Neuropsychopharmacology (2012) 37, 2285–2298
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Reduced Levels of Serotonin 2A Receptors Underlie Resistance of *Egr3*-Deficient Mice to Locomotor Suppression by Clozapine

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The immediate-early gene early growth response 3 (*Egr3*) is associated with schizophrenia and expressed at reduced levels in postmortem patients' brains. We have previously reported that *Egr3*-deficient ($Egr3^{-/-}$) mice display reduced sensitivity to the sedating effects of clozapine compared with wild-type (WT) littermates, paralleling the heightened tolerance of schizophrenia patients to antipsychotic side effects. In this study, we have used a pharmacological dissection approach to identify a neurotransmitter receptor defect in $Egr3^{-/-}$ mice that may mediate their resistance to the locomotor suppressive effects of clozapine. We report that this response is specific to second-generation antipsychotic agents (SGAs), as first-generation medications suppress the locomotor activity of $Egr3^{-/-}$ and WT mice to a similar degree. Further, in contrast to the leading theory that sedation by clozapine results from anti-histaminergic effects, we show that H1 histamine receptors are not responsible for this effect in C57BL/6 mice. Instead, selective serotonin 2A receptor ($5HT_{2A}R$) antagonists ketanserin and MDL-11939 replicate the effect of SGAs, repressing the activity in WT mice at a dosage that fails to suppress the activity of $Egr3^{-/-}$ mice. Radioligand binding revealed nearly 70% reduction in $5HT_{2A}R$ expression in the prefrontal cortex of $Egr3^{-/-}$ mice compared with controls. $Egr3^{-/-}$ mice also exhibit a decreased head-twitch response to $5HT_{2A}R$ agonist 1-(2,5-dimethoxy 4-iodophenyl)-2-amino propane (DOI). These findings provide a mechanism to explain the reduced sensitivity of $Egr3^{-/-}$ mice to the locomotor suppressive effects of SGAs, and suggest that $5HT_{2A}Rs$ may also contribute to the sedating properties of these medications in humans. Moreover, as the deficit in cortical $5HT_{2A}R$ in $Egr3^{-/-}$ mice aligns with numerous studies reporting decreased $5HT_{2A}R$ levels in the brains of schizophrenia patients, and the gene encoding the $5HT_{2A}R$ is itself a leading schizophrenia candidate gene, these findings suggest a potential mechanism by which putative dysfunction in *EGR3* in humans may influence risk for schizophrenia.

Neuropsychopharmacology (2012) **37,** 2285–2298; doi:10.1038/npp.2012.81; published online 13 June 2012

Keywords: 5HT2A receptor; clozapine; Egr3; immediate early gene; locomotor activity; schizophrenia/antipsychotics

INTRODUCTION

The immediate-early gene early growth response 3 (*Egr3*) is associated with schizophrenia risk (Kim *et al*, 2010; Yamada *et al*, 2007; Zhang R *et al*, 2012) and expressed at reduced levels in the brains of patients with the mental illness (Mexal *et al*, 2005; Yamada *et al*, 2007). Animal studies also support a role for *Egr3* in schizophrenia pathogenesis. We have previously reported that *Egr3*-deficient ($Egr3^{-/-}$) mice display locomotor hyperactivity, a phenotype associated with schizophrenia (Gainetdinov *et al*, 2001), which is reversed by treatment with either haloperidol or clozapine (Gallitano-Mendel *et al*, 2008). However, the response of the mice to these two medications was distinctly different. Whereas the dose of haloperidol that normalized the hyperactivity of $Egr3^{-/-}$ mice did not affect the locomotion of wild-type (WT) control animals, the dosage of clozapine required to normalize the activity of $Egr3^{-/-}$ mice profoundly suppressed the locomotor activity in their WT littermates (Gallitano-Mendel *et al*, 2008). This relative resistance to the locomotor suppression effects of clozapine, compared to controls, parallels the heightened tolerance of schizophrenia patients to the side effects of antipsychotics (Cutler, 2001). The cause of this effect in either humans or $Egr3^{-/-}$ mice is not known. Like in humans, identification of the neurobiological cause of abnormal behaviors in gene-deficient mice can be remarkably challenging.

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Received 22 December 2011; revised 6 April 2012; accepted 26 April 2012

Indeed, prior histological studies failed to identify differences in levels of neurotransmitter receptors in the brains of *Egr3*$^{-/-}$ mice to explain these abnormalities (Tourtellotte and Milbrandt, 1998). This points to a need for methods to identify receptor differences that underlie behavioral and pharmacological abnormalities in genetically altered mice.

Clozapine remains one of the leading antipsychotic medications to date, yet its mechanism of action remains unknown. This is due, in part, to its complex receptor binding profile. Clozapine binds to a wide range of receptors in the brain, including numerous subtypes of dopamine, serotonin, histamine, adrenergic, and muscarinic receptors (reviewed in Meltzer and Huang (2008), Roth *et al* (2004), and Stahl (2008)). The mechanism underlying the sedating effects of clozapine in humans is also unknown, although it is frequently attributed to antagonism of the histamine H1 receptor (Casey, 1997). We hypothesized that, by systematically testing a range of pharmacological compounds, which bind to subsets of receptors in the clozapine binding profile (ie, a 'pharmacological dissection'), we could identify the receptor subtype responsible for the resistance of *Egr3* $^{-/-}$ mice to the locomotor activity suppression by clozapine. This should shed light on the mechanism underlying the sedating effects of clozapine in humans. In addition, this method should identify a neurotransmitter receptor defect in *Egr3*$^{-/-}$ mice, providing a clue into a neurobiological abnormality of schizophrenia patients, while also revealing the next step of our hypothesized pathway of schizophrenia susceptibility genes (Gallitano-Mendel *et al*, 2008).

In this study, we show several novel findings resulting from this approach. First, the H1 histamine receptor is not responsible for the resistance of *Egr3*$^{-/-}$ mice to locomotor suppression by clozapine. Second, we demonstrate that the locomotor activity response of *Egr3*$^{-/-}$ mice appears to distinguish second-generation antipsychotics (SGAs, also known as 'atypical antipsychotics') from first-generation antipsychotics (FGAs, also known as 'typical antipsychotic'). Third, we show that selective antagonists for the serotonin 2A receptor ($5HT_{2A}$R) suppress the locomotor activity of WT, but not *Egr3*$^{-/-}$, mice and thus mimic the effect of clozapine. Finally, we find that *Egr3*$^{-/-}$ mice have a nearly 70% decrease in $5HT_{2A}$R binding in the prefrontal cortex (PFC) and display a blunted behavioral head-twitch response to $5HT_{2A}$R agonist 1-(2,5-dimethoxy 4-iodophenyl)-2-amino propane (DOI). These findings suggest that action at the $5HT_{2A}$R contributes to the locomotor suppressive effects of clozapine and other SGAs in mice, and may play a role in the sedating effects of these medications in humans. Furthermore, the reduced levels of $5HT_{2A}$Rs we identified in *Egr3*$^{-/-}$ mice parallel the results of numerous *in vivo* and post-mortem studies that report decreased levels of $5HT_{2A}$Rs in the frontal cortex of schizophrenia patients, including first-break, untreated individuals (Dean and Hayes, 1996; Erritzoe *et al*, 2008; Garbett *et al*, 2008; Hurlemann *et al*, 2008; Lopez-Figueroa *et al*, 2004; Matsumoto *et al*, 2005; Ngan *et al*, 2000; Rasmussen *et al*, 2010; Serretti *et al*, 2007). Taken together, these findings suggest a possible mechanism through which human *Egr3* may influence susceptibility to schizophrenia.

MATERIALS AND METHODS

Animals

Previously generated *Egr3*$^{-/-}$ mice (Tourtellotte and Milbrandt, 1998) were backcrossed to C57BL/6 mice for more than 20 generations. Animals were housed on a 14/10 h light/dark schedule with *ad libitum* access to food and water. A large breeding colony of *Egr3*$^{+/-}$ × *Egr3*$^{+/-}$ mice was maintained to produce study animals. Studies were conducted on adult male littermate progeny of these matings.

Behavioral Testing

Behavioral testing was performed during daytime hours under ambient light conditions. Progeny male +/+ and −/− animals were identified as 'matched pairs' at the time of genotyping and added to experimental cohorts at a minimum of 2 months of age. A total of 10 independent cohorts of animals were used throughout the course of all behavioral studies. To accommodate IACUC recommendations for reduction of animal numbers, mice were used in an average of three tests before being euthanized by CO_2 asphyxiation. All animals were killed by the age of 12 months, if not before. Between tests, mice underwent a washout period of greater than, or equal to, five drug half-lives, and were re-randomized into treatment groups, to minimize possible confounds from repeated use. Furthermore, testing for each drug was replicated in a second, independent cohort of mice. Locomotor activity effects were robust and replicable, reducing the likelihood of possible confounds secondary to re-use of animals. Specific sample sizes per group, per experiment are included in the figure legends for each study.

