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The Presynaptic Component of the Serotonergic System is Required for Clozapine's Efficacy

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Clozapine, by virtue of its absence of extrapyramidal side effects and greater efficacy, revolutionized the treatment of schizophrenia, although the mechanisms underlying this exceptional activity remain controversial. Combining an unbiased cheminformatics and physical screening approach, we evaluated clozapine's activity at >2350 distinct molecular targets. Clozapine, and the closely related atypical antipsychotic drug olanzapine, interacted potently with a unique spectrum of molecular targets. This distinct pattern, which was not shared with the typical antipsychotic drug haloperidol, suggested that the serotonergic neuronal system was a key determinant of clozapine's actions. To test this hypothesis, we used *pet1*$^{-/-}$ mice, which are deficient in serotonergic presynaptic markers. We discovered that the antipsychotic-like properties of the atypical antipsychotic drugs clozapine and olanzapine were abolished in a pharmacological model that mimics NMDA-receptor hypofunction in *pet1*$^{-/-}$ mice, whereas haloperidol's efficacy was unaffected. These results show that clozapine's ability to normalize NMDA-receptor hypofunction, which is characteristic of schizophrenia, depends on an intact presynaptic serotonergic neuronal system.

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INTRODUCTION

Schizophrenia, a devastating illness affecting nearly 1% of the world's population, appears to be a genetically heterogeneous disorder with no single major risk allele (Purcell *et al*, 2009; Shi *et al*, 2009; Stefansson *et al*, 2009; Stefansson *et al*, 2008). This extreme genetic heterogeneity will make it difficult, if not impossible, to develop new medications that specifically target the presumably dysregulated pathways. Despite the polygenic nature of the illness, however, the symptoms of schizophrenia can be reduced, to various degrees of efficacy, by antipsychotic medications (Lieberman *et al*, 2005). Of the various medications currently available to treat schizophrenia, clozapine is uniquely effective (Kane *et al*, 1988). Indeed, clozapine—a drug discovered >50 years ago (Hippius, 1999)—has long been known to effectively treat schizophrenia without associated extrapyramidal symptoms (EPS) (Angst *et al*, 1971). Clozapine has also been demonstrated to be uniquely effective in reducing suicidality (Meltzer *et al*, 2003; Meltzer and Okayli, 1995). Clozapine has a number of serious side effects, including a nearly 1% risk of fatal agranulocytosis (Senn *et al*, 1977), limiting its use to treatment-resistant or treatment-intolerant patients. The discovery of clozapine's characteristic ability to treat schizophrenia without inducing EPS led to the development of the so-called 'atypical' antipsychotic drugs, which are now among the most widely prescribed neuropsychiatric medications (Lieberman *et al*, 2005).

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For many years, it has been appreciated that clozapine and related atypical antipsychotics have extraordinarily robust pharmacological profiles, with potent interactions at a large number of mainly postsynaptically localized biogenic amine receptors, including several serotonin (Meltzer *et al*, 1989; Peroutka *et al*, 1981; Roth *et al*, 1992; Roth *et al*, 1994), dopamine (Creese *et al*, 1976; Van Tol *et al*, 1991), adrenergic (Enna *et al*, 1976), and muscarinic (Bolden *et al*, 1992; Miller and Hiley, 1974) G-protein-coupled receptors (GPCRs) (Roth *et al*, 2004). It is currently unknown, and the subject of considerable controversy, which, if any, of these pharmacological properties are essential for either the unique spectrum of efficacy or the unusual side-effect profile of clozapine. One aspect of their actions that has recently become clear, however, is the observation that only clozapine and the closely related atypical antipsychotic drugs are effective in genetic and pharmacological models that mimic the NMDA-receptor hypofunction of schizophrenia (Abbas *et al*, 2009; Bakshi *et al*, 1994; Belforte *et al*, 2010; Mohn *et al*, 1999) Without a clearer understanding of the molecular and neuronal substrates responsible for these actions, it will remain difficult, if not impossible, to develop new antipsychotic drugs with either improved efficacies or fewer side effects (Conn and Roth, 2008).

