophrenics, thereby suggesting that treatment with atypical antipsychotic drugs partially reverses alterations in the functionally active conformations of the 5-HT_{2A}R in schizophrenia frontal cortex. Similar findings have been previously observed by using other hallucinogenic 5-HT_{2A}R radioligands, with up-regulation of [^{3}H]LSD binding in postmortem frontal cortex of untreated, but not treated, schizophrenic subjects (Whitaker et al., 1981; Joyce et al., 1993; Gurevich and Joyce, 1997). In markedly contrast, however, a series of experiments focused on PET imaging convincingly demonstrate that [^{18}F]altanserin binding is decreased in frontal cortex of drug-naïve first-episode schizophrenic patients (Rasmussen et al., 2010). A potential explanation for these apparently discrepant findings is the different functional outcomes of LSD-like drugs and altanserin, as well as their affinity for all the structural conformations of the 5-HT_{2A}R. Biochemical findings in postmortem frontal cortex suggest that, opposite to DOI and LSD, the ligand altanserin presents a higher affinity for the inactive G protein-uncoupled conformation of the 5-HT_{2A}R in schizophrenic subjects. Thus, heterotrimeric G protein uncoupling increases the fraction of high-affinity sites of altanserin binding to the 5-HT_{2A}R in schizophrenic subjects—indicating that altanserin behaves as a 5-HT_{2A}R inverse agonist in schizophrenic subjects, but not in controls (see also Supplementary Fig. 2). The alterations in both altanserin (Fig. 6) and DOI (Fig. 5) displacing [^{3}H]ketanserin binding that we found in postmortem frontal cortex of schizophrenic subjects point toward up-regulation in the fraction of 5-HT_{2A}Rs that are susceptible of being uncoupled from heterotrimeric G proteins. Together, these data suggest that up-regulation in the fraction of active G protein-coupled 5-HT_{2A}R will lead to both increased binding of DOI (Fig. 5), and, possibly, decreased binding of altanserin (Rasmussen et al., 2010) in schizophrenic subjects. Further work studying 5-HT_{2A}R-G protein coupling is definitely needed to validate this hypothesis, as well as the molecular mechanisms underlying the differences in the effect of Gpp(NH)p in schizophrenic subjects and controls.

4.3. The 5-HT_{2A}R and suicide

Numerous abnormalities have been reported in the serotonergic system in suicide victims. In this study, there were no differences in [^{3}H]ketanserin binding to the 5-HT_{2A}R in a group of suicide victims with psychiatric disorders such as dysthymic disorder, alcoholism, and anorexia nervosa, among others. Similarly, previous work has shown that [^{3}H]ketanserin binding is unaffected in postmortem frontal cortex of suicide victims with major depression (Owen et al., 1983; Crow et al., 1984; Cheetham et al., 1988; Arranz et al., 1994; Lowther et al., 1994; Stockmeier et al., 1997; Rosel et al., 2000). Few studies using [^{3}H]ketanserin reported that the 5-HT_{2A}R is altered in suicide victims without psychiatric diagnosis (Gross-Isseroff et al., 1990a; Turecki et al., 1999). [^{3}H]Ketanserin binding has also been shown to correlate with lifetime aggression in suicide (Oquendo et al., 2006). Our preliminary findings in non-suicide antipsychotic-free schizophrenic subjects suggest that up-regulation of [^{3}H]ketanserin binding sites is related to schizophrenia and not to suicidal behavior (Fig. 2A and 2B). However, a possible explanation for the absence of changes in [^{3}H]ketanserin

binding that we found in suicides (Fig. 3C) may be related to the psychiatric diagnoses of the subjects included in the study. We still cannot exclude the possibility of dysregulations in [^{3}H]ketanserin binding that differ (up-regulation, down-regulation and/or absence of change) in the variety of diagnoses of the suicide victims, which limits the validity of our findings in non-schizophrenic suicide completers (Supplementary Table 3). Adding more complexity, overrepresentation of suicide among the schizophrenia groups could potentially reflect special characteristics of the schizophrenic subjects examined that not fully represent the spectrum of the disorder. Nonetheless, it appears that suicide does not statistically interact with 5HT$_{2A}$ receptor changes neither in antipsychotic-free nor in antipsychotic-treated subjects. The apparent consistency of this finding suggests that increase in [^{3}H]ketanserin binding is more likely related to schizophrenia status than to suicidal behavior in the disease process. However, further work is clearly needed to determine the impact, if any, of suicide on 5-HT$_{2A}$R binding.

