ANTAGONIST FUNCTIONAL SELECTIVITY: 5-HT$_2$A
SEROTONIN RECEPTOR ANTAGONISTS DIFFERENTIALLY
REGULATE 5-HT$_2$A RECEPTOR PROTEIN LEVEL IN VIVO

Prem N. Yadav, Wesley K. Kroeze, Martilias S. Farrell and Bryan L. Roth

Departments of Pharmacology (.PN.Y.,W.K.K., M.S.F., B.L.R..), Medicinal Chemistry (B.L.R.) and Psychiatry (B.L.R.), and Program in Neurosciences and NIMH Psychoactive Drug Screening Program(B.L.R.), University of North Carolina - Chapel Hill Medical School, Chapel Hill, NC 27759
Running title: Functional Selectivity of 5-HT2A receptor antagonists

Corresponding Author:

Bryan L. Roth MD, PhD
Department of Pharmacology
UNC Chapel Hill Medical School
4072 Genetic Medicine Building
120 Mason Farm Road
Chapel Hill, NC 27599
Fax: 919-843-5788
Tel: 919-966-7535 (Office)
Email: bryan_roth@med.unc.edu

Number of text pages: 26

Number of tables: 0

Number of figures: 5

Number of references: 39

Number of words:
  - Abstract: 157
  - Introduction: 666
  - Discussion: 485

Abbreviations: 5-HT_{2A} (serotonin 2A); PCP (phencyclidine), GPCR (G Protein Coupled Receptor)

Recommended section: Neuropharmacology
ABSTRACT

Dysregulation of the serotonin2A (5-HT2A) receptor is implicated in both the etiology and treatment of schizophrenia. Although the essential role of 5-HT2A receptors in atypical antipsychotic drug actions is widely accepted, the contribution of 5-HT2A down-regulation to their efficacy is not known. We hypothesized that down-regulation of cortical 5-HT2A receptors contributes to the therapeutic action of atypical antipsychotic drugs. To test this hypothesis, we assessed the effect of chronically administered antipsychotics (clozapine, olanzapine, and haloperidol), and several 5-HT2A antagonists (ketanserin, altanserin, M100907, M11939, SR46349B and pimavanserin), on the phencyclidine (PCP)-induced hyperlocomotor response and cortical 5-HT2A receptor levels in C57BL/6J mice. Clozapine and olanzapine, but not haloperidol, induced receptor down-regulation and attenuated PCP-induced locomotor responses. Of the selective 5-HT2A antagonists tested, only ketanserin caused significant receptor protein down-regulation, whereas SR46349B up-regulated 5-HT2A receptors and potentiated PCP-hyperlocomotion; the other 5-HT2A receptor antagonists were without effect. The significance of these findings with respect to atypical antipsychotic drug action is discussed.
INTRODUCTION

According to classical concepts of pharmacology, competitive receptor antagonists occupy the ligand binding site and ‘antagonize’ receptor activity by precluding agonist binding: “In competitive antagonism, agonist and antagonist, simultaneously present in solution, are each considered to compete for receptors to the exclusion of the other…” (Stephenson, 1956). This notion of blockade of G-protein coupled receptor (GPCR) activation by “steric hindrance” has been supported by recent crystallographic studies of the β-adrenergic receptor showing that agonists and antagonists occupy overlapping (but not identical) binding sites (Rosenbaum et al., 2007; Rosenbaum et al., 2011; Warne et al., 2011). The notion that some GPCR antagonists can also stabilize the inactive state of the receptor, and thereby function as inverse agonists, was first put forth by Costa and Herz in studies of opioid receptors (Costa and Herz, 1989). It is now well accepted that so-called antagonists could have negative intrinsic activity and thus function as inverse agonists, or lack activity and thus function as neutral antagonists. By definition, however, an ‘antagonist’ is devoid of efficacy.

