

# Antagonist Functional Selectivity: 5-HT<sub>2A</sub> Serotonin Receptor Antagonists Differentially Regulate 5-HT<sub>2A</sub> Receptor Protein Level In Vivo

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## ABSTRACT

Dysregulation of the 5-HT<sub>2A</sub> receptor is implicated in both the etiology and treatment of schizophrenia. Although the essential role of 5-HT<sub>2A</sub> receptors in atypical antipsychotic drug actions is widely accepted, the contribution of 5-HT<sub>2A</sub> down-regulation to their efficacy is not known. We hypothesized that down-regulation of cortical 5-HT<sub>2A</sub> 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-HT<sub>2A</sub> antagonists [ketanserin, altanserin,  $\alpha$ -(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidinemethanol (M100907),  $\alpha$ -phenyl-1-(2-phenylethyl)-4-piperidinemethano (M11939),

4-[(2Z)-3-[[2-(dimethylamino)ethoxy]amino]-3-(2-fluorophenyl)prop-2-en-1-ylidene]cyclohexa-2,5-dien-1-one (SR46349B), and pimavanserin], on the phencyclidine (PCP)-induced hyperlocomotor response and cortical 5-HT<sub>2A</sub> 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-HT<sub>2A</sub> antagonists tested, only ketanserin caused significant receptor protein down-regulation, whereas SR46349B up-regulated 5-HT<sub>2A</sub> receptors and potentiated PCP-hyperlocomotion; the other 5-HT<sub>2A</sub> receptor antagonists were without effect. The significance of these findings with respect to atypical antipsychotic drug action is discussed.

## Introduction

According to classic 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 activation by “steric hindrance” has been supported by recent crystallographic studies of the  $\beta$ -adrenergic receptor, showing that agonists and antagonists occupy overlapping (but not identical) binding sites (Rosenbaum et al., 2007, 2011; Warne et al., 2011). The notion that some G protein-coupled receptor antagonists can also stabilize the inactive state of

the receptor and thereby function as inverse agonists was first put forth by Costa and Herz (1989) in studies of opioid receptors. 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. However, by definition 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 (1979) and Peroutka and Snyder (1980) showed that chronic administration of antidepressants with potent 5-HT<sub>2A</sub> antagonist activity induces a down-regulation of 5-HT<sub>2A</sub> receptor levels. This property was subsequently demonstrated to be shared by a number of drugs with potent 5-HT<sub>2A</sub> 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<sub>2A</sub> antagonists (Leysen et al., 1986). There have also

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**ABBREVIATIONS:** M11939,  $\alpha$ -phenyl-1-(2-phenylethyl)-4-piperidinemethano; M100907,  $\alpha$ -(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidine methanol; SR46349B, 4-[(2Z)-3-[[2-(dimethylamino)ethoxy]amino]-3-(2-fluorophenyl)prop-2-en-1-ylidene]cyclohexa-2,5-dien-1-one; PCP, phencyclidine; SBB, standard binding buffer; WGA, wheat germ agglutinin.

been reports that some 5-HT<sub>2A</sub> antagonists can up-regulate 5-HT<sub>2A</sub> receptors after chronic administration (Rinaldi-Carmona et al., 1993) or have no effect (Gray and Roth, 2001). Antagonist-induced 5-HT<sub>2A</sub> 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 (Roth and Chuang, 1987; Galandrin et al., 2007).

The mechanism(s) responsible for the paradoxical antagonist-induced receptor "down-regulation" are still unclear. Several studies have demonstrated that 5-HT<sub>2A</sub> antagonists can induce receptor internalization in vitro (Berry et al., 1996; Willins et al., 1999; Bhatnagar et al., 2001) and in vivo (Willins et al., 1998, 1999). In addition, receptor down-regulation is apparently independent of changes in receptor gene transcription (Roth and Ciaranello, 1991). Two independent studies have indicated that antagonist-induced internalization occurs via clathrin-dependent processes in vitro (Bhatnagar et al., 2001; Hanley and Hensler, 2002).

In addition, although there have been a large number of reports of antagonist-induced down-regulation of 5-HT<sub>2A</sub> receptors, all prior studies have relied on radioligand binding to quantify changes in total receptor protein. There are a number of possible explanations for alterations in radioligand binding after 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 to quantify 5-HT<sub>2A</sub> 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<sub>2A</sub> antibodies are neither sufficiently specific nor sensitive for biochemical studies in vivo (Xia et al., 2003). In recent studies, however, we identified and validated a 5-HT<sub>2A</sub> receptor antibody in wild-type and 5-HT<sub>2A</sub> knockout 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<sub>2A</sub> antagonists differentially regulate 5-HT<sub>2A</sub> receptor protein levels in vivo. In addition, we are able to identify three classes of 5-HT<sub>2A</sub> antagonists: those that induce receptor down-regulation, those that induce receptor up-regulation, and those that have no effect on receptor protein levels.

