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Clozapine-Induced Locomotor Suppression is Mediated by 5-HT_{2A} Receptors in the Forebrain

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The need for safer, more effective therapeutics for the treatment of schizophrenia is widely acknowledged. To optimally target novel pharmacotherapies, in addition to establishing the mechanisms responsible for the beneficial effects of antipsychotics, the pathways underlying the most severe side effects must also be elucidated. Here we investigate the role of serotonin 2A (5-HT_{2A}), serotonin 2C (5-HT_{2C}), and dopamine 2 receptors (D_2) in mediating adverse effects associated with canonical first- and second-generation antipsychotic drugs in mice. Wild-type (WT) and 5-HT_{2A} knockout (KO) mice treated with haloperidol, clozapine, and risperidone were assessed for locomotor activity and catalepsy. WT mice showed a marked reduction in locomotor activity following acute administration of haloperidol and high-dose risperidone, which was most likely secondary to the severe catalepsy caused by these compounds. Clozapine also dramatically reduced locomotor activity, but in the absence of catalepsy. Interestingly, 5-HT_{2A} KO mice were cataleptic following haloperidol and risperidone, but did not respond to clozapine's locomotor-suppressing effects. Restoration of 5-HT_{2A} expression to cortical glutamatergic neurons re-instated the locomotor-suppressing effects of clozapine in the open field. In sum, we confirm that haloperidol and risperidone caused catalepsy in rodents, driven by strong antagonism of D_2. We also demonstrate that clozapine decreases locomotor activity in a 5-HT_{2A}-dependent manner, in the absence of catalepsy. Moreover, we show that it is the cortical population of 5-HT_{2A} that mediate the locomotor-suppressing effects of clozapine.
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INTRODUCTION

Schizophrenia is characterized by profound disruptions in thinking, affect, language, perception, and sense of self. This impacts upon normal functioning through impaired capacity to earn a livelihood, and while some patients respond well to currently available antipsychotic drugs, others remain symptomatic. Findings from the CATIE study (Lieberman *et al*, 2005) revealed that up to 74% of patients prescribed atypical antipsychotics discontinued treatment as a result of either ineffectiveness of the antipsychotic or intolerability of the side effects, which include sedation, cardiometabolic risks, extrapyramidal symptoms (EPS), agranulocytosis, and cognitive dulling (Leucht *et al*, 2009).

Clozapine—the prototypical atypical antipsychotic—remains one of the most efficacious antipsychotics available (McEvoy *et al*, 2006; Meltzer *et al*, 2003). Clozapine has been shown to bind upwards of 50 receptor targets *in vitro* (Roth *et al*, 2004) showing strong affinity for dopaminergic, serotonergic, muscarinic, adrenergic, and histaminergic receptors; however, the full spectrum of its *in vivo* receptor affinity is subject to some discussion (Beninger *et al*, 2010; Coward, 1992; McCormick *et al*, 2010; Roth *et al*, 2004; Schotte *et al*, 1993). Although it pioneered second-generation drugs, which have lower liability to cause EPS and tardive dyskinesias, these compounds are hampered by side effects such as weight gain, hyperlipidemia, agranulocytosis, and sedation. The severity of these side effects is such that clozapine is prescribed for treatment only as a last resort. A better understanding of how clozapine and other atypical antipsychotics produce both therapeutic and unwanted effects remains a field of active investigation.

The most popular explanation for the improved efficacy in treating negative symptoms and the low EPS produced by atypical antipsychotic compounds is the addition of strong serotonin 2A receptor (5-HT_{2A}) antagonism. Antagonism of 5-HT_{2A} in mesocortical pathways is hypothesized to be one of the mechanisms underlying the improvement of negative symptoms produced by atypical antipsychotics, whereas the attenuated EPS have been proposed to be the result of 5-HT_{2A} antagonist-driven dopamine release in the striatum (Meltzer and Massey, 2011). However, studies investigating these hypotheses have provided

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mixed results in both humans and mice, and alternative hypotheses have been proposed to explain the reduction in EPS, including an anti-cholinergic mechanism or the action of α2-adrenoceptor antagonism (Kalkman *et al*, 1998a, b; Parada *et al*, 1997).

To shed light upon the manner by which atypical antipsychotics circumvent the induction of EPS, we used a combined pharmacological and genetic strategy. If the prevailing hypotheses held true, then administering haloperidol either in combination with a 5-HT$_{2A}$ antagonist or to a 5-HT$_{2A}$ knockout mouse (KO) should shift the phenotype from catalepsy/EPS to something more closely resembling the effects of atypical antipsychotics. Moreover, using a STOP-floxed 5-HT$_{2A}$ transgenic mouse, we should be able to pinpoint the neural loci controlling this behavioral shift.

Contrary to our hypothesis, we found that 5-HT$_{2A}$ signaling alone does not appear to be critical to the shift away from EPS in second-generation drugs. Interestingly, we found that the receptor plays a key role in the regulation of locomotor suppression produced by atypical antipsychotics, like clozapine, and that this response relied upon the expression of 5-HT$_{2A}$ specifically in the forebrain.

MATERIALS AND METHODS

Animals

Animals were housed on a 12 h light/dark cycle with food and water available *ad libitum*. All testing was carried out during the light cycle on 5-HT$_{2A}$ KO mice and wild-type (WT) littermate controls on a 129SvEv/Tac background originally obtained from Taconic (Taconic Farms). Mice heterozygous for a transgenic STOP cassette flanked by LoxP sites located upstream of the *htr2a* gene (Weisstaub *et al*, 2006) were crossed, generating WT, KO, and heterozygous littermates, identified by PCR genotyping. Mice were weaned at 3–4 weeks of age and behavioral testing began at 12 weeks.

Restricted expression of specific populations of 5-HT$_{2A}$ was achieved as described previously. Briefly, STOP-floxed *htr2a* transgenic mice were crossed with mice expressing Emx1-Cre to selectively express 5-HT$_{2A}$ in Emx1-positive cells (predominantly pyramidal forebrain neurons as has been demonstrated previously; Weisstaub *et al*, 2006). A *DAT*-Cre mouse line was used to selectively restore 5-HT$_{2A}$ expression to DAT-containing nigrostriatal neurons.

Drugs

Antipsychotics were tested at a range of doses (Figure 1) before selecting a single dose for subsequent testing: clozapine (5 mg/kg), haloperidol (0.4 mg/kg), risperidone (0.4 mg/kg), AC90179 (10 mg/kg), which shows high potency as an inverse agonist and a competitive antagonist at the 5-HT$_{2A}$, and SB242084 (1 mg/kg), a 5-HT$_{2C}$ inverse agonist with some activity at the 5-HT$_{2B}$ receptor or vehicle (1% Tween-80 at pH 6.0).