Contradicting these fundamental notions of pharmacology have been observations dating to the late 1970s that some so-called ‘antagonists’ can display various patterns of efficacy. Thus, Bergstrom and Kellar (Bergstrom and Kellar, 1979) and Peroutka and Snyder (Peroutka and Snyder, 1980) showed that chronic administration of antidepressants with potent 5-HT$_{2A}$ antagonist activity induces a down-regulation of 5-HT$_{2A}$ serotonin receptor levels. This was subsequently demonstrated to be a property shared by a number of drugs with potent 5-HT$_{2A}$ antagonist activity,
including typical and atypical antipsychotic drugs (Andree et al., 1986), typical and atypical antidepressants (Blackshear and Sanders-Bush, 1982; Brunello et al., 1982), and even relatively selective 5-HT\textsubscript{2A} antagonists (Leysen et al., 1986). There have also been reports that some 5-HT\textsubscript{2A} antagonists can up-regulate 5-HT\textsubscript{2A} receptors after chronic administration (Rinaldi-Carmona et al., 1993) or have no effect (Gray and Roth, 2001). Antagonist-induced 5-HT\textsubscript{2A} down-regulation might have therapeutic relevance (Elphick et al., 2004; Harvey et al., 2004; O’Connor and Roth, 2005). We now know that antagonist-induced receptor down-regulation can be readily explained by the concept of functional selectivity (Urban et al., 2007) or biased agonism (Galandrin et al., 2007) (Roth and Chuang, 1987).

The mechanism(s) responsible for the paradoxical antagonist-induced receptor “down-regulation” are still unclear. Several studies have demonstrated that 5-HT\textsubscript{2A} antagonists can induce receptor internalization \textit{in vitro} (Berry et al., 1996; Willins et al., 1999; Bhatnagar et al., 2001) and \textit{in vivo} (Willins et al., 1998; Willins et al., 1999). Additionally receptor down-regulation is apparently independent of changes in receptor gene transcription (Roth and Ciarnello, 1991). Two independent studies have indicated that antagonist-induced internalization occurs via clathrin-dependent processes \textit{in vitro} (Bhatnagar et al., 2001; Hanley and Hensler, 2002).

Additionally, although there have been a large number of reports of antagonist-induced down-regulation of 5-HT\textsubscript{2A} receptors, all prior studies have relied on \textit{radioligand binding to quantify changes in total receptor protein}. Obviously, there are a number of possible explanations for alterations in radioligand binding following drug administration that could account for changes in receptor protein levels.
independent of receptor down-regulation (e.g., residual drug, pseudo-irreversible binding, and receptor inactivation). Ideally, one would like to be able quantify 5-HT$_{2A}$ receptor protein via a conventional biochemical technique—for instance Western blotting—to begin to definitively address the question of antagonist functional selectivity.

It has long been known that available 5-HT$_{2A}$ antibodies are neither sufficiently specific nor sensitive for biochemical studies in vivo (Xia et al., 2003). Recently, however, we identified and validated a 5-HT$_{2A}$ receptor antibody in wild-type and 5-HT$_{2A}$ knock-out mice, and showed that it was suitable for immunochemical and biochemical studies (Magalhaes et al., 2010; Yadav et al., 2011). Similar results were reported by another group (Weber and Andrade, 2010). Using this new reagent, we are now able to report that 5-HT$_{2A}$ antagonists differentially regulate 5-HT$_{2A}$ receptor protein levels in vivo. Additionally, we are able to identify three classes of 5-HT$_{2A}$ antagonists: those that induce receptor down-regulation, those that induce receptor up-regulation and antagonists that have no effect on receptor protein levels.
MATERIALS AND METHODS

Mice

Male C57BL/6J mice were either obtained from Jackson Laboratories (Bar Harbor, ME) or bred for 2-3 generation in house to obtain sufficient number of male mice (24-30) between 8-10 weeks of age to test drugs. All experiments were approved by the Institutional Animal Care and Use Committee at the University of North Carolina, Chapel Hill. Mice were housed under standard conditions – 12 hour light/dark cycle and food and water ad libitum.