Activity Monitoring

The effect of pharmacological agents on the locomotor activity of WT and *Egr3*$^{-/-}$ mice was measured using the SmartFrame system (Kinder Scientific, Poway, CA) (Table 1). Drug dosages were selected following literature review and subsequent dose–response testing in pilot groups of WT C57BL/6 mice to establish the dosage that suppressed locomotor activity in WT mice (Supplementary Figure S1). The suppressive dosage, vehicle control, and intermediate dosages were then tested in a large cohort of matched *Egr3*$^{-/-}$ and WT littermates. All studies were replicated in a second, separate cohort of animals. The second of the replicate studies is presented in the Results section.

Activity was evaluated in transparent ($47.6 \times 25.4 \times 20.6\,cm^3$ high) polystyrene enclosures using a computerized photobeam system (MotorMonitor Kinder Scientific). Animals were placed in the enclosures 20 min after drug administration, and activity was monitored for 1 h. Locomotor activity was calculated using a number of movements (total photobeam breaks) as the dependent variable for total activity. The term 'Reduced' is used for statistically significant decreases in locomotor activity, compared with vehicle-treated animals of the same genotype, which remain >1000 movements per h. Decreases in locomotor behavior below 1000 movements per h are labeled 'suppressed'.

Table 1 Effect of Drugs Targeting Receptors Bound by Clozapine on the Activity of WT and *Egr3*$^{-/-}$ Mice

Drug	**High affinity target receptors***	**Dosage (mg/kg)**	**Activity**	
			WT	***Egr3*$^{-/-}$**
Antipsychotics				
Clozapine	A1A, H1, A1B; 5-HT2A; M1-2; 5-HT6-7; M3; A2B; M4; 5-HT2C; A2C; D4; M5	3.5, 5, 7	Suppressed	Reduced
Chlorpromazine	H1; A1A-B; D2; 5-HT2A; D3; 5-HT6; M5; 5-HT7; D4; 5-HT2C; A2B-C; M1; M3	5, 10	Suppressed	Suppressed
Haloperidol	D2; A1A-B; D3-4; 5-HT2A; D1	0.03, 0.1, 0.3	Suppressed	Suppressed
Olanzapine	5-HT2A; H1; 5-HT6; M5; D4; 5-HT2C; M1; A2C; H2; M3; D1-3; M2; A2B; D5	1, 2, 3	Suppressed	Reduced
Quetiapine	H1; A1A-B; A2C	10, 20	Suppressed	Reduced
Ziprasidone	5-HT2A; 5-HT1B; D2; 5-HT7; 5-HTD; A1B; D3; A1A; 5-HT2B; D1; A2B; 5-HT6; 5-HT2C; 5-HT1A; A2C	2.5, 5, 7, 10	Suppressed	Reduced
H1 antagonists				
Diphenhydramine	H1	2, 5, 7	No change	N/A
Promethazine	H1	10, 50	Suppressed	Suppressed
Pyrilamine	H1	10, 50	No change	N/A
5-HT2A antagonists				
ACP-103	5-HT2A	15	Suppressed	Reduced
Ketanserin	5-HT2A; H1; 5-HT2C	2.5, 5, 10	Suppressed	Reduced
MDL-11939	5-HT2A, 5-HTIB	2.5, 5, 10	Suppressed	Reduced
Additional drugs targeting other receptors				
Ifenprodil	NMDA/NR2B selective	20, 40	No change	N/A
Medetomidine	α2 agonist	0.01, 0.1, 1	Suppressed	Suppressed
MPEP	Glutamate antagonist	10, 30	No change	N/A
Scopolamine	M1–5 antagonist	0.5, 5, 15	No change	N/A
Terazosin	α1 antagonist	10, 50, 100	Suppressed	Suppressed

Dose–response curves for each drug were tested in WT mice. If the agent was found to suppress the locomotor activity in a 60 min test, further tests were performed in matched *Egr3*$^{-/-}$ and WT mice to determine whether the response differed between the two genotypes. *Reported receptor ligands that bind with $K_i < 100$ nM according to PDSP website (Roth, 2008). Receptor subtypes are listed in the order of binding affinity, from highest to lowest. See Table 2 for complete binding profiles and K_i values. *Suppressed*: Activity decreased below 1000 movements in 1 h of monitored locomotor activity. *Reduced*: Locomotor hyperactivity significantly reduced ($p < 0.05$); compared with vehicle-treated mice of the same genotype, but well above 1000 movements per hour. *No change***:** No significant change in a 1 h locomotor activity test compared with vehicle-treated controls. *N/A*: No change detected in dose–response curve with WT mice, thus no subsequent tests with *Egr3*$^{-/-}$ mice were conducted. References: Bespalov *et al* (2007), Buckton *et al* (2001), Cosi *et al* (2005), Crawley (1981), Dougherty and Aloyo (2011), Fox *et al* (2010), Fukushiro *et al* (2007), Kamei *et al* (2005), Kehne *et al* (1996), Kinkead *et al* (2005), Kyncl (1986), Lynch *et al* (2011), MacDonald *et al* (1991), Moore *et al* (1992), O'Dell *et al* (2000), Oduola *et al* (2004), Philibin *et al* (2005), Rasmussen and Fink-Jensen (2000), Redrobe and Bourin (1997), Simon *et al* (2000), Vanderwolf (1991), Vanover *et al* (2006), Votava *et al* (2008), Yan *et al* (2007), Zarnowski *et al* (1994), and Zhu *et al* (2004).

Data are depicted as graphs of average total activity in response to drug dosage for each genotype. In addition, the same data are also graphed to show the percentage decrease in activity compared with vehicle-treated controls of the same genotype. For the latter graph, each animal was compared to the average of all vehicle-treated animals of the same genotype to generate individual 'percent change in activity' values. These values were then averaged for all mice in a treatment group (defined by genotype and drug dosage) to produce bar graphs. Error bars in all graphs denote standard error of the mean for each treatment group.

Video Recording

After completion of testing, a subset of WT and *Egr3*$^{-/-}$ mice were video recorded following administration of FGA chlorpromazine (10 mg/kg), SGA olanzapine (3 mg/kg), and 5HT$_{2A}$R-specific antagonist MDL-11939 (10 mg/kg). Littermate animals were administered either drug or vehicle and allowed to acclimate for 30 min before the removal of the cage lid for brief recording with a hand-held video camcorder. Gentle shaking of the cage by the investigator was used to stimulate the activity of immobile mice.

Drowsiness, Motor impairment, and Stereotypic Behavior Assessment

The effect of haloperidol (3 mg/kg) and clozapine (7 mg/kg) on drowsiness, motor impairment, and stereotypic behavior was assessed in a cohort of WT and *Egr3*$^{-/-}$ mice. Behavior was scored for a period of 2 min at 30 and 60 min post-drug administration. Abnormal movements were scored according to a behavioral checklist for stereotypy and dyskinesia, adapted from McNamara *et al.* (2006) and Khan *et al.* (2004). The categories scored included: grooming episodes, head bobbing, and myoclonic twitches of the abdomen, head/facial, and limb regions. The total number of head bobs, and face, trunk, or limb twitches in the 2 min period were summed to produce a total stereotypy count. Grooming episodes were rare across all groups, and therefore not included. A second assessment was performed using a sedation and motor impairment rating scale adapted from Aitchison *et al.* (2000). Scores represent the average of two independent observers blind to both genotype and treatment. A detailed protocol and rating scales are included in Supplementary Materials.

DOI-Induced Head-Twitch Response

Head-twitch response to DOI (1 mg/kg) was assessed as described previously (Gonzalez-Maeso *et al*, 2003). Animals were placed in a transparent polystyrene cage 15 min following administration of drug or vehicle and video recorded for 30 min at close range by a camera suspended above the cage. Head twitches were independently scored by two observers blind to genotype and treatment, and scores were averaged for statistical analysis.

Radioligand Binding Assay

Radioligand binding with $[^3H]$ketanserin was used to measure the level of expression of 5-HT_{2A}R in the PFC of drug-naive *Egr3*$^{-/-}$ and WT littermate control mice. Animals were killed via CO_2 asphyxiation, brains were immediately removed, and the PFC was dissected from a coronal slice spanning from Bregma: 1.95, Interaural: 5.78 and Bregma: 0.00, Interaural: 3.80, using the Coronal C57BL/6J Atlas from the Mouse Brain Library (Rosen *et al*, 2000). Collected tissue was snap-frozen on dry ice and stored at −80 °C until binding studies were performed. $[^3H]$Ketanserin (DuPont-NEN, Boston, MA) binding (0.0625–10 nM; 10 concentrations) to $5HT_{2A}$R was measured at equilibrium in 500 μl aliquots (50 mM Tris-HCl; pH 7.4) of membrane preparations (10–57 μg protein per tube), which were incubated at 37 °C for 60 min as described previously (Gonzalez-Maeso *et al*, 2008). Nonspecific binding was determined in the presence of 10 μM methysergide (Tocris Bioscience, Ellisville, MO), and ranged from 27 ± 2 to 61 ± 4% of total binding in all groups. The study was performed in two independent groups of animals; $n = 8$ animals per genotype for each study.