Locomotor Activity

Animals were treated intraperitoneally 30 min before being placed in the MedAssociates locomotor arena, and were then tracked for 30 min using the MedAssociates software. Diphenhydramine displayed a shorter window of efficacy on behavior. As a result, mice were placed in the locomotor arena immediately following drug administration.

Catalepsy Assessment

Catalepsy was assessed using the step down task. Step down task was performed by placing the forepaws or hindpaws on a bar 0.8 cm in diameter fixed at 3.5 cm from the bench top.

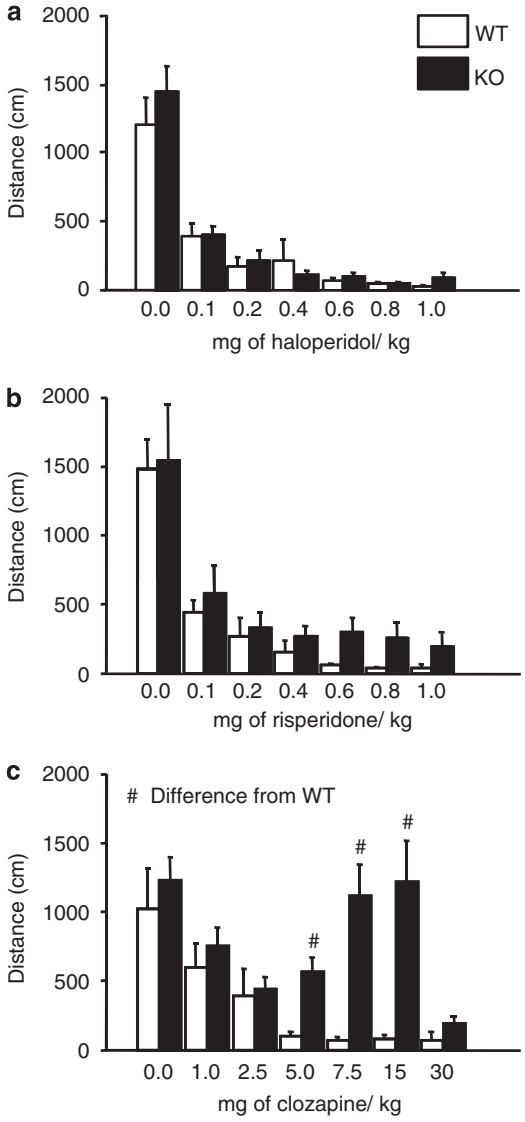

Figure 1 Mice were treated with antipsychotic drugs across a range of doses and locomotor activity in the open field was assessed for 30 min. Haloperidol, at doses of 0.1 mg/kg and higher, significantly decreased locomotor activity in both wild-type (WT) and serotonin 2A (5-HT$_{2A}$) knockout (KO) mice (a: untreated, $N = 26$ WT, 34 KO; haloperidol treated, $N = 11$ KO, 9 WT). Risperidone at both low and high doses also decreased activity comparably in both WT and KO mice (b: untreated, $N = 26$ WT, 34 KO; haloperidol treated, $N = 11$ KO, 9 WT). Clozapine treatment resulted in extreme sedation in WT mice from 5 mk/kg upwards (c: untreated, $N = 26$ WT, 34 KO; haloperidol treated, $N = 12$ KO, 8 WT). In KO mice, clozapine did not induce this extreme level of sedation until 30 mg/kg. Data are shown as mean ± SEM. $^{\#}P < 0.05$ difference from WT.

Mice were injected intraperitoneally 30 min before testing. Time to step down was measured for three consecutive trials for an average step down time.

Neurological Reflex/Motor Behavior

Righting reflex was assessed using a subjective scoring matrix. A score of 0 was allocated to mice that righted immediately (too quickly to time accurately), 1 for animals that turned noticeably more slowly (righted in approximately 1 s), a score of 2 was given to animals that displayed great difficulty in turning over (righted in 1 + s), and a score of 3 indicated mice that did not right at all.

RESULTS

Dose Response to Haloperidol, Risperidone, and Clozapine

Clozapine, haloperidol, and risperidone display distinct pharmacology. Haloperidol, a typical antipsychotic, demonstrates highest affinity for the D_2 receptor and σ_1 receptor, robustly antagonizing signaling at relatively low doses (Roth *et al*, 2004; Schotte *et al*, 1996). Risperidone possesses a broader spectrum, showing high *in vitro* affinity for 5-HT$_{2A}$, 5-HT$_7$, D_2, α_1, and H_1, and is reported to act like an atypical antipsychotic at lower doses, but more like a typical antipsychotic at higher doses (Roth *et al*, 2004; Schotte *et al*, 1996). Clozapine has a complex pharmacology, binding to upwards of 50 receptors with demonstrable affinity for 5-HT$_{2A}$, 5-HT$_{2C}$, D_2, H_1, and M_1 receptors (Roth *et al*, 2004; Schotte *et al*, 1996; Yadav *et al*, 2011).

Haloperidol dramatically reduced activity across both genotypes (Figure 1a: effect of treatment, $F_{(7,224)} = 28.706$, $P < 0.0001$; effect of genotype, $F_{(1,224)} = 0.465$, $P = 0.4958$; genotype × treatment interaction, $F_{(7,224)} = 0.338$, $P = 0.9359$). A significant effect of genotype was detected in the risperidone dose–response curve in addition to the treatment effect; however, the genotypes were not differentially affected by the treatment (Figure 1b: effect of treatment, $F_{(7,224)} = 21.972$, $P < 0.0001$; effect of genotype, $F_{(1,224)} = 4.065$, $P < 0.05$; genotype × treatment interaction, $F_{(7,224)} = 0.084$, $P = 0.9990$). Clozapine affected WT and 5-HT$_{2A}$ KO mice differentially (Figure 1c: effect of treatment, $F_{(7,224)} = 13.169$, $P < 0.0001$; effect of genotype, $F_{(1,224)} = 22.166$, $P < 0.0001$; genotype × treatment interaction, $F_{(7,224)} = 2.698$, $P < 0.05$): decreasing mice locomotor activity in WT mice in a dose dependent manner. 5-HT$_{2A}$ KO displayed a U-shaped dose–response curve, which may be attributable to the increased 5-HT$_{2C}$ antagonism at higher doses of clozapine.