Drugs

Clozapine (8-Chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo[b,e][1,4]diazepin) and Olanzapine (2-Methyl-4-(4-methyl-1-piperazinyl)-10H-thieno[2,3-b][1,5]benzodiazepine) was purchased from from Sigma-Aldrich (St Louis, MO, USA) and Haloperidol ((4-[4-(4-Chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butaneone hydrochloride)) was obtained from RBI (Natick, MA, USA). Ketanserin (3-[2-[4-(4-Fluorobenzoyl)-1-piperidinyl]ethyl]-2,4[1H,3H]-quinazolinedione tartrate), Altanserin (3-[2-[4-(4-Fluorobenzoyl)-1-piperidinyl]ethyl]-2,3-dihydro-2-thioxo-4(1H)-quinazolinone hydrochloride) and M11939 (Alpha-Phenyl-1-(2-phenylethyl)-4-piperidinemethanol) were purchased from Tocris Bioscience (Ellisville, MO, USA). M100907 (Alpha-(2,3-dimethoxyphenyl)-1-(2-(4-fluorophenylethyl))-4-piperidine methanol) and SR46349B (4-[(2Z)-3-[[2-(dimethylamino)ethoxy]amino]-3-(2-fluorophenyl)prop-2-en-1-ylidene]cyclohexa-2,5-dien-1-one) was obtained from Sanofi Aventis (Bridgewater, NJ,
USA). Pimavanserine was kindly provided by Dr. Herb Meltzer (Department of Psychiatry, Vanderbilt University, TN, USA).

**Chronic drug treatment and behavior testing regimen**

C57/BL6 mice (8-10 weeks old) were first tested for acute vehicle (0.9% NaCl solution) or phencyclidine (PCP, 6.0 mg/kg, *i.p.*) induced locomotor response. After 8-10 days of washout, same cohort of mice were used for chronic drug treatments (one injection/day between 2-4 PM, for 14 days) either with vehicle (5ml/kg of solution containing 0.9% NaCl, 50 mM Tartaric acid, 1% DMSO) or M100907 (5.0 mg/kg) or M11939 (5.0 mg/kg) or SR46349B (5.0 mg/kg) or ketanserin (10.0 mg/kg), altanserin (10.0 mg/kg) or pimavanserin (5.0 mg/kg) or clozapine (10.0 mg/kg) or olanzapine (10.0 mg/kg) or haloperidol (0.5 mg/kg). The dosage of each drugs used in this study were decided on the basis of literature doses to induce a regulatory adaptation of 5-HT$_{2A}$ receptors (Gray and Roth, 2001) or the maximum tolerated doses which are in excess of those shown previously to induce antipsychotic-like activity. Thus, the doses of M100907 and M11939 chosen (5 mg/kg) exceed the maximum effective doses used in behavioral studies (0.1 mg/kg for M100907 and M11939)(Costall and Naylor, 1995; Martin et al., 1997). Likewise for ketanserin the dose chosen (10 mg/kg) exceeds the effective dose for normalization of MK801-induced hyperactivity (0.12 mg/kg)(O’Neill et al., 1998) and was the maximum tolerated dose for this drug. For altanserin we chose a dose of 10 mg/kg which was the maximum tolerated dose and in excess of that previously used for behavioral studies of 5-HT2A receptors (0.5 mg/kg)(Stoessl et al., 1990). For pimavanserin, the dose chosen (5 mg/kg) was the maximum tolerated dose and again exceeded the dose used previously in behavioral studies (3 mg/kg)(Horiguchi et al.,
2011). To determine the chronic effects of drug on PCP induced locomotor response, after 12-14 hrs of last drug administration same cohort of mice were challenged with PCP (6.0 mg/kg, i.p.) and locomotor response were measured. Further, mice were euthanized right after locomotore testing and cortical tissue were isolated for the measurement of 5-HT$_{2A}$ receptor levels.

**Radioligand binding assays**

For saturation binding assays, brain regions were rapidly microdissected, frozen on dry ice, and then stored at -80°C. A Tissue TearorTM (BioSpec Products, OK) was used to homogenize tissue (15 seconds, 15,000 rpm) in ice-cold standard binding buffer (SBB - 50 mM Tris-HCl, pH 7.4; 10 mM MgCl$_2$; 0.1 mM EDTA). Homogenized tissue was centrifuged for 20 min at 27,000 x g (4°C); crude membrane pellets were collected and washed two more times in a total of 20 ml of ice-cold SBB. After the last wash, the membrane pellet was either used immediately for binding, or stored at -80°C until use. Saturation binding assays were performed with the homogenized brain tissue and [$^3$H]-ketanserin, then incubated in SBB for 1.5 hours as detailed (Abbas et al., 2009). Nonspecific binding was determined by incubating the reactions with 10 μM ritanserin. Reactions were harvested by vacuum filtration through glass filters (3X ice-cold 50 mM Tris, pH 7.4; pH 6.9 at room temperature) and measured by liquid scintillation using a Perkin-Elmer Tri-Carb 2800TR scintillation counter. Non-linear saturation analysis was done using Graphpad Prism 4.01 to obtain $B_{max}$ values, and Bradford protein assays were (Bio-Rad, Hercules, CA) performed in order to normalize $B_{max}$ determinations to the amount of protein in each assay.
Western blotting