Concluding Remarks

In summary, we find that [^{3}H]ketanserin binding is increased in postmortem frontal cortex of antipsychotic-free schizophrenic subjects. We demonstrate up-regulation of high-affinity binding sites for the hallucinogenic 5-HT$_{2A}$R agonist DOI in schizophrenia frontal cortex. Our findings also suggest that altanserin behaves as a 5-HT$_{2A}$R inverse agonist in schizophrenic subjects, but not in controls. These data may help explain the differences previously reported with use of ligands that bind with different affinities to distinct 5-HT$_{2A}$R conformations. The 5-HT$_{2A}$R has been shown to form heterocomplexes with GPCRs such as the metabotropic glutamate 2 receptor (Gonzalez-Maeso et al., 2008; Fribourg et al., 2011). At present, it is unknown if the relationships between the 5-HT$_{2A}$R and other neurotransmitter receptors as GPCR heterocomplexes are involved in schizophrenia and other neuropsychiatric disorders: a subject under active investigation in our laboratory. Our findings may be potentially useful for a better understanding of the biochemical alterations responsible for schizophrenia and other psychotic disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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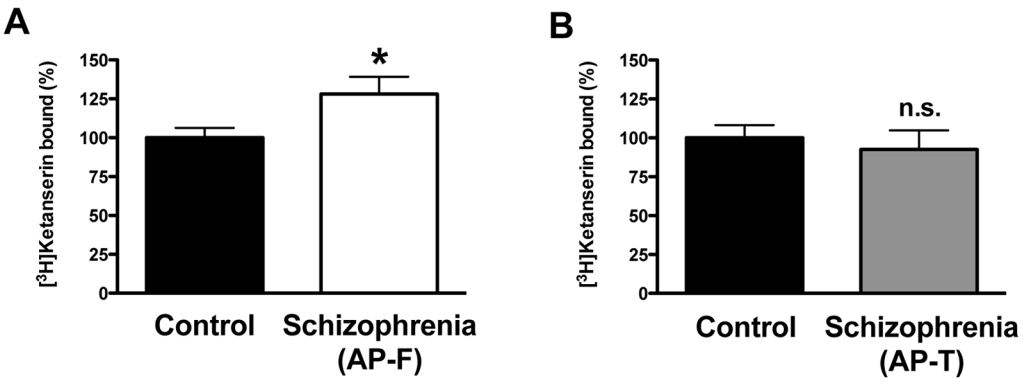

Fig. 1.

Density of 5-HT_{2A}R shown as [^{3}H]ketanserin specific binding (10 nM) in frontal cortex of schizophrenic subjects. (**A**) Antipsychotic-free (AP-F) schizophrenic subjects (n=29) and controls (n=29). (**B**) Antipsychotic-treated (AP-T) schizophrenic subjects (n=16) and controls (n=16). Data are shown as percentage of [^{3}H]ketanserin specific binding relative to individually matched controls. *p<0.05; n.s., not significant; Student's *t*-test. See Supplementary Tables 1 and 2 for demographic information. All values represent means ±SEM.