Drug Preparation and Administration

Chlorpromazine, clozapine, haloperidol, ketanserin, and DOI were obtained from Sigma Aldrich (St Louis, MO). Olanzapine, quetiapine, and ziprasidone were obtained through the NIMH Chemical Synthesis and Drug Supply Program (Bethesda, MD). MDL-11939 was obtained from Tocris Bioscience (Ellisville, MO). Chlorpromazine and DOI were dissolved in saline. Olanzapine, quetiapine, and haloperidol were dissolved in a small amount of glacial acetic acid and further diluted in sterile water. Clozapine and MDL-11939 were dissolved in HCl and diluted in sterile water. Ketanserin was diluted in DMSO and sterile water. Ziprasidone was diluted in 45% 2-hydroxypropyl-β-cyclodextrin. Concentrated aliquots of each drug were stored at −20 °C. Aliquots were thawed at 37°C and diluted to their final concentration in sterile saline on the day of testing. Solutions were buffered as necessary to achieve a final pH of 6.5–7.5. K_i determinations were generously provided by the National Institute of Mental Health's Psychoactive Drug Screening Program, Contract No. HHSN-271-2008-00025-C (NIMH PDSP; Bethesda, MD).

For each drug tested, vehicle was prepared in an identical manner without the addition of drug. Drug or vehicle was administered via intraperitoneal injection in a 10 ml/kg volume.

Data Analysis

Statistical analyses, including analysis of variance (ANOVA), Student's *t*-test, and standard error of the mean (SEM) were performed in SPSS (Chicago, IL) and Microsoft Excel. Locomotor activity and DOI-induced head-twitch behavior were evaluated using a two-way ANOVA. Behavioral assessment data were examined in SPSS using repeated-measures multivariate ANOVA (MANOVA) with treatment and genotype as a between-subjects factor and time as a repeated measure. Data are represented as means ± SEM in all graphs.

RESULTS

We have previously reported that *Egr3*$^{-/-}$ mice are resistant to the locomotor inhibitory effects of the antipsychotic medication clozapine ((Gallitano-Mendel *et al*, 2008), see online video at http://www.nature.com/npp/journal/v33/n6/extref/1301505x3.mov). This response is not due to the baseline hyperactivity (a schizophrenia-like rodent phenotype) displayed by *Egr3*$^{-/-}$ mice as the animals do not show this response to the antipsychotic haloperidol. We have previously shown that haloperidol normalizes the hyperactivity of *Egr3*$^{-/-}$ mice to WT vehicle-treated levels at a dosage that has no effect on the locomotor activity of WT mice, and higher dosages of haloperidol reduce the activity of both WT and *Egr3*$^{-/-}$ mice to the same degree (Gallitano-Mendel *et al*, 2008). In contrast, clozapine profoundly suppresses the activity of WT mice at a dosage that reduces the hyperactivity of *Egr3*$^{-/-}$ mice only to WT vehicle-treated levels (Gallitano-Mendel *et al*, 2008). Thus, the locomotor inhibition produced by haloperidol is not the same as that resulting from clozapine, and *Egr3*$^{-/-}$ mice distinguish this difference.

In this study, we have employed a 'pharmacological dissection' approach, using increasingly selective drugs to target specific receptor subtypes in the clozapine binding profile, to identify the receptor abnormality that is responsible for the decreased sensitivity of *Egr3*$^{-/-}$ mice to locomotor suppression by clozapine, compared with haloperidol. To identify drugs that mimic the effects of clozapine, we first established the dosage of each test drug that suppressed locomotor activity (ie, decreased activity below 1000 movements per h) in a pilot group of WT mice

(Supplementary Figure S1). We then tested whether that dosage also reduced the locomotor activity of *Egr3*$^{-/-}$ mice.

Agents Selective for Receptors Commonly Associated with Sedation Fail to Replicate the Effect of SGAs in *Egr3*$^{-/-}$ Mice

We began by targeting the receptor systems to which the sedating effects of clozapine and other SGAs are commonly attributed: the histamine H1 receptor and α-adrenergic receptors (Alves *et al*, 2010; Casey, 1997; Mengod *et al*, 1996; Parsons and Ganellin, 2006). Dose–response pilot experiments in WT C57Bl/6 mice (the background strain of *Egr3*$^{-/-}$ mice) revealed that the selective H1 receptor antagonist pyrilamine (also known as mepyramine) does not reduce locomotor activity, even at doses up to 50 mg/kg, the highest dose used in mice found in the literature (Figure 1 and Table 1) (Parsons and Ganellin, 2006; Shishido *et al*, 1991). A pilot study with diphenhydramine, another relatively selective H1 antagonist (Parsons and Ganellin, 2006), yielded similar results (Table 1). Promethazine, a less selective H1 receptor antagonist (Wishart *et al*, 2008; Wishart *et al*, 2006) and member of the phenothiazine family, suppressed activity at the highest administered dose (50 mg/kg), but did so equally in both WT control and *Egr3*$^{-/-}$ mice, failing to reproduce the response to clozapine (Table 1). Tests with terazosin, an α1-adrenergic receptor antagonist, and medetomidine, an α2-specific agonist used as a sedative in veterinary medicine (Alves *et al*, 2010), likewise showed similar levels of locomotor suppression in *Egr3*$^{-/-}$ and WT mice (Table 1). These findings indicate that neither H1 histamine receptors nor α-adrenergic receptors alone are responsible for the resistance of *Egr3*$^{-/-}$ mice to the locomotor inhibitory effects of clozapine.

Egr3$^{-/-}$ Mice Exhibit Resistance to Locomotor Suppression by SGAS, but not FGAS

As the receptors commonly implicated in sedation did not appear to be responsible for the resistance of *Egr3*$^{-/-}$ mice to this effect of clozapine, we returned to our earlier finding that *Egr3*$^{-/-}$ mice do not display resistance to the locomotor inhibitory effects of the FGA haloperidol (Gallitano-Mendel *et al*, 2008). In other words, haloperidol reduces the hyperactivity of *Egr3*$^{-/-}$ mice to normal levels at a dosage that does not affect the activity of WT mice, and higher doses of the medication inhibit activity in WT and *Egr3*$^{-/-}$ mice in an equivalent manner (Gallitano-Mendel *et al*, 2008). We hypothesized that this may be because haloperidol is a high potency antipsychotic that is also less sedating than other FGA medications. We therefore tested whether chlorpromazine (0, 5, and 10 mg/kg), a low-potency FGA that is highly sedating, would produce a similar behavioral effect on *Egr3*$^{-/-}$ mice. Figure 2a shows that chlorpromazine reduced the activity of *Egr3*$^{-/-}$ mice at the same dosage as WT mice, an effect similar to that of haloperidol, and markedly different than that of clozapine. A two-way ANOVA revealed a main effect of chlorpromazine ($F(2,54) = 21.1$; $p < 0.001$) and genotype ($F(1,54) = 45.5$; $p < 0.001$) on locomotor activity, and a treatment by genotype interaction ($F(2,54) = 7.1$; $p < 0.05$). Figure 2b shows that each dose of chlorpromazine reduced activity to a similar degree, on average, in both WT (77% with 5 mg/kg and 95% with 10 mg/kg) and *Egr3*$^{-/-}$ mice (74% with 5 mg/kg and 89% with 10 mg/kg) (differences were not significant by Bonferroni-corrected Student's *t*-test). On visual inspection both WT control and *Egr3*$^{-/-}$ mice appeared immobile following the highest dose (10 mg/kg) of chlorpromazine (Supplementary Video S2).

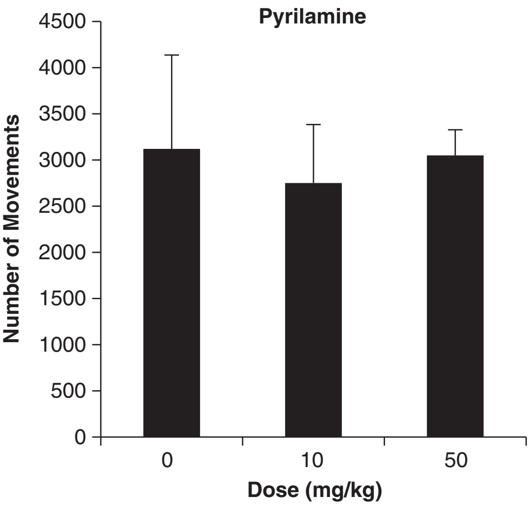

Figure 1 Histamine H_1 antagonism is not responsible for resistance of *Egr3*-deficient (*Egr3*$^{-/-}$) mice to locomotor suppression by clozapine. Locomotor activity was monitored for 60 min in wild-type (WT) mice following administration of highly specific H_1 receptor antagonist pyrilamine. Pyrilamine was not sedating at 10 or 50 mg/kg, the highest dose reported in the literature (Roth, 2008) ($n = 3$ per group).