From these dose–response curves, a dose of 5 mg/kg was chosen for clozapine, 0.4 mg/kg for haloperidol, and 0.4 mg/kg for risperidone.

Antipsychotic-Induced Catalepsy Occurs Independently of 5HT$_{2A}$ Receptors

Typical antipsychotics provoke a cataleptic response in mice, which is considered to be analogous to EPS in human patients. This response is widely attributed to the robust antagonism of D_2 receptors in both mice and humans; however, it has also been suggested to be susceptible to modulation by 5-HT$_{2A}$.

No significant effect of 5-HT$_{2A}$ genotype was observed in the dose–response analysis of catalepsy (Figure 2a: haloperidol—effect of treatment, $F_{(7,704)} = 235.118$, $P < 0.0001$; effect of genotype, $F_{(1,2.513)} = 0.465$, $P = 0.1134$; risperidone—effect of treatment, $F_{(7,704)} = 169.293$, $P < 0.0001$; effect of genotype, $F_{(1,704)} = 0.001$, $P = 0.9737$).

With the selected doses of antipsychotics, a significant statistical effect of treatment was observed in response to antipsychotic drugs (Figure 2b: ANOVA effect of genotype, $F_{(1,260)} = 0.338$, $P = 0.5614$; effect of treatment, $F_{(3,260)} = 102.44$, $P < 0.0001$). Haloperidol and risperidone induced catalepsy on the step down task (Figure 2b: haloperidol compared with vehicle-treated animals, $P < 0.0001$; risperidone *vs* vehicle, $P < 0.0001$). Clozapine did not cause catalepsy in mice ($P = 0.4815$) nor did the administration of AC90179 (Figure 2c: effect of genotype, $F_{(1,243)} = 1.108$, $P = 0.2935$; effect of treatment, $F_{(3,243)} = 116.673$, $P < 0.0001$; AC90179 *vs* vehicle, $P = 0.2082$) or SB242084 (Figure 2d: effect of genotype, $F_{(1,265)} = 0.101$, $P = 0.7509$; effect of treatment, $F_{(3,265)} = 124.749$, $P < 0.0001$; SB242084 *vs* vehicle, $P = 0.2126$). Interestingly, while 'risperidone with SB242084' and 'haloperidol with AC90179 and SB242084' both induced significant catalepsy relative to vehicle-treated mice (Figure 2e: effect of genotype, $F_{(1,243)} = 0.2224$, $P = 0.2224$; effect of treatment, $F_{(3,243)} = 66.491$, $P < 0.0001$; risperidone + SB242084 *vs* vehicle, $P < 0.0001$; haloperidol + AC90179 + SB242084 *vs* vehicle, $P < 0.0001$), they were nonetheless significantly less cataleptic than haloperidol alone (risperidone + SB242084 *vs* haloperidol, $P < 0.0001$; haloperidol + AC90179 + SB242084 *vs* haloperidol, $P < 0.0001$).

5HT$_{2A}$ KO Mice Show Altered Locomotor Response to Clozapine, but not Risperidone or Haloperidol

Antipsychotic treatment significantly reduced locomotor activity (Figure 3a: effect of treatment, $F_{(3,71)} = 10.841$, $P < 0.0001$). There was also a significant overall effect of 5-HT$_{2A}$ genotype ($F_{(1,71)} = 12.231$, $P < 0.001$) and an interaction ($F_{(3,71)} = 10.841$, $P < 0.001$). *Post hoc* analyses revealed that in WT mice, haloperidol ($P < 0.0001$), risperidone ($P < 0.0001$), and clozapine ($P < 0.01$) all caused significant reduction of locomotor activity (Figure 3a).

Haloperidol and risperidone decreased activity equally in both WT and 5-HT$_{2A}$ KO mice. This is most likely a corollary of the catalepsy induced by these drugs at the doses used. Clozapine, in the absence of catalepsy-like behavior, showed a gene × treatment interaction ($F_{(1,35)} = 8.386$, $P < 0.01$): 5-HT$_{2A}$ KO mice were resistant to the locomotor suppression produced by clozapine (Figure 3a: $P < 0.01$ compared to WT mice).

The 5-HT$_{2C}$ receptor antagonist SB242084 significantly increased locomotor activity (Figure 3b: effect of treatment, $F_{(3,78)} = 18.865$, $P < 0.0001$; effect of genotype, $F_{(1,78)} = 9.865$, $P < 0.01$; SB242084 *vs* vehicle, $P < 0.0001$; AC90179 *vs* vehicle, $P = 0.7613$). In 5-HT$_{2A}$ KO mice, co-administration of AC90179 and SB242084 produced the same increased activity observed following SB242084 alone ($P < 0.05$). In WT mice, the combined antagonism of