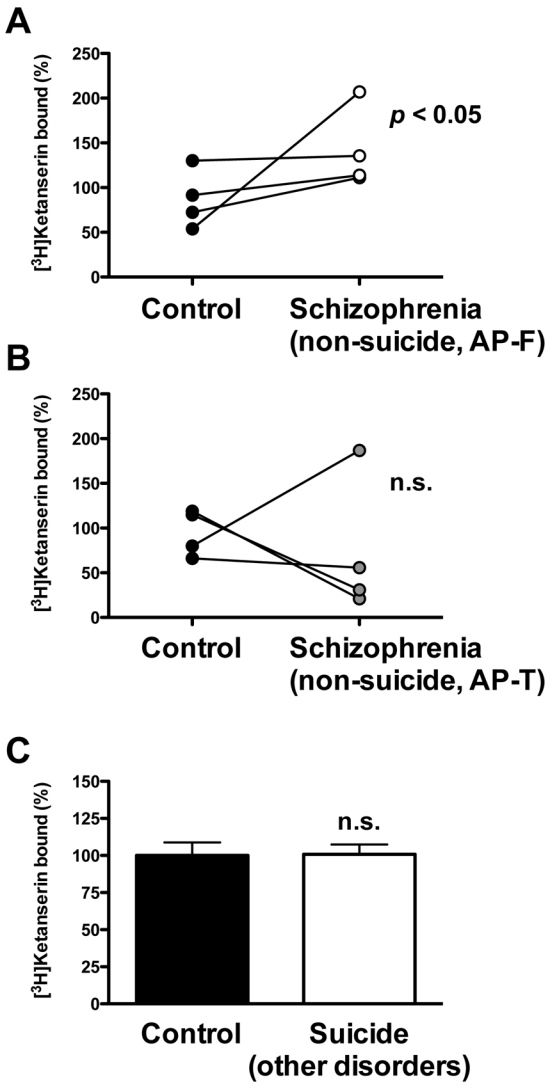

Fig. 2.

(**A**) Individual representation of [^{3}H]ketanserin specific binding (10 nM) in frontal cortex of non-suicide—antipsychotic-free (AP-F) schizophrenic subjects (n=4) and controls (n=4). (**B**) Individual representation of [^{3}H]ketanserin specific binding in frontal cortex of non-suicide—antipsychotic-treated (AP-T) schizophrenic subjects (n=4) and controls (n=4). (**C**) [^{3}H]Ketanserin specific binding in frontal cortex of suicide victims with other psychiatric disorders (n=13) and controls (n=13). Data are shown as percentage of [^{3}H]ketanserin specific binding relative to individually matched controls. $p<0.05$; n.s., not significant; Student's *t*-test. See Supplementary Tables 1, 2 and 3 for demographic information. All values represent means±SEM.

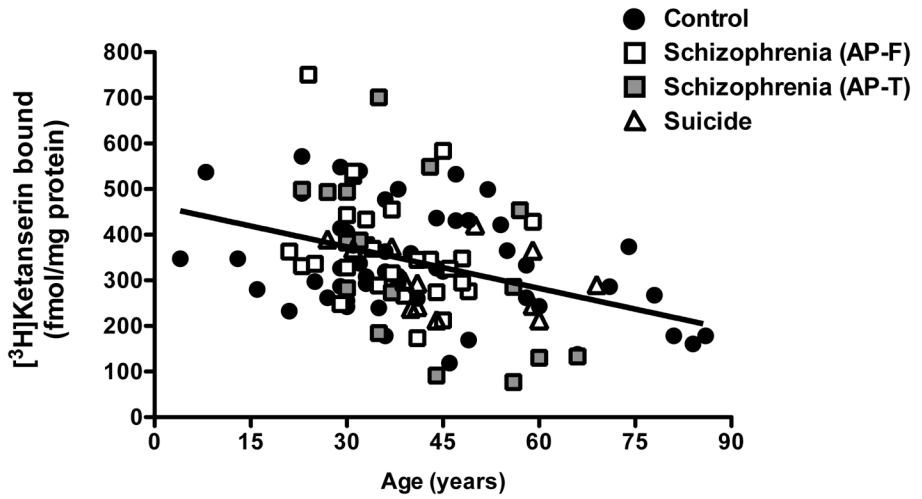

Fig. 3.