We then repeated this assay with other SGAs to assess whether *Egr3*$^{-/-}$ mice would show the same locomotor response to other medications within the same classification as clozapine. Figure 3a shows that olanzapine, an SGA designed to mimic the receptor binding activity of clozapine, suppressed the locomotor activity of WT mice to nearly zero, while the activity of *Egr3*$^{-/-}$ mice was decreased only to vehicle-treated WT levels (also see Supplementary Video S3). This result was identical to that of clozapine (Gallitano-Mendel *et al*, 2008). A two-way ANOVA evaluating locomotor activity following administration of olanzapine (0, 1, 2, and 3 mg/kg) revealed a main effect of treatment ($F(3,56) = 17.7$; $p < 0.001$) and genotype ($F(1,56) = 155.7$; $p < 0.001$), and a treatment by genotype interaction ($F(3,56) = 4.6$; $p < 0.01$). Figure 3b shows that each dose of olanzapine reduced the activity more in WT mice than in *Egr3*$^{-/-}$ mice. Compared to vehicle-treated mice, a 3 mg/kg dose of olanzapine reduced activity by 98%, on average, in WT mice, but only 51% in *Egr3*$^{-/-}$ mice ($p < 0.001$, Student's *t*-test).

Similar analyses following administration of two additional SGAs replicated these effects. Figures 3c–f show that *Egr3*$^{-/-}$ mice are similarly resistant to the locomotor suppressive effects of quetiapine and ziprasidone as to clozapine and olanzapine. The two-way ANOVA following administration of quetiapine (0, 10, and 20 mg/kg) revealed a main effect of treatment ($F(2,36) = 20.4$; $p < 0.001$) and genotype ($F(1,36) = 92.5$; $p < 0.001$), and a treatment by

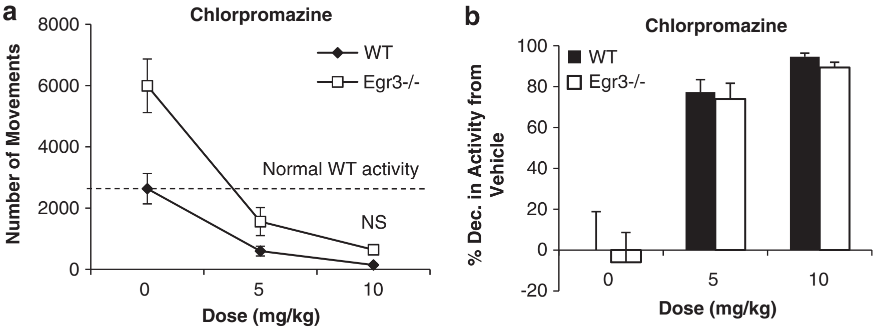

Figure 2 First-generation antipsychotics (FGAs) reduce activity to a similar degree in *Egr3*-deficient (*Egr3*$^{-/-}$) and wild-type (WT) mice. The locomotor activity of *Egr3*$^{-/-}$ and WT mice was monitored for 60 min following administration of chlorpromazine, a low-potency, highly sedating FGA. As previously reported with haloperidol (Gallitano-Mendel *et al*., 2008), *Egr3*$^{-/-}$ mice demonstrate a similar susceptibility to locomotor suppression by chlorpromazine as WT controls (see also Supplementary Video S2). (a) Vehicle-treated *Egr3*$^{-/-}$ mice are hyperactive in comparison to vehicle-treated WT mice. Chlorpromazine reduced locomotor activity in a dose-dependent manner to a similar degree in both *Egr3*$^{-/-}$ and WT mice ($n = 10$ per group). (b) The average activity of vehicle-treated mice for each genotype was used to calculate the percent decrease from basal activity for each animal (see Methods). The average percent decrease in activity is presented for both *Egr3*$^{-/-}$ and WT mice treated with either 0, 5, or 10 mg/kg chlorpromazine.

genotype interaction (F(2,36) = 4.0; $p<0.05$) (Figure 3c). Figure 3d shows that 20 mg/kg of quetiapine reduced activity more in WT than *Egr3*$^{-/-}$ mice when compared to vehicle-treated mice of the respective genotype ($p<0.001$, Student's *t*-test). Ziprasidone treatment (0, 2.5, and 5 mg/kg) also revealed main effects of treatment (F(2,41) = 18.1; $p<0.001$) and genotype (F(1,41) = 62.6, $p<0.001$), and a treatment by genotype interaction (F(2,41) = 4.5, $p<0.05$) (Figure 3e). Figure 3f shows that, like the other SGAs, in comparison to vehicle-treated mice, each dose of ziprasidone reduce the activity of WT mice to a greater degree than the activity of *Egr3*$^{-/-}$ mice ($p<0.005$ for 2.5 mg/kg dose, $p<0.001$for 5 mg/kg dose, Student's *t*-test). These findings suggest that *Egr3*$^{-/-}$ mice differ from WT mice in their response to the locomotor suppressive effect of SGAs, but not to that of FGAs.

The SGAs differ from FGAs in producing a significantly lower incidence of extra-pyramidal side effects (Pierre, 2005). This response in mice is identified by 'stereotypic movements' (or 'stereotypy'). As the activity-monitoring test we employed does not differentiate sedation from other causes of immobility, we evaluated the behavioral response of *Egr3*$^{-/-}$ and WT mice to the SGA clozapine, and the FGA haloperidol, using rating scales for drowsiness, motor impairment, and stereotypic behaviors. Figures 4a and b show that WT mice are significantly more sensitive to both the drowsiness and motor impairment caused by clozapine than are *Egr3*$^{-/-}$ mice. Repeated-measures MANOVA revealed a main effect of both treatment and genotype and a treatment by genotype interaction on drowsiness (F(1,28) = 21.5 ($p< 0.001$), F(1,28) = 7.6 ($p<0.05$), F(1,28) = 10.4 ($p<0.005$), respectively) and motor impairment (F(1,28) = 33.8 ($p<0.001$), F(1,28) = 6.7 ($p<0.05$), F(1,28) = 6.7 ($p<0.05$)). In contrast, WT and *Egr3*$^{-/-}$ mice did not differ in the number of stereotypic movements they displayed following administration of clozapine (Figure 4c). Analysis of stereotypy data (Figure 4c) revealed a main effect of clozapine treatment (F(1,28) = 15.5 ($p<0.005$)), but not genotype ($p>0.05$). Within-subjects analysis revealed a time by genotype interaction on stereotypy (F(1,28) = 5.2; $p<0.05$), indicating that the two genotypes varied in the timing of their stereotypic movements across the test period, with WT mice displaying more stereotypy at 30 than 60 min, and *Egr3*$^{-/-}$ mice showing a more level number of stereotypic movements between the two time points. However, this timing effect is unlikely to account for drug-induced differences in locomotor-activity between *Egr3*$^{-/-}$ and WT mice as both time points are included in the 60 min activity monitoring session.

Like clozapine, haloperidol also induced a different degree of drowsiness in *Egr3*$^{-/-}$ mice than in WT mice. However, the effect was the opposite to that of clozapine, with *Egr3*$^{-/-}$ mice showing more drowsiness than WT mice following haloperidol administration (Figure 4d). Haloperidol caused motor impairment in both *Egr3*$^{-/-}$ and WT mice, although the difference in the response of the two genotypes was not evident until 60 min after drug administration (Figure 4e). Repeated-measures MANOVA on haloperidol treatment revealed a main effect of both treatment and genotype on drowsiness (F(1,32) = 33.8 ($p<0.001$) and F(1,32) = 17.2 ($p<0.001$), respectively) and motor impairment (F(1,32) = 116.3 ($p<0.001$) and F(1,32) = 4.6 ($p<0.05$), respectively) and a treatment by genotype interaction on drowsiness (F(1,32) = 15.5 ($p<0.001$)), but not on motor impairment ($p>0.05$). Analysis of stereotypy data (Figure 4f) revealed a main effect of treatment (F(1,32) = 70.0 ($p<0.001$)), but not of genotype (F(1,32) = 0.007 ($p = 0.9$)). Within-subject analysis revealed a main effect of time on drowsiness (F(1,32) = 5.3; $p<0.05$) and a three-way time by dose by genotype interaction (F(1,32) = 5.33; $p<0.05$). These results indicate that *Egr3*$^{-/-}$ mice differ from WT mice in their sensitivity to sedating and motor-impairing effects of antipsychotic medications, but they do not differ in their sensitivity to the stereotypic effects of these drugs. This suggests that the different motor effects of FGAs *vs* SGAs on *Egr3*$^{-/-}$ mice are not stereotypic in nature.