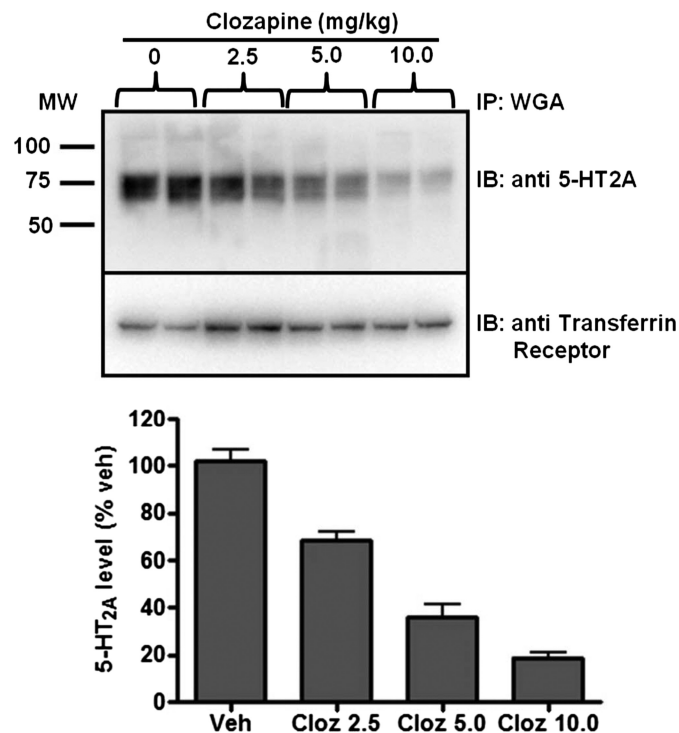
## Materials and Methods

**Mice.** Male C57BL/6J mice were either obtained from The Jackson Laboratory (Bar Harbor, ME) or bred for two to three generations in-house to obtain a sufficient number of male mice (24–30) between 8 and 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-h 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] were purchased from Sigma-Aldrich (St. Louis, MO), and haloperidol [(4-[4-(4-

chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone hydrochloride] was obtained from Sigma/RBI (Natick, MA). Ketanserin [3-[2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl]-2,4[1*H*,3*H*]-quinazolin-3-one tartrate], altanserin [3-[2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl]-2,3-dihydro-2-thioxo-4(1*H*)-quinazolinone hydrochloride], and  $\alpha$ -phenyl-1-(2-phenylethyl)-4-piperidinemethano (M11939) were purchased from Tocris Bioscience (Ellisville, MO).  $\alpha$ -(2,3-Dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidine methanol (M100907) and 4-[(2*Z*)-3-[[2-(dimethylamino)ethoxy]amino]-3-(2-fluorophenyl)prop-2-en-1-ylidene]cyclohexa-2,5-dien-1-one (SR46349B) was obtained from sanofi-aventis (Bridgewater, NJ). Pimavanserin was kindly provided by Dr. Herb Meltzer (Department of Psychiatry, Vanderbilt University, Nashville, TN).

**Chronic Drug Treatment and Behavior Testing Regimen.** C57/BL6 mice (8–10 weeks old) were first tested for short-term vehicle (0.9% NaCl solution)- or phencyclidine (PCP) (6.0 mg/kg i.p.)-induced locomotor response. After 8 to 10 days of washout, the same cohort of mice were used for long-term drug treatments (one injection/day between 2:00 and 4:00 PM for 14 days) either with vehicle (5 ml/kg solution containing 0.9% NaCl, 50 mM tartaric acid and 1% dimethyl sulfoxide) or with 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 drug used in this study was decided on the basis of literature doses to induce a regulatory adaptation of 5-HT<sub>2A</sub> receptors (Gray and Roth, 2001) or the maximal tolerated doses that 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 maximal 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)