Age-related changes in [^{3}H]ketanserin specific binding (10 nM) in frontal cortex of antipsychotic-free (AP-F) schizophrenics (n=29), antipsychotic-treated (AP-T) schizophrenics (n=16), suicide victims with other psychiatric disorders (n=13), and controls (n=55). Pearson's correlation: $R=-0.37$; $p<0.0001$.

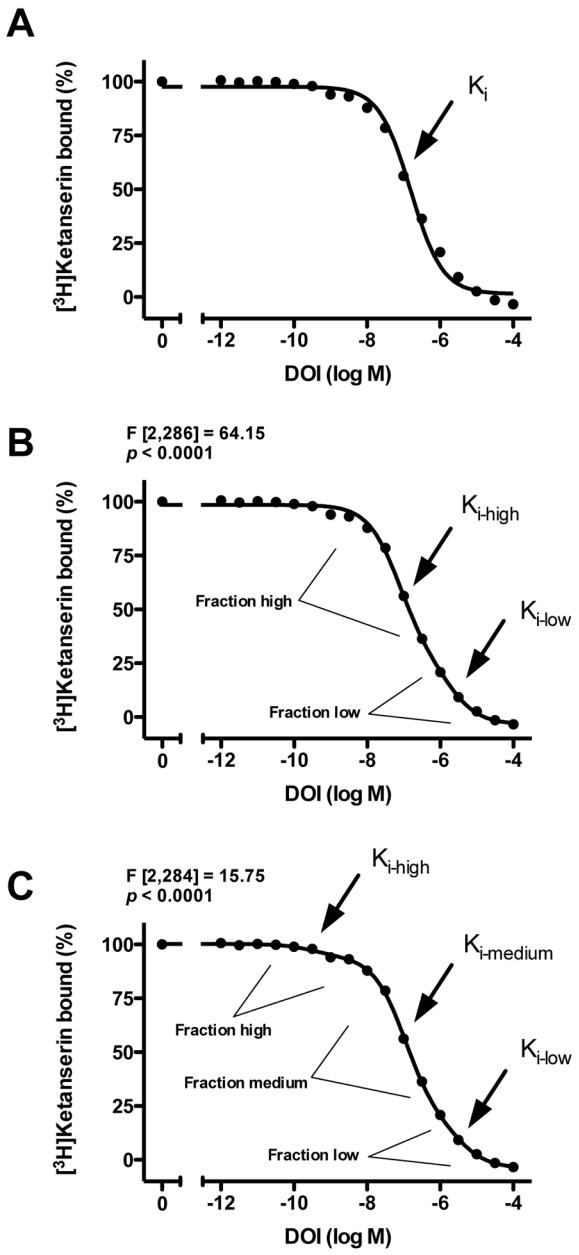

Fig. 4.

(**A,B,C**) Displacement curves of [^{3}H]ketanserin specific binding (2 nM) by DOI in frontal cortex of control subjects (n=24). The same dataset was analyzed as monophasic (**A**), biphasic (**B**) and triphasic (**C**) displacement curve. Data were best fit to a biphasic (**B**) compared to monophasic (**A**) displacement curve. Data were best fit to a triphasic (**C**) compared to biphasic (**B**) displacement curve. All values represent means±SEM. Standard errors are not depicted when they are smaller than the symbols.