For 5-HT$_{2A}$ receptor immunoblotting, frontal cortex crude membrane preparations were resuspended in cold lysis buffer (50 mM Tris-Cl, pH 7.4; 150 mM NaCl, 10% glycerol, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, plus protease inhibitor cocktail (Roche Diagnostics) and incubated on ice for 1 hr. Detergent-soluble proteins were collected after 20 min of centrifugation at 4°C and 12,500g, and 500 μg (1.0 mg/ml) of protein was incubated with 25-30 μl (packed volume) of wheat germ agglutinin (WGA)-conjugated agarose beads (Vector laboratories, Inc. CA) for a minimum of 2 hrs at 4°C on a rotary mixer. After three washes with 500 μl of lysis buffer, WGA-bound proteins were eluted with 40 μl of 1X SDS-sample buffer, and whole eluates were resolved by SDS-PAGE, followed by electrotransfer of protein from gel to PVDF membrane. Membranes were blocked for one hr using buffer containing 3% non-fat dry milk, 1 % BSA and 5% glycerol in TBST. Rabbit polyclonal anti-5-HT$_{2A}$ receptor antibody (Neuromics, Edina, MN) was used at a 1:500 dilution for 12-18 hrs at 4°C for measurement of 5-HT$_{2A}$ receptor immunoreactivity. For loading control, same membrane was stripped and reprobed with anti-transferrin receptor mouse monoclonal antibody (Invitrogen Corp., CA). We have previously verified the specificity of the anti-5-HT$_{2A}$ receptor antibody using 5-HT$_{2A}$ WT and KO mice cortex tissue by immonoblotting (Magalhaes et al., 2010).

Locomotor and stereotypic activity

Locomotor activity was assessed in photocell-based activity chambers under standardized environmental conditions, using an AccuScan activity monitor (AccuScan...
Instruments, Columbus, OH) with a 25.8 × 25.8 cm Plexiglas chamber and a beam spacing of 1.52 cm as described (Abbas et al., 2009). Mice were injected \textit{i.p.} with vehicle (0.9\% NaCl solution) or PCP (6 mg/kg, dissolved in saline solution) after 20-30 min of habituation in activity chambers followed by measurement of horizontal activity for 60-90 min. Horizontal activity was measured as the total distance covered in centimeters as the total of all vectored X-Y coordinate changes.

**Statistical analysis.** For quantitation of immunoblots, comparison of \( B_{\text{max}} \) data, and other two-group comparisons, two-tailed unpaired \( t \) tests were used to ascertain statistical significance. All behavioral data were analyzed by two-way ANOVA followed by Bonferroni post tests for comparing multiple groups. Comparisons were considered significant if \( p \) was less than 0.05.
RESULTS

Olanzapine and clozapine but not haloperidol down-regulate 5-HT$_{2A}$ receptors. In initial studies we performed dose-response investigating the ability of the atypical antipsychotic drug clozapine to induce cortical 5-HT$_{2A}$ receptor down-regulation in C57BL/6J mice. To quantify 5-HT$_{2A}$ receptor protein levels we used our recently validated Western blot procedure (Magalhaes et al., 2010; Yadav et al., 2010) along with parallel radioligand binding studies with $^3$H-ketanserin. As can be seen in dose-response studies (Figure 1), 10.0 mg/kg clozapine induced maximal down-regulation of 5-HT$_{2A}$ receptor protein, and thus this dose was chosen for further studies.

We next evaluated three drugs we had previously shown to induce a differential pattern of receptor internalization in vitro and in vivo: clozapine, olanzapine and haloperidol (Willins et al., 1999). As can be seen, chronic treatment with clozapine and olanzapine, but not haloperidol, induced a down-regulation of 5-HT$_{2A}$ receptor protein as assessed by both Western blot (Figs. 2A,B) and radioligand binding (Figs. 2C,D) studies. As is seen in Figs. 2E-H, chronic olanzapine and clozapine, but not haloperidol or vehicle, was associated with an attenuation of the PCP-induced locomotor response.