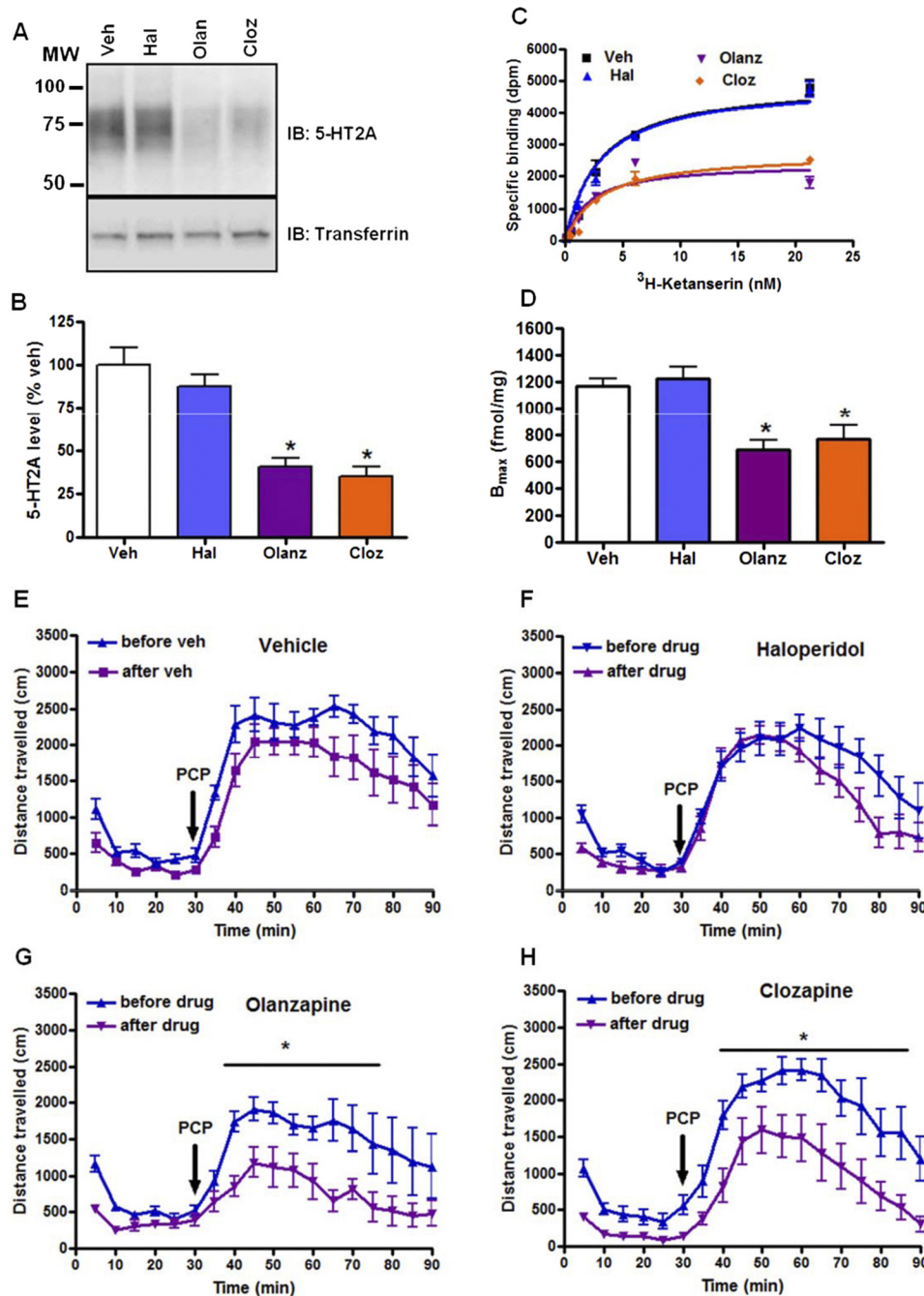


**Fig. 1.** Dose-dependent down-regulation of 5-HT<sub>2A</sub> receptor by clozapine. Top, C57BL/6J mice were treated with vehicle (Veh) or clozapine (Cloz) (2.5–10.0 mg/kg i.p.) for 14 days, and 5-HT<sub>2A</sub> receptor levels were determined by immunoblotting (IB) of WGA immunoprecipitates (IP) from frontal cortex membrane lysates (top panel). The same blots were stripped and probed for transferrin receptor as loading controls (bottom panel). Bottom, quantitation of immunoblots by densitometry of 5-HT<sub>2A</sub> immunoreactivity ( $n = 4$  mice/treatment group). MW, molecular weight.

exceeds the effective dose for normalization of MK801-induced hyperactivity (0.12 mg/kg) (O'Neill et al., 1998) and was the maximal tolerated dose for this drug. For altanserin, we chose a dose of 10 mg/kg, which was the maximal tolerated dose and in excess of that previously used for behavioral studies of 5-HT<sub>2A</sub> receptors (0.5 mg/kg) (Stoessl et al., 1990). For pimavanserin, the dose chosen (5 mg/kg) was the maximal 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, 12 to 14 h after the last drug administration, the same cohort of mice were challenged with PCP (6.0 mg/kg i.p.), and locomotor responses were measured. Furthermore, mice were euthanized right after locomotor testing, and cortical tissue were isolated for the measurement of 5-HT<sub>2A</sub> receptor levels.