5HT$_{2A}$R Antagonists Parallel the Effect of Clozapine on *Egr3*$^{-/-}$ Mice

One of the leading features distinguishing SGAs from FGAs is the high affinity SGAs display for the 5HT$_{2A}$R (Meltzer *et al*, 2003). We therefore tested whether this receptor was responsible for the resistance of *Egr3*$^{-/-}$ mice to the

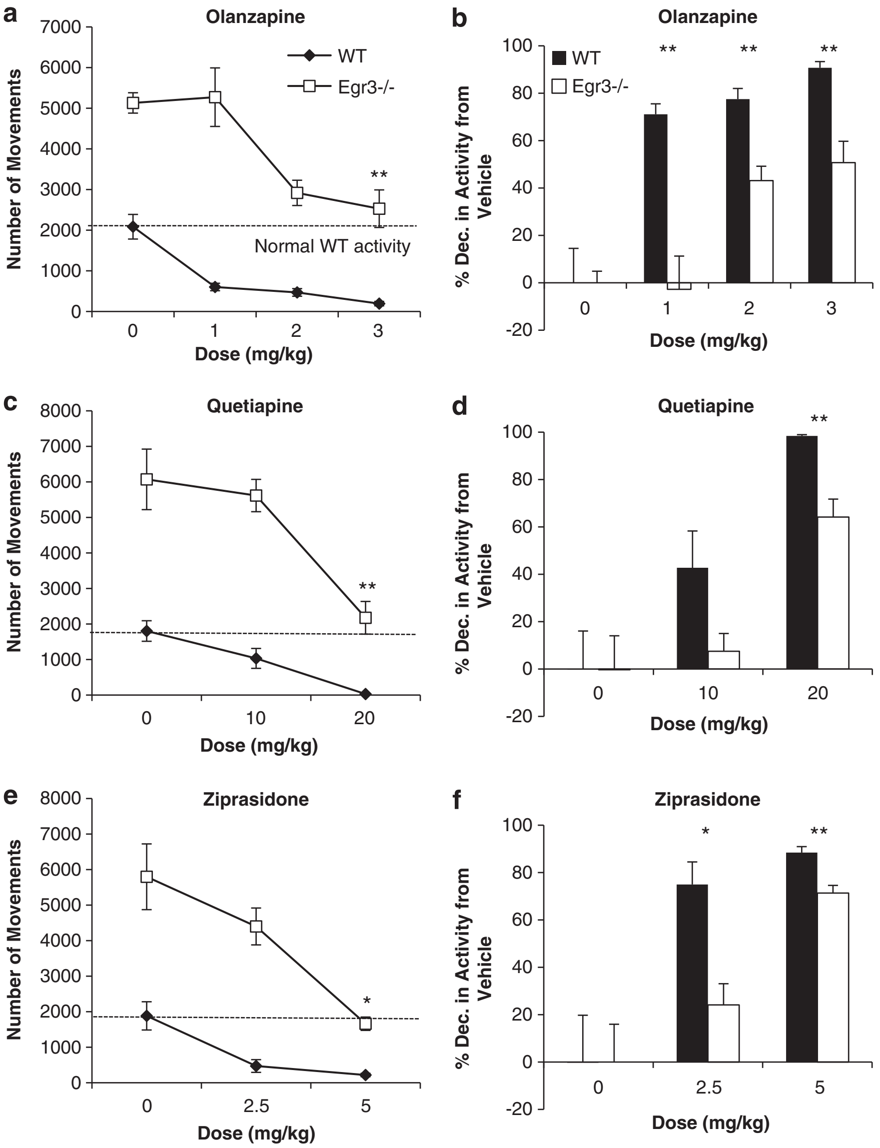

Figure 3 *Egr3*-deficient (*Egr3*$^{-/-}$) mice are resistant to locomotor inhibition by second-generation antipsychotic agents (SGAs). The locomotor activity of *Egr3*$^{-/-}$ and WT mice was monitored for 60 min following administration of SGAs. (a) Olanzapine suppressed the activity of WT controls at a dosage of 1 mg/kg, while a dosage of 3 mg/kg reduced the activity of *Egr3*$^{-/-}$ mice to normal WT activity levels ($n = 8$ per group) (see also Supplementary Video S3). (b) The average activity of vehicle-treated mice for each genotype was used to calculate the percent decrease from basal activity for each animal (see Materials and Methods). The average percent decrease is presented for both *Egr3*$^{-/-}$ and WT mice treated with 0, 1, 2, or 3 mg/kg olanzapine. (c) Quetiapine reduced the activity of *Egr3*$^{-/-}$ mice to vehicle-treated WT activity levels, while abolishing almost all locomotor activity in WT mice, at 20 mg/kg ($n = 7$ per group). (d) The average percent decrease in activity from vehicle group is presented for both *Egr3*$^{-/-}$ and WT mice treated with 0, 10, or 20 mg/kg quetiapine. (e) Ziprasidone (2.5 mg/kg) suppressed the activity of WT mice, while 5 mg/kg was required to reduce the hyperactivity of *Egr3*$^{-/-}$ mice to normal WT levels ($n = 10$ per group). (f) The average percent decrease in activity from vehicle group is presented for both *Egr3*$^{-/-}$ and WT mice treated with 0, 2.5, or 5 mg/kg ziprasidone. *Significant *post hoc* comparisons of simple main effects between *Egr3*$^{-/-}$ and WT mice at the dose leading to an extreme suppression in activity in WT controls (a, c, and e), or Student's *t*-test after Bonferroni correction for multiple comparisons (b, d, and f) (*$p < 0.05$; **$p < 0.001$).

locomotor suppressive effects of these medications by examining the effect of drugs with relatively selective affinity for the $5HT_{2A}R$. First, we examined the effect of the $5HT_{2A}R$ antagonist ketanserin. Figure 5a shows that 5 mg/kg ketanserin suppressed locomotor activity in WT mice, while the locomotor hyperactivity of *Egr3*$^{-/-}$ mice was not even reduced to vehicle-treated WT levels. A two-way ANOVA on activity following treatment with $5HT_{2A}R$ antagonist ketanserin (0, 2.5, and 5 mg/kg) revealed a main effect of treatment ($F(2, 42) = 4.5$; $p < 0.05$) and genotype ($F(1,42) = 65.8$; $p < 0.001$), and no treatment by genotype interaction ($F(2,42) = 1.1$; $p > 0.05$). Figure 5b shows that ketanserin reduced activity more in WT mice than *Egr3*$^{-/-}$ mice. Compared to vehicle-treated mice, a 5 mg/kg dose of ketanserin reduced activity by 78%, on average, in WT mice, but only 17% in *Egr3*$^{-/-}$ mice ($p < 0.001$, Student's *t*-test).

Ketanserin binds with high affinity to $5HT_{2A}Rs$, but also binds to other serotonin receptors, as well as H1 and D1 dopamine receptors, with lower affinity, as summarized in

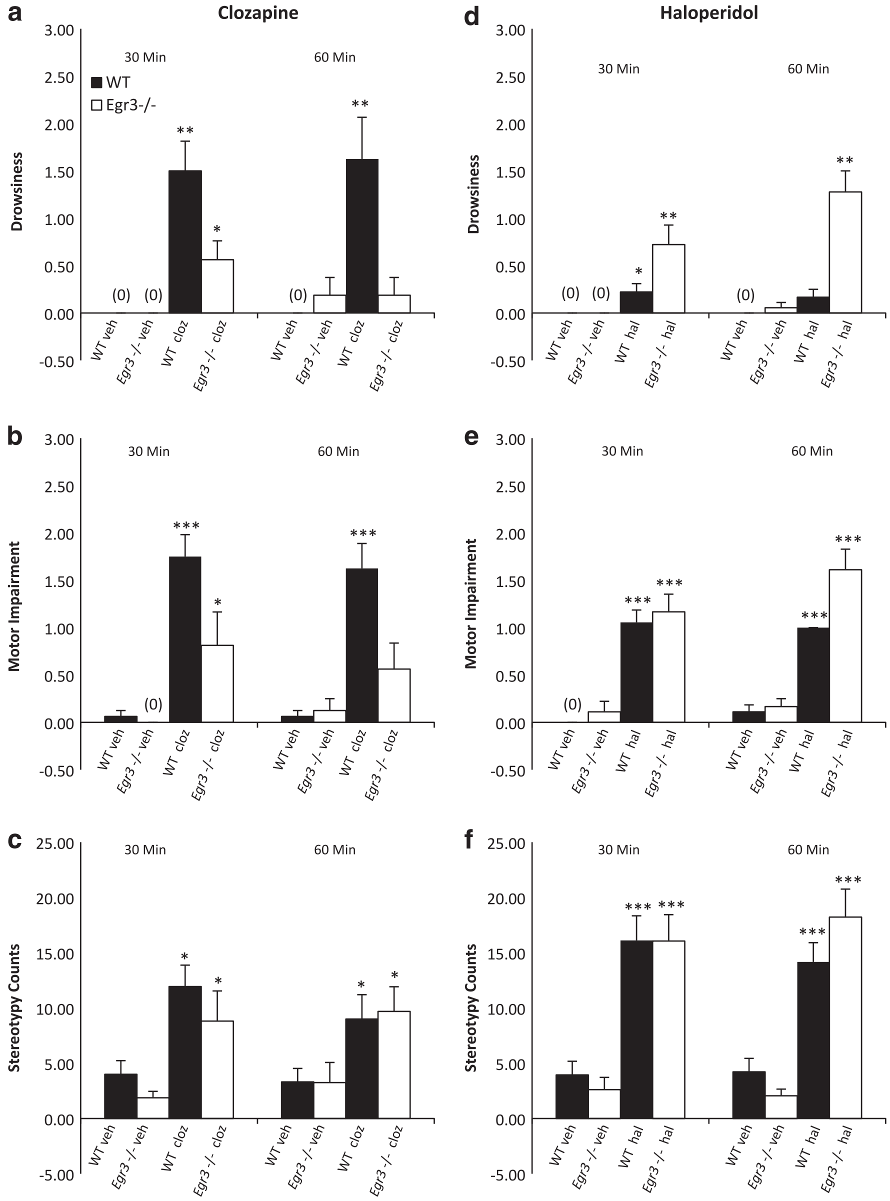

Figure 4 Stereotypic behavior does not account for the differential response of *Egr3*-deficient ($Egr3^{-/-}$) mice to first-generation antipsychotics (FGAs) *vs* second-generation antipsychotics (SGAs). Drowsiness, motor impairment, and stereotypy scores were assessed at 30 and 60 min following administration of the drug (clozapine, 7 mg/kg, a, b, and c; or haloperidol, 3 mg/kg, d, e, and f) or corresponding vehicle. $Egr3^{-/-}$ and wild-type (WT) mice responded differently to clozapine than to haloperidol in measures of drowsiness (a, d) and motor impairment (b, e), but not stereotypy (c, f) ($n = 8$ per group for clozapine; $n = 9$ per group for haloperidol). *Significant comparisons between vehicle and drug treatment groups within genotype (*$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; by Student's *t*-test).