Selective and preferential 5-HT$_{2A}$ antagonists differentially modulate 5-HT$_{2A}$ receptor protein levels following chronic administration. We next evaluated a series of selective 5-HT$_{2A}$ antagonists for their ability to induce 5-HT$_{2A}$ receptor down-regulation. As shown in Figures 3A-C, chronic treatment with M100907 and MDL11939 had no effect
on either 5-HT$_{2A}$ receptor protein or radioligand binding. Additionally, no effect was seen on PCP-induced locomotor responses (Figs. 3D-F).

By contrast, the prototypical 5-HT$_{2A}$ antagonist ketanserin, but not the closely related compound altanserin, induced 5-HT$_{2A}$ receptor protein down-regulation (Figs. 4A-C). Significantly, chronic treatment with these compounds had no significant effect on PCP-induced locomotor responses (Figs. 4D-F).

We next tested two 5-HT$_{2A}$ antagonists that have been shown in small-scale trials to have efficacy in treating psychosis, SR46349B (Meltzer et al., 2004) and pimavanserin (Meltzer et al., 2010). In the case of SR46349B, an up-regulation of 5-HT$_{2A}$ receptor protein was noted, whereas chronic pimavanserin treatment had no effect (Figs. 5A-F). These results indicate that reasonably selective 5-HT$_{2A}$ antagonists may display strikingly different effects on receptor protein levels: up-regulation (SR46349B), down-regulation (ketanserin) or no effect (M100907, altanserin, pimavanserin, MDL11939). SR46349B-mediated up-regulation was associated with a potentiation of PCP-induced locomotor responses (Fig. 5G), whereas chronic pimavanserin treatment had no effect (Fig. 5H).
DISCUSSION

The main finding of these studies is that 5-HT$_{2A}$ receptor antagonists can be classified into three major categories based on their differing abilities to regulate 5-HT$_{2A}$ receptor protein expression: (1) up-regulating antagonists (SR46349B); (2) down-regulating antagonists (ketanserin, clozapine, olanzapine); and (3) antagonists that do not alter receptor expression in male C57BL/6J mice at the doses used in this study (M100907, MDL11939, pimavanserin, altanserin). We also report that the SR46349B-induced receptor up-regulation is associated with an apparently augmented PCP-induced locomotor response. Although it has been widely reported that 5-HT$_{2A}$ antagonists display differential abilities to induce alterations in 5-HT$_{2A}$ receptor radioligand binding, this is the first study demonstrating an actual change in 5-HT$_{2A}$ receptor protein levels following chronic drug administration.

For many years it has been suggested that 5-HT$_{2A}$ down-regulation might be associated with the therapeutic actions of many drugs (Gray and Roth, 2001; Elphick et al., 2004; O’Connor and Roth, 2005). Indeed, down-regulation of 5-HT$_{2A}$ receptors is likely part of the mechanism by which certain 5-HT$_{2A}$ antagonists apparently protect against JC virus infections (Elphick et al., 2004; O’Connor and Roth, 2005). It has also been suggested that 5-HT$_{2A}$ receptor down-regulation itself might contribute to atypical antipsychotic drug actions. Indeed, as this paper clearly shows, chronic treatment with clozapine and olanzapine—two prototypical atypical antipsychotic drugs, induces substantial down-regulation of 5-HT$_{2A}$ receptor protein. This down-regulation was associated with an attenuated ability of PCP to induce a locomotor response. By contrast, chronic treatment with the typical antipsychotic drug haloperidol was without
effect on either PCP-induced locomotion or 5-HT$_{2A}$ receptor levels. However, atypical antipsychotic clozapine and olanzapine have marked polypharmacology, with affinities for dopamine, serotonin, muscarinic, adrenergic receptors (Roth et al., 2004). Thus, it is quite complicated and the subject considerable controversy to explain unique salutary effects of clozapine and other related atypicals (Roth et al., 2004; Allen and Roth, 2011).