**Radioligand Binding Assays.** For saturation binding assays, brain regions were rapidly microdissected, frozen on dry ice, and

then stored at  $-80^{\circ}\text{C}$ . A Tissue-Tearor (BioSpec Products, Bartlesville, OK) was used to homogenize tissue (15 s, 15,000 rpm) in ice-cold standard binding buffer (SBB) (50 mM Tris-HCl, pH 7.4, 10 mM MgCl<sub>2</sub>, and 0.1 mM EDTA). Homogenized tissue was centrifuged for 20 min at 27,000g ( $4^{\circ}\text{C}$ ); 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  $-80^{\circ}\text{C}$  until use. Saturation binding assays were performed with the homogenized brain tissue and [<sup>3</sup>H]ketanserin and then incubated in SBB for 1.5 h as detailed (Abbas et al., 2009). Nonspecific binding was determined by incubating the reactions with 10  $\mu\text{M}$  ritanserin. Reactions were harvested by vacuum filtration through glass filters ( $3\times$  ice-cold 50 mM Tris, pH 7.4; pH 6.9 at room temperature) and measured by liquid scintillation using a Tri-Carb 2800TR scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA). Nonlinear saturation



**Fig. 2.** Chronic clozapine and olanzapine but not haloperidol down-regulate 5-HT<sub>2A</sub> receptor and attenuate PCP-induced hyperlocomotor responses in C57BL/6J mice. **A**, immunoblot (IB) 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  $t$  test). **C**, representative saturation isotherms obtained by binding of [<sup>3</sup>H]ketanserin with equal amounts of cortical membrane proteins (25  $\mu\text{g}$ ) from vehicle (Veh) or clozapine (Cloz) or olanzapine (Olanz) or haloperidol (Hal) treatment groups. **D**,  $B_{\text{max}}$  estimates were obtained by performing [<sup>3</sup>H]ketanserin saturation binding on cortical membrane homogenates. Data are presented as mean  $\pm$  S.E.M. ( $n = 6$ /group; \*,  $p < 0.05$  unpaired  $t$  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 traveled in 5-min bins over 60 min after PCP administration ( $\pm$ S.E.M.). \*,  $p < 0.05$ , two-way analysis of variance, followed by Bonferroni post-tests for multiple comparisons. MW, molecular weight.

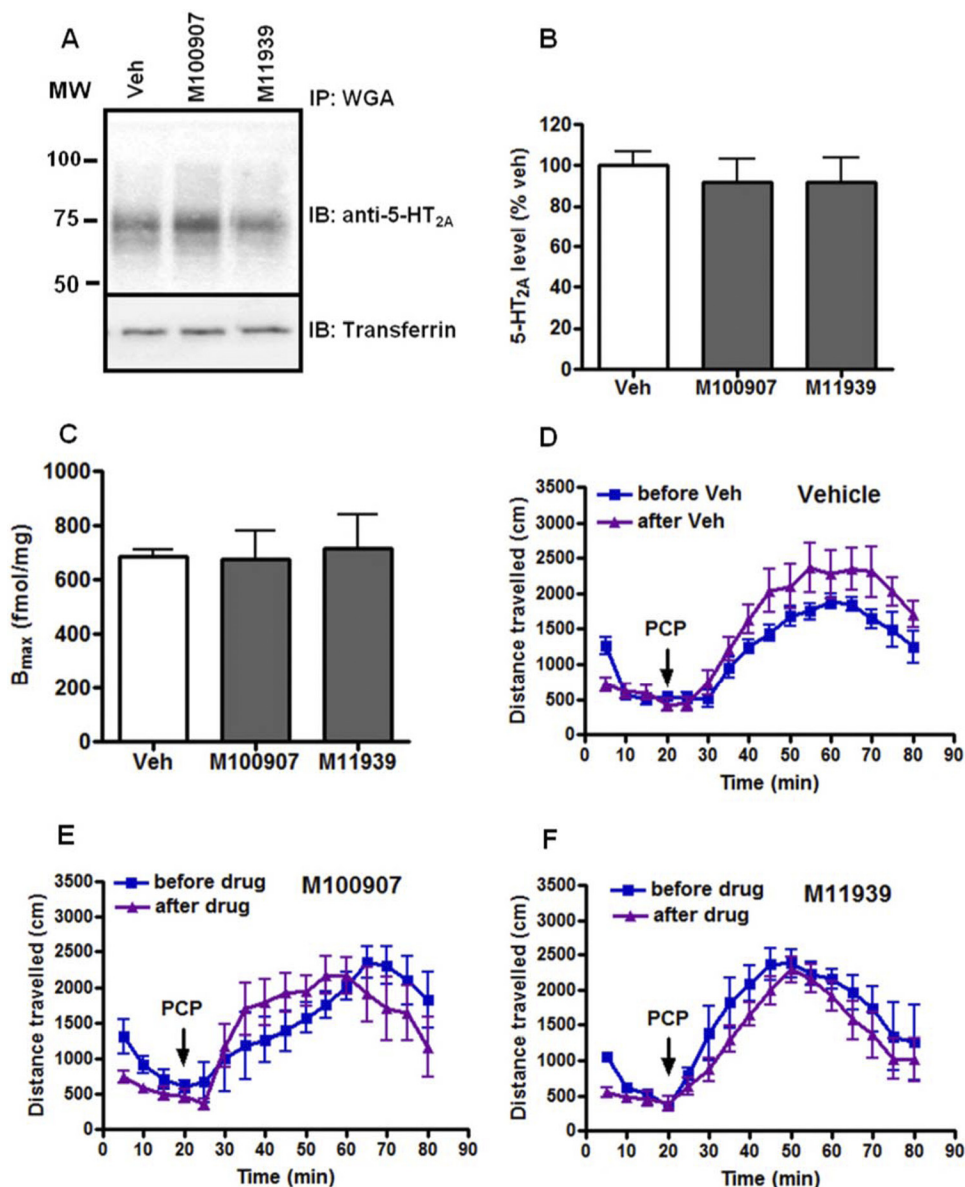
analysis was done with GraphPad Prism 4.01 (GraphPad Software Inc., San Diego, CA) to obtain  $B_{max}$  values, and Bradford protein assays (Bio-Rad Laboratories, Hercules, CA) were performed to normalize  $B_{max}$  determinations to the amount of protein in each assay.