Table 2. We therefore also examined the effect of the potent, selective 5HT$_{2A}$R antagonist MDL-11939 (0, 2.5, 5, and 10 mg/kg). Figure 5c shows that 5 mg/kg of MDL-11939 produced the same locomotor inhibitory effect on WT mice as ketanserin. An additional increase in dosage (to 10 mg/kg) did not further suppress locomotor activity in either WT or $Egr3^{-/-}$ mice. The two-way ANOVA revealed a main effect of treatment ($F(3,56) = 63.8$; $p < 0.001$) and genotype ($F(1,56) = 4.5$; $p < 0.01$), but no treatment by genotype interaction ($F(3,56) = 0.7$; $p > 0.05$). While the locomotor suppression produced by MDL-11939 in WT mice was not as extreme as that of the highest doses of antipsychotics, the resistance of $Egr3^{-/-}$ mice to its suppressive effect was greater than to the SGAs, as MDL-11939 (up to 10 mg/kg) failed to reduce the hyperactivity of $Egr3^{-/-}$ mice to normal WT levels. Visual inspection of animals revealed a marked difference in activity between treated WT and $Egr3^{-/-}$ mice (Supplementary Video S4).

$Egr3^{-/-}$ Mice have a Deficit of 5HT$_{2A}$Rs in the Prefrontal Cortex

To determine whether dysfunction of 5HT$_{2A}$Rs may be the mechanism underlying the resistance of $Egr3^{-/-}$ mice to the locomotor inhibitory effects of 5HT$_{2A}$R-specific agents, we conducted a radioligand binding assay to determine the

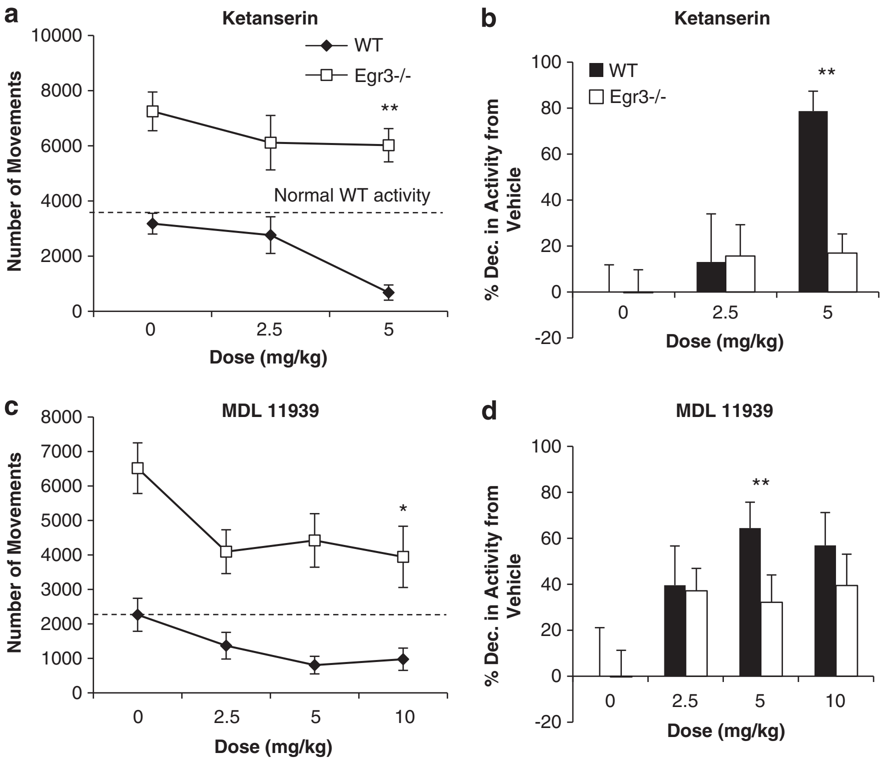

Figure 5 Serotonin 2A receptor ($5HT_{2A}R$) antagonists suppress the locomotor activity of wild-type (WT), but not *Egr3*-deficient (*Egr3*$^{-/-}$) mice. Locomotor activity was monitored for 60 min following administration of $5HT_{2A}$R-specific agents or vehicle. (a) Ketanserin suppresses the locomotor activity of WT mice at 5 mg/kg, a dose that decreases the hyperactivity of *Egr3*$^{-/-}$ mice, but does not reduce it to normal WT levels ($n = 7$–9 per group). (b) The average activity of vehicle-treated mice for each genotype was used to calculate the percent decrease from basal activity for each animal (see Methods). The average percent decrease in activity from vehicle group is presented for both *Egr3*$^{-/-}$ and WT mice treated with 0, 2.5, or 5.0 mg/kg ketanserin. (c) MDL-11939 (10 mg/kg) suppresses the activity in WT mice, but fails to reduce the hyperactivity of *Egr3*$^{-/-}$ mice to normal WT activity levels ($n = 8$ per group) (see also Supplementary Video S4). (d) The average percent decrease in activity from vehicle group is presented for both *Egr3*$^{-/-}$ and WT mice treated with 0, 2.5, 5, and 10 mg/kg MDL-11939. *Significant *post hoc* comparisons of simple main effects between *Egr3*$^{-/-}$ and WT mice at the dose leading to an extreme reduction in activity in WT controls (a and c) or Student's *t*-test after Bonferroni correction for multiple comparisons (b and d) (*$p < 0.005$; **$p < 0.001$).

level of expression of $5HT_{2A}R$ in *Egr3*$^{-/-}$ mice. In the murine brain, $5HT_{2A}$Rs are expressed in the frontal cortex along an anterior to posterior gradient, and show very little expression in other brain regions (Lein *et al*, 2007; Meltzer *et al*, 2010). The PFC expresses high levels of $5HT_{2A}$Rs, and is also a key region implicated in schizophrenia pathogenesis in humans. We therefore dissected this region to compare receptor levels in *Egr3*$^{-/-}$ and WT using radioligand binding with [^{3}H]ketanserin, a selective $5HT_{2A}R$ ligand (Figure 6a). There was no change in the receptor binding affinity. Figure 6b shows that the maximum number of $5HT_{2A}R$/[^{3}H]ketanserin binding sites is reduced by nearly 70% in the prefrontal cortex of *Egr3*$^{-/-}$ mice compared with WT controls ($F(2,170) = 14.77$; $p < 0.001$, Student's *t*-test).

Egr3$^{-/-}$ mice also displayed a decreased behavioral response to DOI (1 mg/kg), a $5HT_{2A}R$ agonist that produces a distinctive head-twitch response in WT mice (Darmani *et al*, 1990; Gonzalez-Maeso *et al*, 2003) (Figure 6c). The two-way ANOVA revealed a main effect of treatment ($F(1,20) = 33.1$; $p < 0.001$) and a treatment by genotype interaction ($F(1,20) = 9.1$; $p < 0.01$). In summary, these results demonstrate a functional deficit of membrane-bound $5HT_{2A}$Rs in the PFC of *Egr3*$^{-/-}$ mice.

DISCUSSION

The aim of this study was to elucidate the mechanism underlying our previously published observation that *Egr3*$^{-/-}$ mice are resistant to the locomotor suppression produced in WT mice by clozapine, a uniquely effective antipsychotic medication that remains one of the leading treatments for schizophrenia (Gallitano-Mendel *et al*, 2008; Kane *et al*, 1988). In addition, this study simultaneously addressed our larger objective, to identify a downstream effector of *Egr3*. Such a gene would be a potential next step in our hypothesized biological pathway influencing schizophrenia susceptibility.