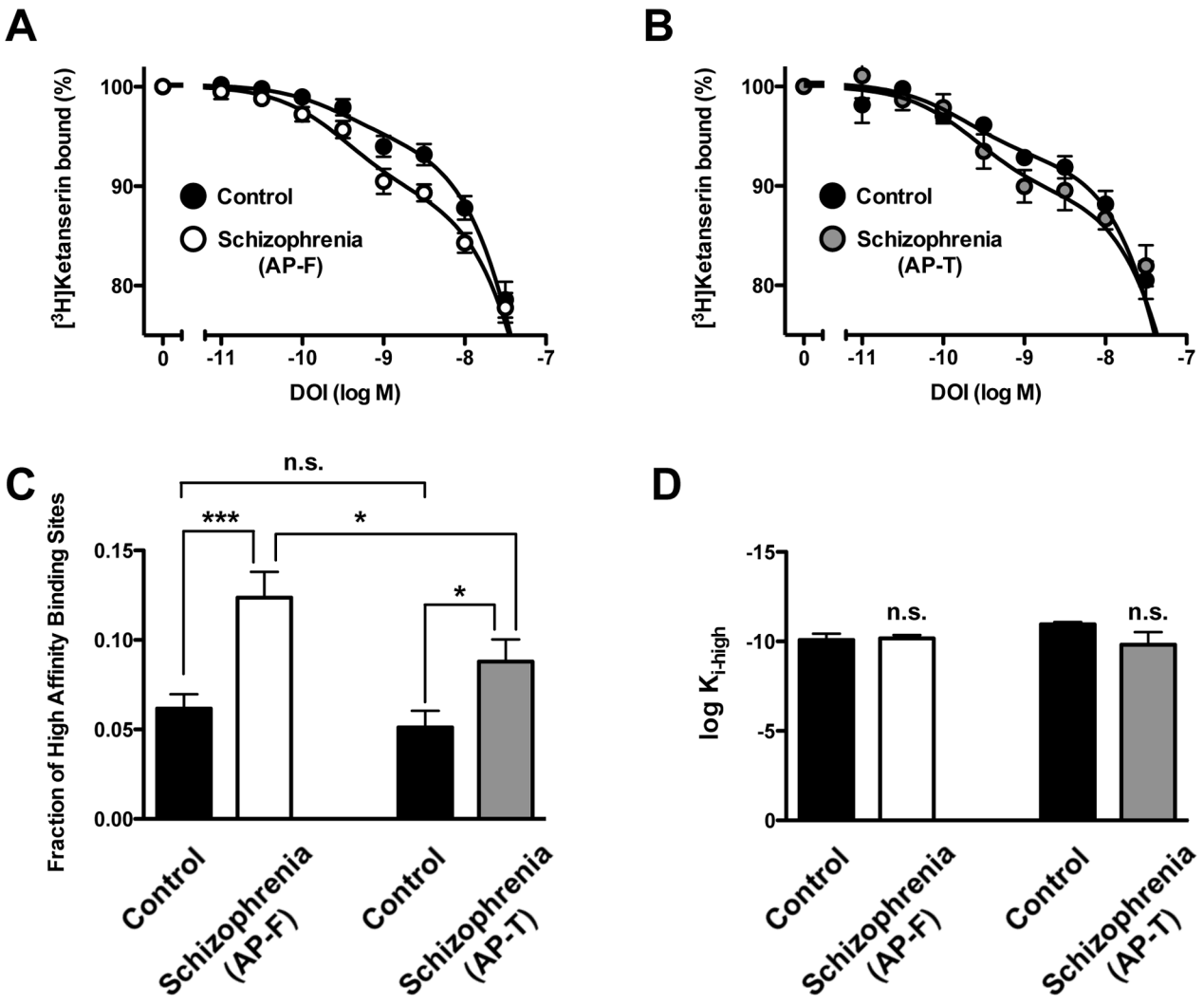

Fig. 5.

(**A**) [^{3}H]Ketanserin specific binding (2 nM) displacement curves by DOI in postmortem frontal cortex of antipsychotic-free (AP-F) schizophrenic subjects and controls. (**B**) [^{3}H]Ketanserin specific binding (2 nM) displacement curves by DOI in postmortem frontal cortex of antipsychotic-treated (AP-T) schizophrenic subjects and controls. (**C**) Fraction of high-affinity sites of DOI displacing [^{3}H]ketanserin specific binding. (**D**) Normalized high-affinity (log $K_{i\text{-}high}$) of DOI displacing [^{3}H]ketanserin specific binding in schizophrenic subjects and individually matched controls. *p<0.05; ***p<0.001; n.s., not significant; Student's *t*-test. See Supplementary Tables 1 and 2 for demographic information. See Table 1 for statistical analysis. All values represent means±SEM.