**Western Blotting.** For 5-HT<sub>2A</sub> 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% sodium deoxycholate, and 0.1% SDS, plus protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and incubated on ice for 1 h. 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 to 30 μl (packed volume) of wheat germ agglutinin (WGA)-conjugated agarose beads (Vector Laboratories, Inc., Burlingame, CA) for a minimum of 2 h 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 1× SDS sample buffer, and whole eluates were resolved by SDS-polyacrylamide gel electrophoresis, followed by electrotransfer of protein from gel to polyvinylidene difluoride membrane. Membranes were blocked for 1 h using buffer containing 3% nonfat dry milk, 1% bovine serum albumin, and 5% glycerol in Tris-buffered saline-Tween 20. Rabbit polyclonal anti-5-HT<sub>2A</sub> receptor antibody (Neuromics, Edina, MN) was used at a 1:500

dilution for 12 to 18 h at 4°C for measurement of 5-HT<sub>2A</sub> receptor immunoreactivity. For a loading control, the same membrane was stripped and reprobed with anti-transferrin receptor mouse monoclonal antibody (Invitrogen, Carlsbad, CA). We have previously verified the specificity of the anti-5-HT<sub>2A</sub> receptor antibody using 5-HT<sub>2A</sub> wild-type and knockout mouse cortex tissue by immunoblotting (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 received intraperitoneal injections of vehicle (0.9% NaCl solution) or PCP (6 mg/kg, dissolved in saline solution) after 20 to 30 min of habituation in activity chambers followed by measurement of horizontal activity for 60 to 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  $B_{max}$  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 analysis of variance fol-



**Fig. 3.** Chronic administration of 5-HT<sub>2A</sub> selective antagonists M100907 or M11939 did not modulate 5-HT<sub>2A</sub> receptor level and PCP-induced hyperlocomotor response in C57BL/6J mice. A, immunoblot (IB) of immunoprecipitates (IP) from cortex membrane lysates. The same blots were stripped and probed for transferrin receptor as loading controls. B, densitometry of immunoblots of immunoprecipitates from  $n = 4$  mice/treatment group. C,  $B_{max}$  estimates were obtained by performing [<sup>3</sup>H]ketanserin saturation binding on cortical membrane homogenates. Data are presented as mean ± S.E.M. ( $n = 6$ /group). D–F, chronic treatments with vehicle (Veh) or M100907 or M11939 (5.0 mg/kg, 14 days) 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-min bins over 60 min after PCP administration (± S.E.M.). MW, molecular weight.

lowed by Bonferroni post-tests for comparing multiple groups. Comparisons were considered significant if  $p < 0.05$ .