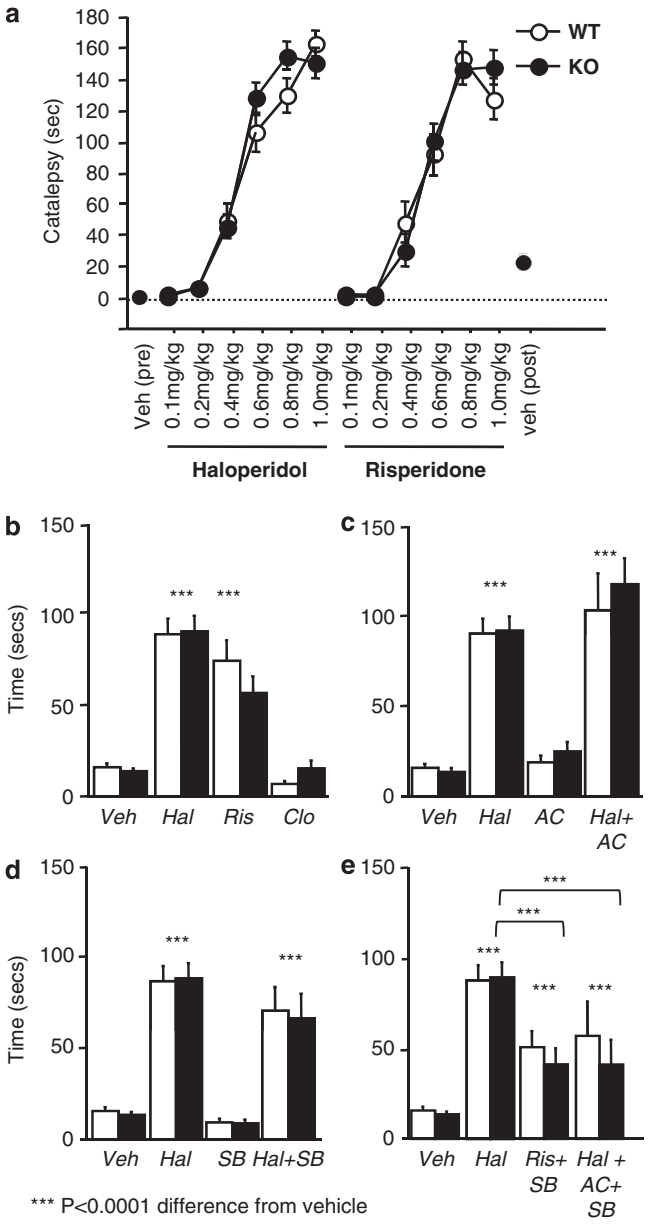

Figure 2 Haloperidol and risperidone caused catalepsy in both wild-type (WT) ($N=19$–33) and knockout (KO) mice ($N=21$–31) in a dose-dependent manner (a). At doses of 0.4 mg/kg haloperidol and risperidone both induced catalepsy relative to vehicle-treated mice. Clozapine 5 mg/kg ($N=22$ WT, 19 KO), however, did not induce catalepsy in either genotype (b). The serotonin 2A (5-HT$_{2A}$) antagonist AC90179 (c) ($N=21$ WT, 23 KO) and the 5-HT$_{2C}$ antagonist SB242084 (d) ($N=34$ WT, 30 KO) alone did not affect catalepsy nor did they alter the cataleptic response to haloperidol ($N=9$–13). Mice treated with risperidone and SB242084 ($N=11$ WT, 10 KO) showed significantly less catalepsy than haloperidol-treated mice (e: $N=10$ WT, 12 KO). They were also significantly different to mice treated with vehicle ($N=10$ WT, 9 KO) (e). Co-administration of SB242084 and AC90179 with haloperidol ($N=10$ WT, 11 KO) also attenuated catalepsy. However, catalepsy levels were still significantly higher than vehicle-treated mice ($N=10$ WT, 9 KO) (e). Data are shown as mean ± SEM. ***$P<0.0001$ difference from vehicle treatment.

the 5-HT$_{2A}$ and 5-HT$_{2C}$ resulted in no significant differences from vehicle-treated mice ($P=0.4052$) indicative of a counterbalancing effect of 5-HT$_{2A}$ and 5-HT$_{2C}$ antagonism on locomotor activity.

Combined D$_2$ and 5-HT$_{2A}$ antagonism caused significant locomotor suppression, independent of genotype, an effect that was similar to that of haloperidol alone (Figure 3c: effect of treatment, $F_{(3,\ 75)}=39.284$, $P<0.0001$; effect of genotype, $F_{(1,75)}=1.898$, $P=0.1724$; haloperidol + AC90179 *vs* haloperidol, $P=0.9647$; haloperidol + AC90179 *vs* risperidone, $P=0.8570$), and was significantly different to vehicle treatment ($P<0.0001$). Interestingly, SB242084 administered in combination with haloperidol resulted in a significant reduction in locomotor activity compared to SB242084 alone (Figure 3d: effect of treatment, $F_{(3,78)}=17.584$, $P<0.0001$; effect of genotype, $F_{(1,78)}=0.145$, $P=0.7042$; haloperidol + SB242084 *vs* SB242084, $P<0.001$), but not significantly different from vehicle ($P=0.6860$) nor haloperidol alone ($P=0.1103$; Figure 3d).

The genotype effect of clozapine administration could be recapitulated by treating mice with risperidone (which shares clozapine's affinity at the D$_2$ and 5-HT$_{2A}$) in combination with SB242084 (Figure 3e: effect of treatment, $F_{(3,74)}=2.002$, $P=0.121$; effect of genotype, $F_{(1,74)}=7.569$, $P<0.01$; treatment × genotype interaction, $F_{(3,74)}=1.473$, $P=0.2288$). WT mice display locomotor suppression comparable to clozapine alone (risperidone + SB242084 *vs* clozapine, $P=0.8255$), which was significantly different from their baseline activity ($P<0.01$), whereas 5-HT$_{2A}$ KO mice, following risperidone and SB242084, were not different from vehicle treatment ($P=0.7472$) or clozapine treatment ($P=0.3536$). This was also achievable by the combination of haloperidol, AC90179, and SB242084, which in WT mice was significantly different from vehicle treatment ($P<0.05$), but not clozapine treatment ($P=0.3290$), while in 5-HT$_{2A}$ KO mice there was no difference between this combination of drugs and vehicle ($P=0.6210$), or clozapine ($P=0.8972$).

Thus, clozapine suppresses locomotor activity in a 5-HT$_{2A}$-dependent manner, in the absence of catalepsy, suggesting an alternate mechanism.

Differential Response to Atypical Antipsychotics in 5-HT$_{2A}$ KO Mice is not due to Changes in Histaminergic or Muscarinic Signaling

To determine the involvement of 5-HT$_{2C}$ in mediating locomotor suppression induced by clozapine, 5-HT$_{2C}$ knockout mice were treated with either saline or clozapine and assessed in the open field. WT and 5-HT$_{2C}$ KO mice displayed comparable levels of locomotor suppression following 5 mg/kg clozapine treatment (Figure 4a: treatment effect, $F_{(1,29)}=15.146$, $P<0.001$; genotype effect, $F_{(1,29)}=0.003$, $P=0.9564$).

Moreover the behavioral response to clozapine was found to generalize to other, similar atypical antipsychotics. Olanzapine dramatically reduced locomotion (Figure 4b: effect of treatment, $F_{(1,33)}=12.671$, $P<0.01$; effect of genotype, $F_{(1,33)}=2.031$, $P=0.1635$; genotype × treatment interaction, $F_{(1,33)}=4.979$, $P<0.05$), an effect that was again observed to be muted in 5-HT$_{2A}$ KO mice ($P<0.0001$).

Given clozapine's affinity for M1 and H1, and the possible interactions between these receptors and the 5-HT$_{2A}$ (Hirano *et al*, 1995; Morisset *et al*, 1999), locomotor response to muscarinic and histaminergic antagonism were assessed to

test the hypothesis that the differential response to atypical antipsychotics may be a product of alterations in these neurotransmitter systems.

Mice showed robust locomotor activation following scopolamine treatment (1 mg/kg) independent of their genotype (Figure 4c: effect of treatment, $F_{(1,36)} = 73.721$, $P < 0.0001$; effect of genotype, $F_{(1,36)} = 0.290$, $P = 0.5937$). Diphenhydramine (6 mg/kg) also increased locomotor activity equally in both WT and 5-HT$_{2A}$ KO mice (effect of treatment, $F_{(1,36)} = 8.899$, $P < 0.01$; effect of genotype, $F_{(1,36)} = 0.011$, $P = 0.9176$).