Interestingly, chronic treatment with three candidate antipsychotic drugs (M100907, SR46349B and pimavanserin) either had no effect (M100907, pimavanserin) or up-regulated 5-HT$_{2A}$ receptors. It is interesting in this regard that not only did none of these drugs attenuate PCP’s hyperlocomotive ability, but also that none of these drugs displayed efficacy in large-scale Phase III clinical trials. Taken together, these findings are consistent with the notion that down-regulation of 5-HT$_{2A}$ receptors may be important for certain therapeutic actions of atypical antipsychotic drugs. However, atypical antipsychotic clozapine and olanzapine have marked polypharmacology, with affinities for dopamine, serotonin, muscarinic, adrenergic receptors (Roth et al., 2004). Thus, it is quite complicated and the subject of considerable controversy to explain unique salutary effects of clozapine and other related atypicals (Allen and Roth, 2011).

The most important implication of these findings is the clear-cut evidence in favor of the hypothesis that antagonists display unique patterns of functional selectivity. The precise molecular mechanisms responsible for the paradoxical down-regulation of 5-HT$_{2A}$ receptors \textit{in vivo} is currently unknown and are the topic of intensive research at present (Yadav et al, \textit{in preparation}).
AUTHORSHIP CONTRIBUTIONS:

Participated in research design: Yadav and Roth.

Conducted experiments: Yadav, Kroeze, and Farrell.

Performed data analysis: Yadav and Roth.

Wrote or contributed to the writing of the manuscript: Yadav, Kroeze and Roth.

REFERENCES

Abbas AI, Yadav PN, Yao WD, Arbuckle MI, Grant SG, Caron MG and Roth BL (2009) PSD-95 is essential for hallucinogen and atypical antipsychotic drug actions at serotonin receptors. J Neurosci 29:7124-7136.


FOOT NOTES:
This work was funded by grants from the National Institutes of Health (U19MH82441; RO1MH61887) to BLR and the Michael Hooker Chair.
Figure Legends

Figure 1. Dose dependent down-regulation of 5-HT\textsubscript{2A} receptor by clozapine. (A) C57BL/6J mice were treated with vehicle or clozapine (2.5-10.0 mg/kg, \textit{i.p.}) for 14 days, and 5-HT\textsubscript{2A} receptor levels were determined by immunoblotting of WGA immunoprecipitates from frontal cortex membrane lysates (upper panel). The same blots were stripped and probed for transferrin receptor as loading controls (lower panel). (B) Quantitation of immunoblots by densitometry of 5-HT2A immunoreactivity (n= 4 mice/treatment group).

Figure 2. Chronic clozapine and olanzapine, but not haloperidol, down-regulate 5-HT\textsubscript{2A} receptor and attenuate PCP-induced hyperlocomotor response in C57BL/6J mice. (A) Immunoblot of immunoprecipitates from cortex membrane lysates. The same blots were stripped and probed for transferrin receptor as loading controls. (B) Densitometry of immunoblots (n=6 mice/treatment group, *p<0.05 by unpaired \textit{t} test). (C) Representative saturation isotherms obtained by binding of [\textsuperscript{3}H]ketanserin with equal amounts of cortical membrane proteins (25 \textmu g) from vehicle (Veh) or clozapine (Cloz) or olanzapine (Olanz) or haloperidol (Hal) treatment groups. (D) B\textsubscript{max} estimates were obtained by performing [\textsuperscript{3}H]ketanserin saturation binding on cortical membrane homogenates. Data are presented as mean ± SEM (n=6/group, *p<0.05 unpaired \textit{t} test). (E-H) Chronic treatments with clozapine or olanzapine (10.0 mg/kg, 14 days) significantly attenuated PCP (6.0 mg/kg)-induced hyperactivity, but haloperidol (0.5 mg/kg) had no significant effect in C57BL/6J mice (n=8/group). Data are expressed as mean total horizontal
distance travelled in 5 minute bins over 60 min after PCP administration (± SEM). *p<0.05, two way ANOVA, followed by Bonferroni post tests for multiple comparisons.