## Results

**Olanzapine and Clozapine but Not Haloperidol Down-Regulate 5-HT<sub>2A</sub> Receptors.** In initial studies, we performed dose-response experiments to investigate the ability of the atypical antipsychotic drug clozapine to induce cortical 5-HT<sub>2A</sub> receptor down-regulation in C57BL/6J mice. To quantify 5-HT<sub>2A</sub> receptor protein levels, we used our recently validated Western blot procedure (Magalhaes et al., 2010; Yadav et al., 2011) along with parallel radioligand binding studies with [<sup>3</sup>H]ketanserin. As can be seen in dose-response studies (Fig. 1), 10.0 mg/kg clozapine induced maximal down-regulation of 5-HT<sub>2A</sub> receptor protein, and thus this dose was chosen for further studies.

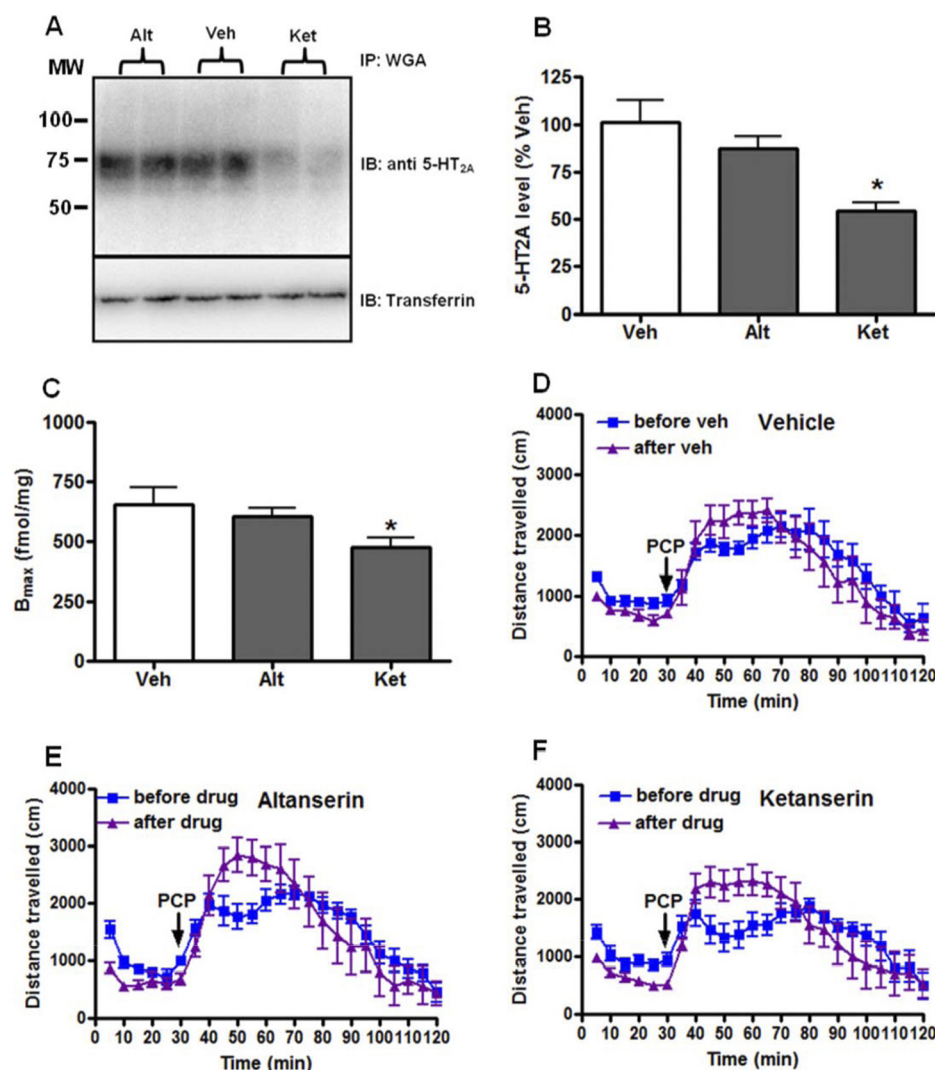
We next evaluated three drugs we had previously shown to induce a differential pattern of receptor internalization in vitro and in vivo: clozapine, olanzapine, and haloperidol (Willins et al., 1999). As can be seen, chronic treatment with clozapine and olanzapine, but not haloperidol, induced a down-regulation of 5-HT<sub>2A</sub> receptor protein as assessed by both Western blot (Fig. 2, A and B) and radioligand binding (Fig. 2, C and D) studies. As is seen in Fig. 2, E to H, chronic olanzapine and

clozapine, but not haloperidol or vehicle, was associated with an attenuation of the PCP-induced locomotor response.