Forebrain Glutamatergic 5HT$_{2A}$ is Sufficient to Recapitulate the WT-Like Response to Atypical Antipsychotics

Transgenic animals with 5-HT$_{2A}$ selectively expressed in restricted neural regions were generated as described previously (Weisstaub *et al*, 2006). The Emx1 lineage of cells includes cortical and hippocampal glutamatergic neurons in addition to subpopulations of oligodendrocytes and astrocytes (Gorski *et al*, 2002; Guo *et al*, 2000), whereas the *DAT*-Cre results in strong recombination in the VTA and SNc, in addition to weak expression in the olfactory bulb and the hypothalamus (Zhuang *et al*, 2005).

Locomotor activity.

Emx1-Cre: As observed previously, we found an overall effect of genotype ($F_{(3,50)} = 4.233$, $P < 0.01$; Figure 5a) and treatment ($F_{(1,\ 50)} = 28.888$, $P < 0.0001$), as well as an interaction ($F_{(3,50)} = 3.125$, $P < 0.05$). Clozapine severely reduced locomotor activity relative to saline treatment in WT and Cre control animals ($P < 0.01$ for WT and $P < 0.001$ for Cre control; Figure 5a). 5-HT$_{2A}$ KO mice were not susceptible to the locomotor-suppressing effects of clozapine, showing no significant differences compared with their KO saline-treated littermates ($P = 0.9503$). 5-HT$_{2A}$ expression in forebrain glutamatergic neurons alone was sufficient to restore the locomotor-suppressing effects of clozapine ($P < 0.01$; Figure 5a).

DAT-*Cre:* Given the important role of dopamine in motor behavior and the high expression of 5-HT$_{2A}$ on dopamine neurons, we had hypothesized that antagonism of this population of 5-HT$_{2A}$ might be responsible for clozapine's cataleptic effects. We again observed an effect of genotype ($F_{(3,\ 99)} = 8.667$, $P < 0.0001$) and a genotype × treatment interaction ($F_{(3,\ 99)} = 2.998$, $P < 0.05$); however, expression of 5-HT$_{2A}$ in *DAT*-Cre-expressing neurons did not restore clozapine's locomotor-suppressing effect (Figure 5b: KO *vs* *DAT*-Cre-driven 5-HT$_{2A}$-expressing mice, $P = 0.1068$).

Righting reflex. Another measure of sedative behavior is the righting reflex. We measured the righting reflex in WT, Cre controls, 5-HT$_{2A}$ KO mice, and mice with 5HT$_{2A}$

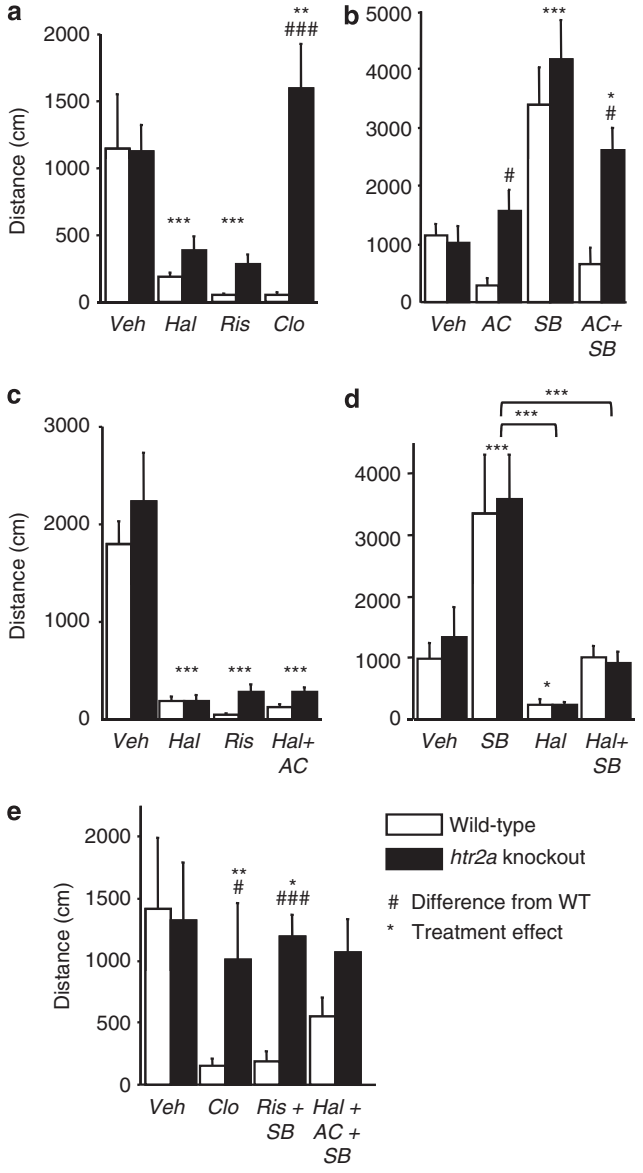

Figure 3 Clozapine, risperidone, and haloperidol reduced locomotor activity in wild-type (WT) mice ($N = 10$ per treatment), relative to vehicle-treated animals ($N = 9$) (a). Serotonin 2A (5-HT$_{2A}$) knockout (KO) mice were sensitive to the locomotor suppression caused by haloperidol ($N = 10$) and risperidone ($N = 10$), but were resistant to the effects of clozapine ($N = 10$) (a). AC90179 alone was not significantly different from vehicle (b) ($N = 10$ per group) and SB242084 increased activity in both genotypes ($N = 10$ per group). Co-administration resulted in a significant increase in activity in KO mice ($N = 13$, $P < 0.05$), while in WT mice AC90179 antagonism of the 5-HT$_{2A}$ cancelled out the hyperlocomotion produced following SB242084 alone ($N = 9$) (b). Mice treated with haloperidol and AC90179 ($N = 10$ per group) were significantly different from vehicle, but not from haloperidol alone (c). Mice treated with SB242084 ($N = 10$ per group) again displayed hyperactivity relative to vehicle-treated mice ($N = 11$ per group) (d). Haloperidol ($N = 10$ WT, 12 KO) decreased distance traveled, while haloperidol plus SB242084 ($N = 13$ WT, 9 KO) was significantly different from haloperidol alone ($P < 0.0001$) and from SB242084 alone ($P < 0.0001$), but not from vehicle treatment ($P < 0.6860$) (d). A significant effect of genotype was seen following clozapine administration ($N = 12$ WT, 9 KO; vehicle-treated mice, $N = 10$ WT, 9 KO). Significant differences were also observed following risperidone + SB242084 ($N = 21$ WT, 22 KO) and a trend was observed following co-injection of haloperidol, AC90179, and SB242084 ($N = 10$ WT, 11 KO) (treatment effect, $P = 0.071$; genotype effect, $P < 0.1085$) (e). Data are shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$ difference from vehicle treatment. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.0001$ indicates a significant difference in behavior of the KO relative to WT mice under the same treatment regimen.