In the past decade, human genetics studies have identified numerous genes that are associated with risk to develop schizophrenia, a severe mental illness that affects 1% of the world's population. However, any individual gene is only able to account for a small percentage of illness risk (Allen *et al*, 2008; Owen *et al*, 2010). One way to unite multiple candidate genes conceptually is to identify those which act in a common biological pathway. *EGR3* is an immediate-early gene transcription factor that is regulated downstream of three major proteins implicated in schizophrenia susceptibility (neuregulin 1; (Hippenmeyer *et al*, 2002;

Table 2 Cloned Receptor Binding Affinities (in nM) of Study Drugs

	5-HTT	5-HT1A	5-HT1B	5-HT1D	5-HT1E	5-HT2A	5-HT2B	5-HT2C	5-HT3	5-HT5A	5-HT6	5-HT7	A1A	A1B	A2A	A2B	A2C	M1	M2	M3	M4	M5	D1	D2	D3	D4	D5	H1	H2	H4	NET
Antipsychotics																															
Clozapine[a]	1624.0	105.0	398.0	2132.0	966.0	13*		29*	241.0	3857.0	17.0	18.0	1.6	7.0	142.0	27.0	34.0	14.0	14.0	25.0	29.0	94.0	189.0	431.0	646*	39.0	235.0	2.0	153.0	820.0	3168.0
Chlorpromazine[a]	1296.0	3115.0	1489.0	452.0	344.0	3.2*		26*	977.0	118.0	12.0	21.0	0.3	0.8	184.0	28.0	46.0	47.0	433.0	47.0	151.0	18.0	112.0	2.0	5*	24.0	133.0	0.2	174.0	5048.0	2443.0
Haloperidol[a]	3256.0	1202.0	165.0	7606.0	> 10 000	73*		> 10 000*	> 10 000	2247.0	3666.0	378.0	12.0	8.0	1130.0	480.0	550.0	> 10 000	> 10 000	> 10 000	> 10 000	657.0	83.0	2.0	12*	15.0	147.0	3002.0	1003.0	> 10 000	2112.0
Olanzapine[a]	3676.0	2063.0	509.0	1582.0	2408.0	3*		24*	202.0	1212.0	6.0	105.0	109.0	263.0	314.0	82.0	29.0	24.0	79.0	51.0	998.0	9.0	58.0	72.0	63*	19.0	90.0	4.9	44.0	> 10 000	> 10 000
Quetiapine[a]	> 10 000	431.0	1109.0	> 10 000	2402.0	366*		1500*	> 10 000	3120.0	1864.0	308.0	22.0	39.0	3630.0	747.0	29.0	858.0	1339.0	1943.0	542.0	1942.0	712.0	567.0	483*	1202.0	1738.0	7.5	> 10 000	> 10 000	> 10 000
Ziprasidone[a]	112.0	76.0	4.0	9.0	1279.0	2.8*	27.2	68*	> 10 000	291.0	61.0	6.0	19.0	9.0	160.0	48.0	77.0	> 10 000	> 10 000	> 10 000	> 10 000	> 10 000	30.0	4.0	17*	105.0	152.0	130.0	3500.0	> 10 000	44.0
H1 antagonists																															
Diphenhydramine[b]																		100.0	120.0	229.0	112.0	260.0						9.6	> 10 000	> 10 000	
Promethazine[b]																												0.24		150.2	
Pyrilamine[b]		> 10 000																										2.5**			
5-HT2A antagonists																															
ACP-103[c]						0.13																									
Ketanserin[b]		2089.0	4350.0	111.0	> 10 000	1.3	217.0	47.9		2800.0	794.3					199.0							190.0	> 10 000			2500.0	1.8			
MDL-11939[b]	2598.0	3416.0	70.5*			6.1	1419.0	263.0		3416.0	2752.0		1876.0	1579.0	1586.0		1186.0						327.2			3934.0		1604.0			> 10 000

Antipsychotics, histamine H1 antagonists, and 5HT$_{2A}$R antagonists and inverse agonist used in pharmacological dissection studies. All data were collected from the NIMH Psychoactive Drug Screening Program's Ki Database (http://pdsp.med.unc.edu/). Owing to large gaps and inconsistencies in reported binding affinities in rodent studies, the table primarily summarizes values obtained with human tissue. Unless marked, values were determined in human receptors (cloned). Cells that are empty indicate no information available. [a]K_i values for antipsychotic drugs were obtained from PDSP website (Roth, 2008) and are PDSP certified values. [b]K_i values were obtained from PDSP website, but PDSP Certified values were not available. Values listed represent the lowest reported value on the PDSP website. [c]K_i reported from Vanover *et al* (2006).

*K_i values determined in rat receptors (cloned).

**K_i values were determined in human brain tissue.

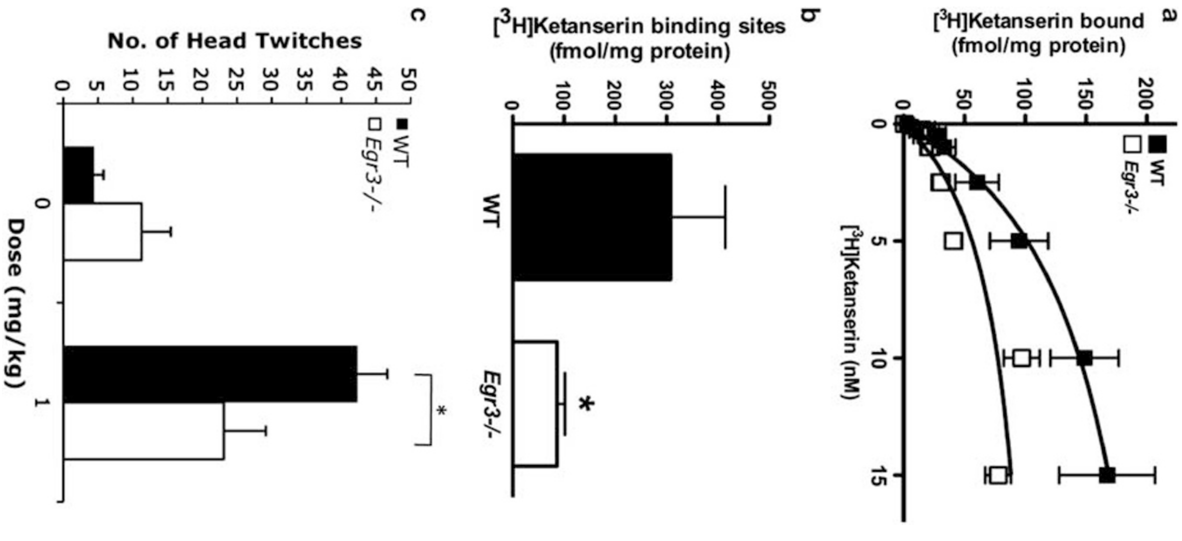

Figure 6 Serotonin 2A receptor (5HT$_{2A}$R) levels are decreased in the prefrontal cortex (PFC) of *Egr3*-deficient (*Egr3*$^{-/-}$) mice. (a) [^{3}H]Ketanserin binding saturation curves in the frontal cortex of WT (black) and *Egr3*$^{-/-}$ (white) mice ($n = 8$ per group). There was no change in binding affinity. (b) Maximum number of binding sites (B_{max}) for [^{3}H]ketanserin obtained from individual saturation curves. (c) *Egr3*$^{-/-}$ mice display a reduced behavioral response to 5HT$_{2A}$R hallucinogenic agonist 1-(2,5-dimethoxy 4-iodophenyl)-2-amino propane (DOI). Head-twitch responses were recorded for 30 min post-administration of DOI (1 mg/kg) or vehicle (*$p < 0.0001$, Student's *t*-test).

Jacobson *et al*, 2004; Stefansson *et al*, 2002), *N*-methyl D aspartate receptors (NMDARs) (Olney *et al*, 1999; Yamagata *et al*, 1994), and calcineurin (Mittelstadt and Ashwell, 1998; Yamada *et al*, 2007)), and was recently identified as the central gene in a network of transcription factors and microRNAs implicated in schizophrenia susceptibility (Guo *et al*, 2010). Moreover, *EGR3* is, itself, associated with schizophrenia (Kim *et al*, 2010; Yamada *et al*, 2007), and expressed at reduced levels in post-mortem brain tissue from schizophrenia patients (Mexal *et al*, 2005; Yamada *et al*, 2007). These findings suggest the need for further investigations of a potential role for *EGR3*, and the biological pathway of genes in which it functions, in psychotic disorders.

Our 'pharmacological dissection' approach proved successful in revealing that $5HT_{2A}$R-specific antagonists parallel the activity of clozapine in suppressing the locomotor activity of WT mice at dosages that fail to reduce the activity of $Egr3^{-/-}$ mice below normal WT activity levels. This is not due to the basal hyperactivity of $Egr3^{-/-}$ mice, as FGAs haloperidol (previously reported) and chlorpromazine (Figure 2) suppress the locomotor activity of $Egr3^{-/-}$ mice at the same dosage as WT mice.

We hypothesized that a defect in the function of $5HT_{2A}$Rs in the $Egr3^{-/-}$ mice could explain their differential sensitivity to $5HT_{2A}$R antagonists. Indeed, receptor binding studies using the $5HT_{2A}$R-selective ligand ketanserin revealed a nearly 70% reduction in $5HT_{2A}$R activity in the PFC of $Egr3^{-/-}$ mice (Figure 6a and b). This reduction of receptors corresponded with the results of a functional assay, the head-twitch response to the $5HT_{2A}$R agonist DOI (figure 6c), a drug-induced behavior that is absent in $5HT_{2A}$R knockout mice. Thus, it appears that the reduced sensitivity of $Egr3^{-/-}$ mice to the locomotor suppressive effects of clozapine and other SGAs may be a result of decreased levels of $5HT_{2A}$Rs in the brains of the mice. Further investigation of the relative affinities of the FGAs and SGAs tested (using values from the PDSP website (Roth, 2008) and Table 2) indicated that the ratio of $5HT_{2A}$R to D2R binding affinity best correlated with the locomotor inhibitory response of $Egr3^{-/-}$ mice.