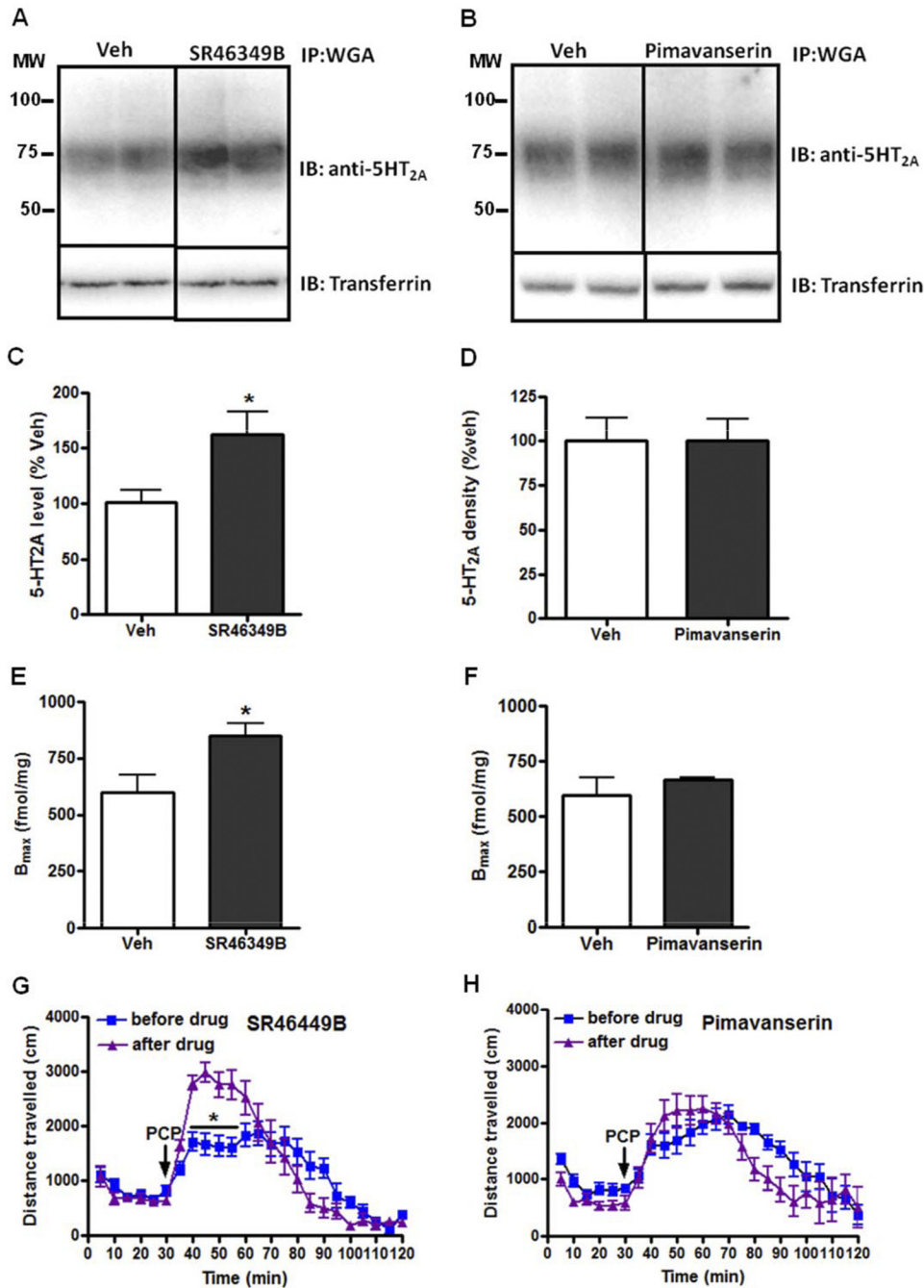
**Selective and Preferential 5-HT<sub>2A</sub> Antagonists Differentially Modulate 5-HT<sub>2A</sub> Receptor Protein Levels after Chronic Administration.** We next evaluated a series of selective 5-HT<sub>2A</sub> antagonists for their ability to induce 5-HT<sub>2A</sub> receptor down-regulation. As shown in Fig. 3, A to C, chronic treatment with M100907 and M11939 had no effect on either 5-HT<sub>2A</sub> receptor protein or radioligand binding. In addition, no effect on PCP-induced locomotor responses was seen (Fig. 3, D–F).

In contrast, the prototypical 5-HT<sub>2A</sub> antagonist ketanserin, but not the closely related compound altanserin, induced 5-HT<sub>2A</sub> receptor protein down-regulation (Fig. 4, A–C). Chronic treatment with these compounds had no significant effect on PCP-induced locomotor responses (Fig. 4, D–F).