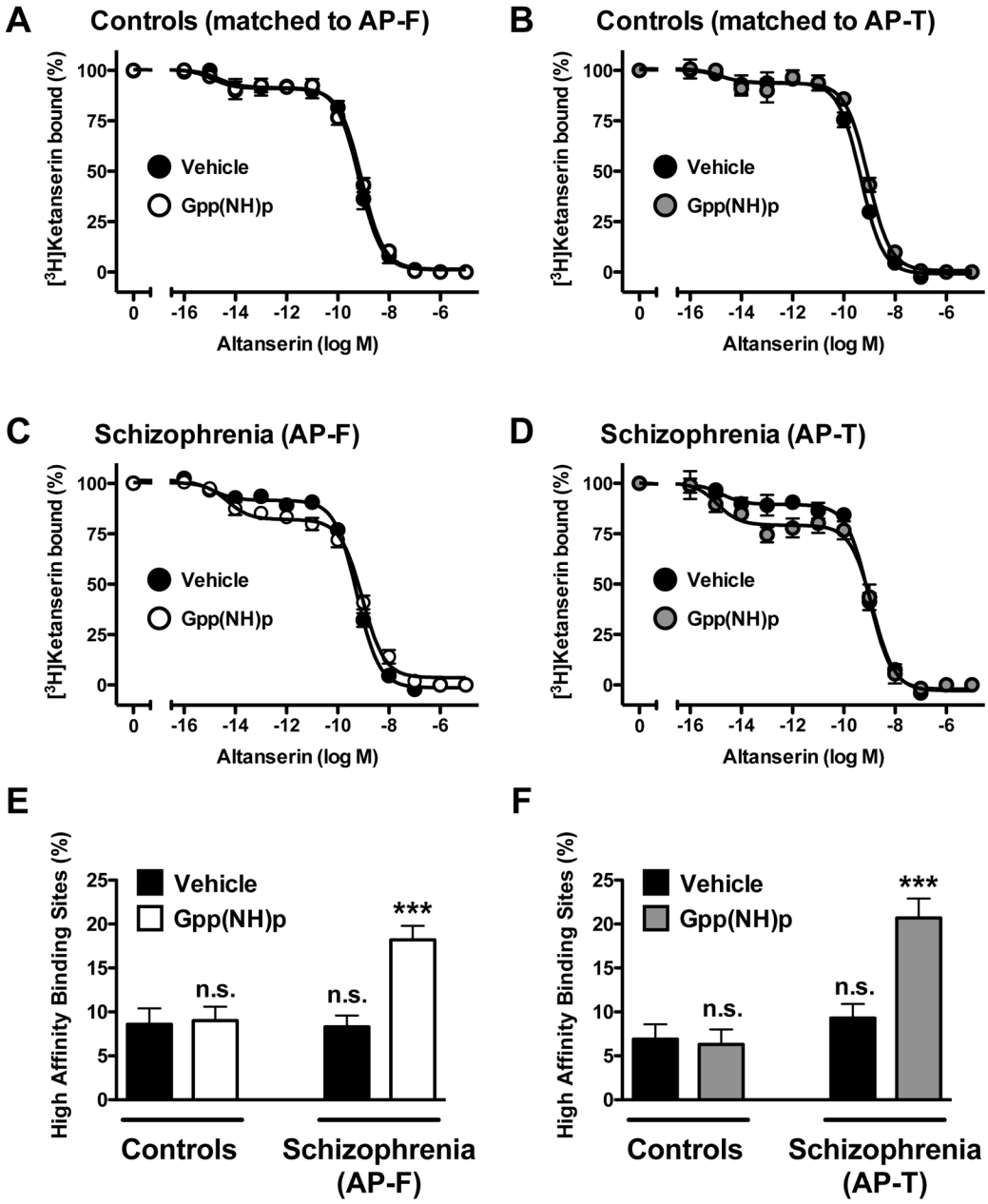

Fig. 6.