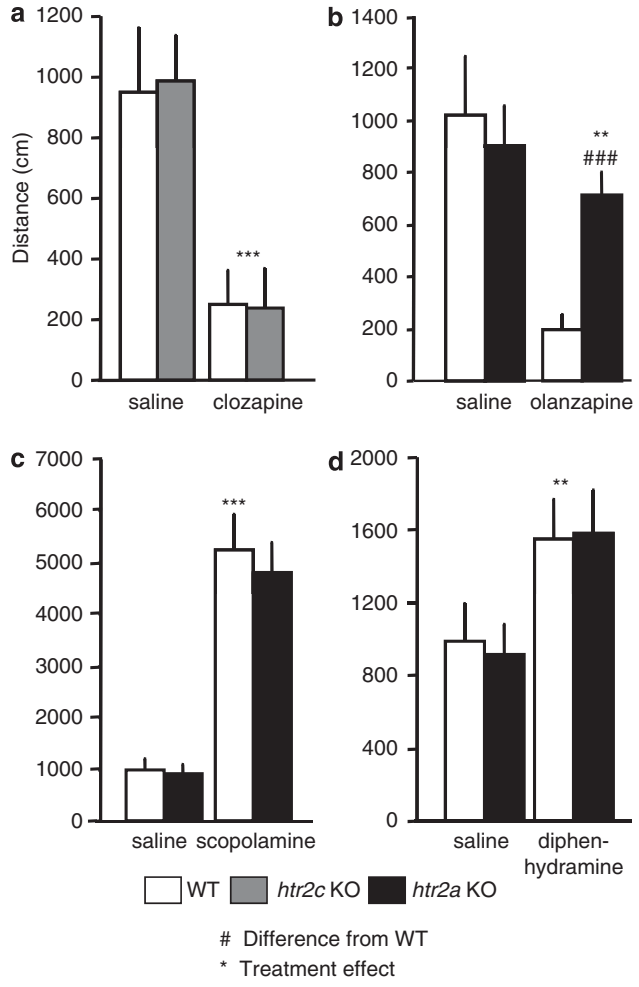

Figure 4 Wild-type (WT) mice and serotonin 2C (5-HT$_{2C}$) knockout (KO) mice showed comparable levels of baseline locomotion and were equally affected by clozapine treatment of 5 mg/kg (a). In 5-HT$_{2A}$ KO mice, olanzapine produces a similar effect to that observed following clozapine, with WT mice demonstrating reduced locomotor behavior relative to saline-treated animals, while 5HT$_{2A}$ KO mice are resistant to this effect (b). Scopolamine (c) and diphenhydramine (d) both produced an increase in locomotion that was not dependent on 5-HT$_{2A}$ receptor signaling. Data are shown as mean ± SEM. $^{**}P<0.01$, $^{***}P<0.0001$ difference from vehicle treatment. $^{\#\#\#}P<0.0001$ indicates a significant difference in behavior of the KO relative to WT mice under the same treatment regimen.

expressed in either Emx1 or DAT cell lineages (Figures 5c and d), following clozapine or saline administration.

EMX1-Cre: A. significant effect of treatment was observed ($F_{(1,\ 50)}=23.747$, $P<0.0001$; Figure 5c) with clozapine exerting a sedative effect on the mice. This effect was not equal across all genotypes with significant genotype ($F_{(3,\ 50)}=5.662$, $P<0.01$) and genotype × treatment interaction ($F_{(3,\ 50)}=5.662$, $P<0.01$) effects observed. The interaction was caused by significant differences in response to clozapine, where 5-HT$_{2A}$ KO mice were not observed to be sedated following clozapine (KO *vs* WT, $P<0.01$; KO *vs* Cre control, $P<0.01$). Moreover, the rescue of 5HT$_{2A}$ in the cortex did not restore WT-like behavior to the mice (Rescue *vs* WT, $P<0.05$; Rescue *vs* Cre control, $P<0.05$; Rescue *vs* KO, $P=0.5698$).

DAT-*Cre:* Expression of 5-HT$_{2A}$ in nigrostriatal neurons did not restore the locomotor-suppressing effects of clozapine (Figure 5d). Again a significant effect of treatment ($F_{(1,\ 99)}=59.515$, $P<0.0001$), genotype ($F_{(3,\ 99)}=25.126$, $P<0.0001$), and a treatment × genotype interaction ($F_{(3,\ 99)}=25.872$, $P<0.0001$) was observed. 5-HT$_{2A}$ KO mice and mice with 5-HT$_{2A}$ expressed only in DAT-containing cells were significantly different to WT mice ($P<0.0001$ and $P<0.0001$, respectively) and to Cre control mice ($P<0.0001$ and $P<0.0001$, respectively). *DAT*-Cre 5-HT$_{2A}$ mice did not differ significantly from 5-HT$_{2A}$ KO mice ($P=0.5192$).

DISCUSSION

Advances in psychopharmacotherapy have seen an increase in efficacy and decreased EPS, but second-generation antipsychotics are nonetheless associated with unpleasant side effects. Although these compounds are highly non-selective, research using animal models is helping to unravel the role of specific receptors in the beneficial effects and the adverse effects. Recent studies demonstrate that 5-HT$_{2A}$ antagonists can attenuate psychotomimetic behavioral responses, by modulating serotonergic tone (Martin *et al*, 1998). That said, it has also been shown that while modulation of the serotonergic tone is necessary for the therapeutic effects of clozapine (Yadav *et al*, 2011), this is not dependent on either 5-HT$_{2A}$ (predominantly postsynaptic) (Yadav *et al*, 2011) or 5HT$_{1A}$ (largely autoreceptors) (Scorza *et al*, 2010). In this context, the central role played by 5-HT$_{2A}$ in mediating the adverse effects of atypical antipsychotics acquires increased significance for future drug discovery.