We next tested two 5-HT<sub>2A</sub> 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<sub>2A</sub> receptor protein was noted, whereas chronic pimavanserin treatment had no effect (Fig. 5, A–F). These results indicate that reasonably selective 5-HT<sub>2A</sub> antagonists may display strikingly different effects on receptor protein



**Fig. 4.** Chronic administration of the 5-HT<sub>2A</sub> antagonist ketanserin (Ket) but not altanserin (Alt) down-regulates the cortical 5-HT<sub>2A</sub> receptor level in C57BL/6J mice. A, measurement of 5-HT<sub>2A</sub> receptor level in frontal cortex membrane lysates by immunoblotting (IB) of immunoprecipitates (IP). The same blots were re-probed for transferrin receptor as loading controls. B, densitometry of immunoblots of immunoprecipitates from  $n = 4$  mice/treatment group. \*,  $p < 0.05$ , unpaired  $t$  test. C,  $B_{max}$  estimates were obtained by performing [<sup>3</sup>H]ketanserin saturation binding on cortical membrane homogenates. Data are presented as mean  $\pm$  S.E.M. ( $n = 6$ /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-min bins over 90 min after PCP administration ( $\pm$ S.E.M.). MW, molecular weight; Veh, vehicle.



**Fig. 5.** Chronic administration of the 5-HT<sub>2A</sub> selective inverse agonist SR46349b, but not pimavanserin, up-regulates 5-HT<sub>2A</sub> receptor level but had no significant effect on PCP-induced hyperlocomotor response in C57BL/6J mice, A and B, immunoblot (IB) of immunoprecipitates (IP) from cortex membrane lysates. The same blots were reprobbed for transferrin receptor as loading controls. C and D, densitometry of immunoblots of immunoprecipitates from  $n = 4$  mice/treatment group. \*,  $p < 0.05$ , unpaired  $t$  test. E and F,  $B_{max}$  estimates were obtained by performing [<sup>3</sup>H]ketanserin saturation binding on cortical membrane homogenates. Data are presented as mean  $\pm$  S.E.M. ( $n = 6$ /group), \*,  $p < 0.05$ , unpaired  $t$  test. G and H, chronic treatment with vehicle or SR46349b or pimavanserin (5.0 mg/kg, 14 days) 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-min bins over 90 min after PCP administration ( $\pm$ S.E.M.). \*,  $p < 0.05$ , unpaired  $t$  test. MW, molecular weight; Veh, vehicle.

levels: up-regulation (SR46349B), down-regulation (ketanserin), or no effect (M100907, altanserin, pimavanserin, and M11939). 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<sub>2A</sub> receptor antagonists can be classified into three major categories on the basis of their differing abilities to regulate 5-HT<sub>2A</sub> receptor protein expression: 1) up-regulating antagonists (SR46349B), 2) down-regulating antagonists (ketanserin, clozapine, and olanzapine), and 3) antagonists that do not alter receptor expression in male C57BL/6J mice at the doses used in this study (M100907, M11939, pimavanserin, and 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<sub>2A</sub> antagonists display differential abilities to induce alterations in 5-HT<sub>2A</sub> receptor radioligand binding, this is the first study demonstrating an actual change in 5-HT<sub>2A</sub> receptor protein levels after chronic drug administration.

For many years, it has been suggested that 5-HT<sub>2A</sub> 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<sub>2A</sub> receptors is probably part of the mechanism by which certain 5-HT<sub>2A</sub> antagonists apparently protect against JC virus infections (Elphick et al., 2004; O'Connor and Roth, 2005). It has also been suggested that 5-HT<sub>2A</sub> receptor down-

regulation itself might contribute to atypical antipsychotic drug actions. Indeed, as this article clearly shows, chronic treatment with clozapine and olanzapine, two prototypical atypical antipsychotic drugs, induces substantial down-regulation of 5-HT<sub>2A</sub> receptor protein. This down-regulation was associated with an attenuated ability of PCP to induce a locomotor response. In contrast, chronic treatment with the typical antipsychotic drug haloperidol was without effect on either PCP-induced locomotion or 5-HT<sub>2A</sub> receptor levels.

Of interest, chronic treatment with three candidate antipsychotic drugs (M100907, SR46349B, and pimavanserin) either had no effect (M100907 and pimavanserin) or up-regulated 5-HT<sub>2A</sub> receptors. It is interesting in this regard that not only did none of these drugs attenuate the hyperlocomotive ability of PCP but also 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<sub>2A</sub> receptors may be important for certain therapeutic actions of atypical antipsychotic drugs. However, atypical antipsychotics clozapine and olanzapine have marked polypharmacology, with affinities for dopamine, serotonin, muscarinic, and 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 atypical antipsychotics (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<sub>2A</sub> receptors in vivo are currently unknown and are the topic of intensive research at present (P. M. Yadav and B. L. Roth, manuscript 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.

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