**Figure 3.** Chronic administration of 5-HT$_{2A}$ selective antagonists M100907 or M11939, did not modulate 5-HT$_{2A}$ receptor level and PCP-induced hyperlocomotor response in C57BL/6J mice. (A) Immunoblot of immunoprecipitates from cortex membrane lysates. The same blots were stripped and probed for transferrin receptor as loading controls. (B) Densitometry of immunoblots of IP from n=4 mice/treatment group. (C) $B_{\text{max}}$ estimates were obtained by performing [3H]ketanserin saturation binding on cortical membrane homogenates. Data are presented as mean ± SEM (n=6/group). (D-F) Chronic treatments with vehicle or M100907 or M11939 (5.0 mg/kg, 14 day) had no significant effect on PCP (6.0 mg/kg)-induced hyperactivity in C57BL/6J mice (n=7/group). Data are expressed as mean total horizontal distance travelled in 5 minute bins over 60 min after PCP administration (± SEM).

**Figure 4.** Chronic administration of the 5-HT2A antagonist ketanserin, but not altanserin, down-regulates cortical 5-HT$_{2A}$ receptor level in C57BL/6J mice. (A) measurement of 5-HT2A receptor level in frontal cortex membrane lysates by immunoblotting of immunoprecipitates. The same blots were reprobed for transferrin receptor as loading controls. (B) Densitometry of immunoblots of IP from n=4 mice/treatment group *p<0.05, unpaired $t$ test. (C) $B_{\text{max}}$ estimates were obtained by performing [3H]ketanserin saturation binding on cortical membrane homogenates. Data are presented as mean ± SEM (n=6/group). (D-F) Chronic treatment with vehicle or ketanserin or altanserin (10.0 mg/kg, 14 days) had no significant effect on PCP (6.0 mg/kg)-induced hyperactivity in C57BL/6J mice (n=8/group). Data are expressed as
mean total horizontal distance travelled in 5 minute bins over 90 min after PCP administration (± SEM).

**Figure 5.** Chronic administration of the 5-HT2A selective inverse agonist SR46349b, but not pimavanserin, up-regulates 5-HT2A receptor level but had no significant effect on PCP-induced hyperlocomotor response in C57BL/6J mice. (A,B) Immunoblot of immunoprecipitates from cortex membrane lysates. The same blots were reprobed for transferrin receptor as loading controls. (C,D) Densitometry of immunoblots of IP from n=4 mice/treatment group, *p<0.05, unpaired t test. (E,F) B_max estimates were obtained by performing [3H]ketanserin saturation binding on cortical membrane homogenates. Data are presented as mean ± SEM (n=6/group) *p<0.05, unpaired t test. (D-F) Chronic treatment with vehicle or SR46349b or pimavanserin (5.0 mg/kg, 14 day) had no significant effect on PCP (6.0 mg/kg)-induced hyperactivity in C57BL/6J mice (N=6-8/group). Data are expressed as mean total horizontal distance travelled in 5 minute bins over 90 min after PCP administration (± SEM), *p<0.05, unpaired t test.
Figure 1.

<table>
<thead>
<tr>
<th>Clozapine (mg/kg)</th>
<th>0</th>
<th>2.5</th>
<th>5.0</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IB: anti 5-HT2A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IB: anti Transferrin Receptor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Graph showing 5-HT2A level (% veh) for different concentrations of Clozapine: Veh, Cloz 2.5, Cloz 5.0, Cloz 10.0.
Figure 5.

(A) Western blot analysis showing the expression levels of 5-HT₂A receptor (IB: anti-5HT₂A) and transferrin (IB: Transferrin) in Veh, SR46349B, and IP:WGA conditions.

(B) Western blot analysis showing the expression levels of 5-HT₂A receptor (IB: anti-5HT₂A) and transferrin (IB: Transferrin) in Veh, Pimavanserin, and IP:WGA conditions.

(C) Bar graph showing the 5-HT₂A level (% Veh) before and after treatment with Veh and SR46349B.

(D) Bar graph showing the 5-HT₂A density (% Veh) before and after treatment with Veh and Pimavanserin.

(E) Bar graph showing the B₅₀ (fmol/mg) before and after treatment with Veh and SR46349B.

(F) Bar graph showing the B₅₀ (fmol/mg) before and after treatment with Veh and Pimavanserin.

(G) Graph showing the distance travelled (cm) before and after treatment with SR46449B and after PCP injection.

(H) Graph showing the distance travelled (cm) before and after treatment with Pimavanserin and after PCP injection.