The focus of this study was the behavioral response to combined D_2, 5-HT$_{2A}$, and 5-HT$_{2C}$ receptor antagonism, common to many atypical antipsychotics and several interesting findings emerge. Primarily, we show that atypical antipsychotic-induced locomotor suppression is driven by a forebrain glutamatergic locus of activity, and that 5-HT$_{2A}$ receptors mediate this effect. The data also support the role of D_2 in mediating catalepsy in mice, and appear to preclude a role for 5-HT$_{2A}$ receptors in controlling this response. Finally, these experiments reproduce the oppositional relationship between 5-HT$_{2A}$ and 5-HT$_{2C}$ in their influence over locomotor behavior (Halberstadt *et al*, 2009).

The Role Played by 5-HT2AR in Catalepsy

Catalepsy in rodents is considered to accurately predict EPS in humans and both behaviors are thought to result from strong antagonism at the D_2. Although some studies have implicated serotonin in the modulation of cataleptic behavior, reports have been mixed, and a rationale for the divergence in these findings has not been proposed. A catalepsy-prone strain of inbred rats display decreased 5-HT$_{2A}$ in the striatum in comparison to the catalepsy-resistant Wistar rat strain (Kulikov *et al*, 2002). Furthermore, the combined 5-HT$_{2A}$/5-HT$_{2C}$ antagonist, ketanserin, has been postulated to overcome the cataleptic effects of haloperidol (Creed-Carson *et al*, 2011). However, a separate study demonstrated that lower doses of a 5-HT$_{2A}$ inverse agonist did not affect cataleptic behavior following

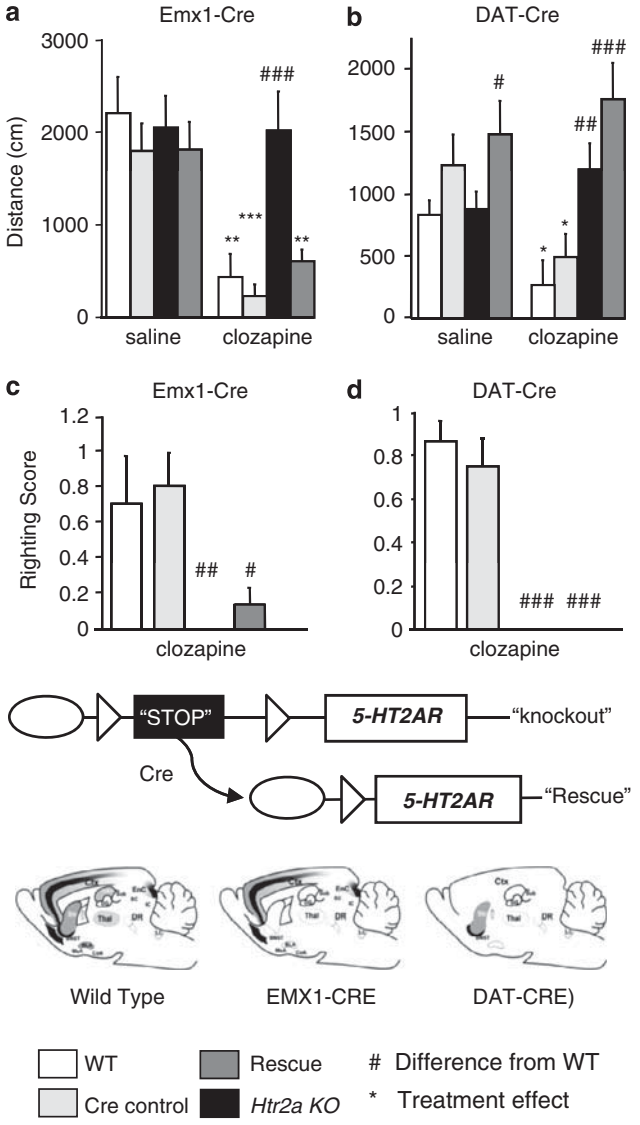

Figure 5 As found previously, clozapine dramatically reduced locomotor activity in wild-type (WT) mice (a) ($N=7$) as well as in Cre control mice ($N=8$). The locomotor behavior of serotonin $_{2A}$ (5-HT$_{2A}$) knockout (KO) mice was unaffected by clozapine treatment ($N=7$); however, mice expressing *htr2ar* in the cortex ($N=7$ saline, 8 clozapine treated) behaved like WT mice, showing decreased locomotor activity following clozapine treatment. (b) At baseline, 5-HT$_{2A}$-*DAT*-Cre rescue mice showed slightly elevated baseline activity. Clozapine attenuated this locomotion in WT and *DAT*-Cre control mice; however, it did not affect the activity of KO ($n=11$) or 5HT$_{2A}$-*DAT* mice ($N=15$). (c) Clozapine impaired the righting reflex in WT mice ($N=7$) and EMX1-Cre control mice ($N=8$). Righting reflex was not impaired in KO mice following clozapine ($N=7$) nor was it impaired in 5-HT$_{2A}$-EMX1-Cre mice ($N=7$). (d) In a subsequent cohort of mice, we again observed impaired righting reflex in WT ($n=15$) and *DAT*-Cre control mice ($N=12$). KO mice were resistant to this response ($N=11$) as were 5-HT$_{2A}$-*DAT*-Cre rescue mice ($N=15$). No significant genotype effects were observed in saline-treated mice as none of the groups displayed impaired righting reflex (data not shown). Data are shown as mean ± SEM. $^{**}P<0.01$, $^{***}P<0.0001$ difference from vehicle treatment. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.0001$ indicates a significant difference in behavior of the KO relative to WT mice under the same treatment regimen.

either haloperidol or risperidone (Gardell *et al*, 2007). Interestingly, both agonism (Prinssen *et al*, 2002) and antagonism (Wadenberg, 1996) of the 5-HT$_{1A}$ have been shown to weaken catalepsy in rodents, whereas the antagonists have been shown to attenuate EPS in primates (Casey, 1993).

Consistent with published work, we show that haloperidol and high-dose risperidone cause catalepsy in rodents, driven by strong antagonism at the D_2 (seen in many first-generation neuroleptics as well as high doses of some second-generation antipsychotics, such as risperidone) (Wiley and Evans, 2008). In support of the findings of Gardell and colleagues, in our dose–response curve we show that WT mice are no more sensitive to catalepsy than 5-HT$_{2A}$ KO mice following haloperidol and risperidone. Nonetheless, we do report some attenuation of the catalepsy response in mice treated with haloperidol in combination with *both* 5-HT$_{2A}$ and 5-HT$_{2C}$ antagonists, whereas neither of these compounds affected haloperidol-induced catalepsy on their own. Taken together, our findings, and those reported previously, suggest that substantial changes in serotonergic signaling through multiple receptors are required to overcome the catalepsy caused by D_2 antagonism following typical antipsychotic administration. This may be achieved either by targeting the presynaptic 5-HT$_{1A}$, controlling 5-HT release and thus the postsynaptic response, or by directly blocking both postsynaptic 5-HT$_2$ receptors. This supports the notion that the primary and most crucial mechanism underlying neuroleptic-induced catalepsy is via the indirect striatopallidal output pathway, driven by D_2 blockade.