(**A,B,C,D**) [^{3}H]Ketanserin specific binding (2 nM) displacement curves by altanserin in postmortem frontal cortex of schizophrenic subjects (**C,D**) and controls (**A,B**) in the presence and in the absence of the non-hydrolyzable GTP analog Gpp(NH)p. The presence of Gpp(NH)p increases the fraction of high-affinity binding of altanserin displacing [^{3}H]ketanserin binding in antipsychotic-free (AP-F) (**C,E**) and antipsychotic-treated (AP-T) (**D,F**) schizophrenics, but not in individually matched controls (**A,B,E,F**). ***p<0.001; n.s., not significant; Student's *t*-test. See Supplementary Tables 1 and 2 for demographic information. See Table 2 for statistical analysis. All values represent means±SEM.

Table 1

[^{3}H]Ketanserin binding displacement curves by DOI in frontal cortex membrane preparations of antipsychotic-free (AP-F) schizophrenics, antipsychotic-treated (AP-T) schizophrenics, and controls.

	Control	Schizophrenia(AP-F)	Control	Schizophrenia (AP-T)
log $K_{i\text{-}high}$	−10.21 ± 0.32	−10.20 ± 0.15 ***	−10.78 ± 0.33	−10.53 ± 0.33 *,+
Fraction high (%)	5.51 ± 1.06	10.34 ± 0.88 ***	−05.75 ± 1.01	8.76 ± 1.51 *,+
log $K_{i\text{-}medium}$	−7.70 ± 0.05	−7.55 ± 0.03 ***	0-7.69 ± 0.04	−7.56 ± 0.07 *,+
Fraction medium (%)	72.5 ± 5.31	73.8 ± 1.68 ***	--78.0 ± 2.74	75.2 ± 4.20 *,+
log $K_{i\text{-}low}$	−6.25 ± 0.16	−5.91 ± 0.19 ***	0-6.06 ± 0.23	−5.94 ± 0.37 *,+

* p<0.05;

*** p<0.001 when compared with individually matched control subjects by Student's *t*-test.

+ p<0.05 when compared with antipsychotic-free schizophrenic subjects by Student's *t*-test.

Data were best fit to a triphasic compared to biphasic displacement curve by F-test (p<0.0001).

See Supplementary Tables 1 and 2 for demographic information. All values represent means ± SEM.

Table 2

[^{3}H]Ketanserin binding displacement curves by altanserin in frontal cortex membrane preparations of antipsychotic-free (AP-F) schizophrenics, antipsychotic-treated (AP-T) schizophrenics, and controls. Experiments were performed in the presence or in the absence of Gpp(NH)p.

	Control		Schizophrenia (AP-F)		Control		Schizophrenia (AP-T)	
	Vehicle	**Gpp(NH)p**	**Vehicle**	**Gpp(NH)p**	**Vehicle**	**Gpp(NH)p**	**Vehicle**	**Gpp(NH)p**
log $K_{i\text{-}high}$	−15.2 ± 0.60	−15.2 ± 0.05	−15.3 ± 0.40	−14.9 ± 0.01 ***	−15.4 ± 0.80	−15.0 ± 0.80	−15.4 ± 0.50	−15.3 ± 0.32 **
Fraction high (%)	8.6 ± 1.80	9.0 ± 1.60	8.3 ± 1.30	18.2 ± 1.61 ***	6.3 ± 1.70	6.3 ± 1.70	9.3 ± 1.60	20.7 ± 2.21 **
log $K_{i\text{-}low}$	−9.8 ± 0.05	−9.4 ± 0.02	−9.9 ± 0.04	−9.3 ± 0.05 ***	−10.1 ± 0.05	−9.4 ± 0.06	−9.7 ± 0.05	−9.2 ± 0.08 **

** p<0.01;

*** p<0.001 when compared with individually matched control subjects (vehicle) by Student's *t*-test.

Data were best fit to a biphasic compared to monophasic displacement curve by F-test (p<0.0001). See Supplementary Tables 1 and 2 for demographic information. All values represent means ± SEM.