Findings in our animal model are notable as numerous studies have identified deficits in $5HT_{2A}$R levels in the brains of schizophrenia patients (Dean and Hayes, 1996; Erritzoe *et al*, 2008; Garbett *et al*, 2008; Hurlemann *et al*, 2008; Lopez-Figueroa *et al*, 2004; Matsumoto *et al*, 2005; Ngan *et al*, 2000; Rasmussen *et al*, 2010; Serretti *et al*, 2007). Moreover, the *HTR2A* gene, which encodes the $5HT_{2A}$R, is a leading candidate schizophrenia gene (Allen *et al*, 2008). Thus, the deficit of $5HT_{2A}$R in $Egr3^{-/-}$ mice suggests a possible mechanism through which *EGR3* (itself a candidate schizophrenia gene) may influence susceptibility to this mental illness. Furthermore, this finding suggests that the $5HT_{2A}$R may act downstream of *EGR3* in what we hypothesize to be a biological pathway of genes influencing schizophrenia risk.

Insights into the Mechanisms of SGA-Induced Locomotor Suppression

To date, the precise mechanism by which clozapine exerts its antipsychotic effects remains unclear. Similarly, the etiology of side effects, such as sedation and weight gain, are also uncertain. Despite this, the sedating effect of clozapine has frequently been attributed to antagonism of H1 histamine receptors (Casey, 1997; Mengod *et al*, 1996; Stahl, 2008). Our results suggest that this is not the case in C57BL/6 mice, as selective H1 antagonists fail to reduce WT locomotor activity even at the highest doses reported used in mice in the literature (Figure 1 and Table 1) (Parsons and Ganellin, 2006; Shishido *et al*, 1991). These findings suggest the possibility that selective antagonism of H1 receptors may not be the mechanism responsible for the sedating effect of SGAs in humans either.

However, it is possible that sedation in humans may differ from the locomotor suppression we see in mice. Species differences in the molecular regulation of psychoactive medications have been reported (Gershon *et al*, 2011). Further investigation in primates and humans are needed to assess whether these results translate across species. Alternatively, H1 antagonism may have a different effect in combination with drug activity at other receptors than it does alone. In fact, studies investigating low-dose administration of psychiatric medications are aimed at determining the relative influence of H1 receptors *vs* other receptors involved in brain activation, in the sedating characteristics of these medications (Casey (1997) and references therein).

Instead, our findings suggest that the locomotor suppressive effect of SGAs in mice may result, at least in part, from the binding of these medications to $5HT_{2A}$Rs. Our data demonstrate that drugs which selectively target this receptor, ketanserin and MDL-11939 (Figure 5), parallel the effect of clozapine, and other SGAs, on $Egr3^{-/-}$ mice. They suppress locomotor activity in WT mice at dosages that partially or completely reverse the hyperactivity of $Egr3^{-/-}$ mice, but do not reduce their activity below that of vehicle-treated WT mice. However, although these agents show a similar divergence in their locomotor suppressive effects on WT and $Egr3^{-/-}$ mice as do the SGAs, they do not suppress the locomotor activity of WT mice to the same degree as the SGAs, which block movements in a 1 h test to nearly zero. Thus, although the reduction in PFC $5HT_{2A}$Rs may be sufficient to reduce sensitivity of $Egr3^{-/-}$ mice to the locomotor suppressive effects of SGAs, the blockade of other receptors in combination with $5HT_{2A}$Rs may be contributing to the activity-suppressing effects of SGAs in WT mice. Finally, the possibility that the $5HT_{2A}$R may contribute to the sedating effects of SGAs in humans is less surprising when one considers that $5HT_{2A}$R-specific antagonists and inverse agonists are being investigated by the pharmaceutical industry as sleep aids (Teegarden *et al*, 2008).

Our findings are consistent with those of McOmish and colleagues (2010), who recently reported that $5HT_{2A}R^{-/-}$ mice show the same resistance to the locomotor suppression produced in WT mice by clozapine that we previously reported in $Egr3^{-/-}$ mice. Using a conditional regional rescue of $5HT_{2A}$R function, they demonstrated that this response results from loss of $5HT_{2A}$Rs in the cortex, and is not caused by receptor loss in the striatum. This suggests that the reduction in $5HT_{2A}$Rs we have identified in the cortex of $Egr3^{-/-}$ mice is, indeed, responsible for their differential response to clozapine and other SGAs, compared with FGAs.

Our study does not address whether the loss of $5HT_{2A}$R expression may contribute to other phenotypes that the $Egr3^{-/-}$ mice display, including schizophrenia-like behavioral abnormalities (Gallitano-Mendel *et al*, 2007; (Gallitano-Mendel *et al*, 2008). However, the nearly 70% reduction in cortical $5HT_{2A}$Rs does not appear to disrupt the effectiveness of clozapine, as we have previously reported that chronic clozapine is able to reverse the aggressive behavior of $Egr3^{-/-}$ mice (Gallitano-Mendel *et al*, 2008). This is consistent with a recent report by Yadav and co-workers (2011), which demonstrated that post-synaptic $5HT_{2A}Rs$ are not essential for clozapine's ability to reverse phencyclidine-induced disruption of sensory–motor gating in mice, an NMDAR hypofunction animal model of schizophrenia.

FGA and SGA Antipsychotics

Inspection of the binding profiles of the FGAs and SGAs, as shown in Table 2, does not reveal a single receptor that can explain the differential susceptibility of $Egr3^{-/-}$ mice to the locomotor suppressive actions of these two classes of drugs. Like SGAs, the FGAs also bind to $5HT_{2A}Rs$. In fact, the affinity of chlorpromazine for the $5HT_{2A}R$ is nearly identical to that of olanzapine and ziprasidone and, according to the PDSP source (Roth, 2008), is greater than that of clozapine. As noted earlier, the ratio of $5HT_{2A}R$ to dopamine D2 receptor affinities appears to most closely align with the differential susceptibility of $Egr3^{-/-}$ mice to locomotor suppressive effects of these drugs. Notably, Meltzer and co-workers (1989, 2003) have hypothesized that the 5HT2AR : D2R ratio is the main characteristic that distinguishes the FGAs from SGAs.

Despite the importance of SGAs, which became first-line treatments for schizophrenia from the late 1990s to early 2000s, there is no simple experimental assay for distinguishing FGAs from SGAs. Such an assay would be beneficial for screening novel candidate molecules for antipsychotic characteristics (Geyer and Ellenbroek, 2003). One screening test has been reported, but it involves extensive behavioral training of animals followed by multiple pharmacological interventions, and is thus difficult and time-intensive (Philibin *et al*, 2005). Our finding that the behavioral response of $Egr3^{-/-}$ mice appears to distinguish SGAs from FGAs suggests that these mice may provide a rapid assay for this purpose.

Further work is needed to identify the etiology of the reduced PFC $5HT_{2A}R$ binding in $Egr3^{-/-}$ mice. In particular, studies aimed at identifying whether the dysfunction is at the level of protein localization or translation, or gene expression, must be undertaken. These studies are challenging as antibodies against the $5HT_{2A}R$ have been notoriously poor for immunohistochemical and western blot methods. While there has been some recent progress in this area, they still provide poor anatomical resolution (Weber and Andrade, 2010) and are less sensitive than radiological receptor binding assays. As *Egr3* is a transcription factor, it is intriguing to hypothesize that it may directly regulate expression of the *Htr2a* gene. However, as an immediate-early gene, *Egr3* expression is stimulus-dependent and its basal expression is low. We have found that systematic induction of *Egr3* expression is necessary to identify putative target genes. These studies are beyond the scope of the current report, but are important areas for future investigation to identify the mechanism by which *Egr3* influences this important receptor.

ACKNOWLEDGEMENTS

We are grateful to L Muppana, MS, for animal colony maintenance and technical assistance; to W Rodriguez for experimental assistance; to B Appelhans, PhD, Rush University Medical Center, for statistics consultation; to H Meltzer, MD, Northwestern University, for his donation of ACP-103 and his advice throughout the project; and to both H Meltzer, MD, and D Kupfer, MD, University of Pittsburgh, for their critical reading of the manuscript. Ki determinations, receptor binding profiles, and agonist and/or antagonist functional data, were generously provided by the National Institute of Mental Health's Psychoactive Drug Screening Program, Contract No. HHSN-271-2008-00025-C (NIMH PDSP). The NIMH PDSP is Directed by Bryan L Roth MD, PhD at the University of North Carolina at Chapel Hill and Project Officer Jamie Driscol at NIMH, Bethesda MD, USA. This work was supported by a NARSAD/Sidney R Baer Jr Foundation Young Investigator Award (to ALG), an Arizona Biomedical Research Commission grant (to ALG), and by NIH Grant R01 MH084894 (to JGM). Additional support from Science Foundation Arizona (to AAW), and from the Howard Hughes Medical Institute through the Undergraduate Science Education program and the School of Life Sciences at Arizona State University, is gratefully acknowledged.

DISCLOSURE

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)