Serotonergic Control of Locomotor Activation and Suppression

Locomotor suppression induced by clozapine in rodents is widely acknowledged; however, there has been a paucity of research invested in addressing the mechanism by which this is achieved. The results herein provide critical insight into how these behaviors occur; however, the unexpected nature of the findings also open up several important avenues for further investigation. *How does 5-HT$_{2A}$regulate these behaviors? Which second messenger systems control this response? Can we dissociate these pathways from those that control therapeutic efficacy?*

Inverse agonism of 5-HT$_{2A}$ and 5-HT$_{2C}$ (with AC90179 and SB242084 respectively), administered in combination with haloperidol, resulted in reduced locomotor activity, similar to that observed following atypical antipsychotics. Although it is not possible to definitively relate the atypical antipsychotic-induced locomotor suppression directly to a human correlate, it is tempting to speculate that the decreased activity in mice may be analogous to the sedative effects of atypical antipsychotics in schizophrenia patients. Muscarinic M_1 receptors, histaminergic H_1 receptors, and α1 adrenergic receptors (Cooper, 1986; Nicholson, 1983; Sallinen *et al*, 2007) have been associated with wakefulness and their respective neurotransmitters are known to be involved in the arousal pathways (Castberg *et al*, 2006; Stahl, 2008; Stahl and Shayegan, 2003). As such, the belief has been that these receptors and their downstream signaling systems are likely to be responsible for atypical antipsychotic-induced sedation, which has a significant impact on the quality of life of schizophrenia patients (Asenjo Lobos *et al*, 2010). In addition, previous reports have shown that blocking dopamine D_2 can cause

locomotor suppression and neurolepsis at high doses (Huang *et al*, 2010; Shireen and Haleem, 2011; Starr and Starr, 1987); however, this is likely associated with the cataleptic nature of D_2 antagonism.

Here, we show that 5-HT$_{2A}$ receptors are required to produce this phenotype. To our knowledge, 5-HT$_{2A}$ has not previously been considered as a target for this behavior. As such, it is important to note that 5-HT$_{2A}$ have been shown to influence H_1 and M_1 signaling (Hirano *et al*, 1995; Morisset *et al*, 1999). However, although we cannot rule it out altogether, our results are indicative of a locomotor suppression that is independent of histaminergic or muscarinic tone, as we do not observe a differential response to diphenhydramine, a histamine receptor antagonist, nor to scopolamine, a muscarinic antagonist, in the 5-HT$_{2A}$ knockout mice, relative to WT mice.

Interestingly, while WT mice display a dose-dependent decrease in locomotor behavior in response to clozapine, 5-HT$_{2A}$ KO mice display a muted, U-shaped, locomotor response curve, which may be attributable to the yin–yang relationship between 5-HT$_{2A}$ and 5-HT$_{2C}$. The data show that antagonism of 5-HT$_{2C}$ produces robust locomotor activation that can be attenuated by antagonism of the 5-HT$_{2A}$ receptor, findings that complement previously published research demonstrating that agonism at the 5-HT$_{2A}$ increases activity in a manner which can be countered by 5-HT$_{2C}$ agonism (Halberstadt *et al*, 2009). With increasing doses of clozapine one would produce increasing antagonism at the 5HT$_{2C}$, resulting in locomotor activation in the absence of 5-HT$_{2A}$ receptors.

We also observed a divergence in the population of 5-HT$_{2A}$ responsible for locomotor suppression *vs* the righting reflex, which may be attributed to subtle differences in the behaviors being tested, the level of motivation required to perform the task, and the underlying neural circuits. In fact, while we demonstrate the importance of cortical 5-HT$_{2A}$ in mediating locomotor suppression, it is generally believed that the righting reflex is predominantly a cerebellar behavior (Johnson *et al*, 1985; Spuhler *et al*, 1982; Tanaka and Okeda, 2000).

Another interesting finding, which may provide some clue as to the underlying mechanism, is the distinction between the effects of chronic absence of the 5-HT$_{2A}$ and the effect of acute antagonism of this receptor. Specifically, that clozapine (a 5-HT$_{2A}$ antagonist) is dependent on the presence of the 5-HT$_{2A}$, but not 5-HT$_{2C}$, for its locomotor-suppressing effects is seemingly counter-intuitive. In this context, it is important to consider how the constitutive absence of 5-HT$_{2A}$ might impact upon neural development, and adult neurochemistry and neuroanatomy. To date, we have not identified any significant compensatory changes caused by the constitutive absence of 5HT$_{2A}$ that could explain the observed genotype difference (Weisstaub *et al*, 2006). That said, more thorough investigations may reveal additional changes in other signaling pathway components. One such possibility would be changes in expression of the immediate early gene (IEG), *Egr3*. The potential importance of *Egr3* in mediating this behavior, and its possible link to the 5-HT$_{2A}$ receptor, is presented in detail in a recent publication and we will thus refrain from further elaboration on the relevance of this point (Williams *et al*, 2012; Gallitano-Mendel *et al*, 2008). However, should this signaling pathway be found to modulate 5-HT$_{2A}$ activity, it would provide a parsimonious explanation for the divergence between the effect of the receptor knockout and the pharmacological inactivation of the receptor. Moreover, it would be consistent with the decreased expression of 5-HT$_{2A}$ observed in the egr3 KO mice.

Conclusions

To date, the neurochemical and neuroanatomical loci underlying many of the side effects associated with clozapine have not been widely explored and academic discourse and drug discovery have moved forward in the absence of this important information. This series of experiments are among the first to identify categorically a neural locus for the locomotor suppression induced by clozapine, while at the same time identifying a novel target for the neurochemical pathways involved (see also Williams *et al*, 2012). The regulation of clozapine-induced locomotor suppression by glutamatergic forebrain 5-HT$_{2A}$-expressing neurons provides insight into the adverse effects of antipsychotics, clarifying our understanding of the neural circuitry underlying the side effects induced by antipsychotics.

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DISCLOSURE

The authors declare no conflict of interest.

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