To discover the molecular and neuronal determinants of clozapine's actions, we utilized our unbiased chemical, biological, and cheminformatic approaches to deconstruct clozapine's molecular and cellular actions (Armbruster and Roth, 2005; Keiser *et al*, 2007; Keiser *et al*, 2009). We discovered that clozapine and the related atypical antipsychotic drug olanzapine, but not haloperidol, displayed a unique spectrum of activity at selected molecular targets. This pattern of activity suggested to us that the serotonin neuronal phenotype was an essential mediator of clozapine's actions. The ETS-domain transcription factor *pet1* has recently emerged as a key determinant of the serotonergic phenotype (Hendricks *et al*, 1999; Hendricks *et al*, 2003; Jensen *et al*, 2008) of neurons, and *pet1*$^{-/-}$ mice almost completely lack the presynaptic component of the serotonergic phenotype, including tryptophan hydroxylase 2 (Tph2), the serotonin transporter (SERT), the vesicular monoamine transporter 2, and aromatic amino acid decorboxylase in dorsal raphe neurons of adult KO mice. These mice express only 20% of the adult level of 5-HT (Hendricks *et al*, 2003). We discovered that genetically induced deletion of the presynaptic component of the serotonergic neuronal system selectively abolished the ability of clozapine to normalize NMDA-receptor hypofunction in mouse pharmacological models of schizophrenia. Importantly, genetic deletion of the main postsynaptic receptor implicated in clozapine's actions (HTR2A) did not abolish clozapine's therapeutic actions. These results demonstrate an unexpectedly essential role for the presynaptic component of the serotonin neuronal system for clozapine's therapeutic actions.

MATERIALS AND METHODS

Mice

A detailed description of how the *pet1*$^{-/-}$ mice were generated is provided elsewhere (Hendricks *et al*, 2003). All experiments were approved by the institutional animal care and use committee at the University of North Carolina, Chapel Hill. Mice were housed under standard conditions—12 h light/dark cycle and food and water *ad libitum*.

Radioligand Binding Assays

For saturation binding assays, brain regions were rapidly microdissected, frozen on dry ice, and then stored at −80 °C. A Tissue TearorTM (BioSpec Products) was used to homogenize tissue (15 s, 15 000 r.p.m.) in ice-cold standard binding buffer (SBB—50 mM Tris-HCl, pH 7.4; 10 mM $MgCl_2$; 0.1 mM EDTA). Homogenized tissue was centrifuged for 20 min at 27 000 *g* (4 °C); crude membrane pellets were collected and washed two more times in a total of 10 ml of SBB. After the last wash, the membrane pellet was either used immediately for binding, or stored at −80 °C until use. Saturation binding assays were performed with the homogenized brain tissue and [^{3}H]-ketanserin (5-HT_{2A}; cortex) or [^{3}H]-WAY100635 (5-HT_{1A}, cortex, midbrain, and hippocampus), and then incubated in SBB for 1.5 h as detailed (Abbas *et al*, 2009). Nonspecific binding was determined by incubating the reactions with 10 μM ritanserin (5-HT_{2A}) or 10 μM buspirone (5-HT_{1A}). Reactions were harvested by vacuum filtration through glass filters (3 × ice-cold 50 mM Tris, pH 7.4; pH 6.9 at room temperature) and measured by liquid scintillation using a Perkin-Elmer Tri-Carb 2800TR scintillation counter. Nonlinear saturation analysis was done using Graphpad Prism 4.01 to obtain B_{max} values, and Bradford protein assays were performed in order to normalize B_{max} determinations to the amount of protein in each assay.

Western Blotting

DOI (1-(4-iodo-2,5-dimethoxyphenyl)propan-2-amine hydrochloride) or vehicle was injected intraperitoneally (i.p.) with light restraint to minimize stress effects, and mice were killed 15 min later by cervical dislocation. Microdissection was performed on ice as quickly as possible. For detection of pERK1/2, tissue was homogenized in 300 μl of ice-cold SBB plus protease and phosphatase inhibitor cocktails (Roche Diagnostics, Complete, no. 11697498001; PhosSTOP, no. 04906845001) and 5% glycerol, and centrifuged for 10 min at 20–25 000 *g* and 4 °C. Then, 25–30 μg of protein from the supernatant was used to measure phospho- and total ERK1/2 using 10% SDS-PAGE followed by immunoblotting. The following antibodies were used for western blots in a 1 : 1000 dilution: rabbit polyclonal anti-p-ERK1/2 antibody (9101L, Cell Signaling), and rabbit polyclonal anti-ERK1/2 antibody (9102L, Cell Signaling). For 5-HT_{2A} receptor immunoblotting, frontal cortex crude membrane preparations were resuspended in cold lysis buffer (50 mM Tris-Cl, pH 7.4; 150 mM NaCl, 10% glycerol, 1% NP-40, 0.5% Na-deoxycholate, 0.5% CHAPS, plus protease inhibitor cocktail (Roche Diagnostics) and incubated on ice for 1 h. Detergent-soluble proteins were collected after 20 min of centrifugation at 4 °C and 12 500 *g*, and 500 μg (1.0 mg/ml) of protein was incubated with 25–30 μl (packed volume) of wheat germ agglutinin (WGA)-conjugated agarose beads (Vector Technology) for a minimum of 2 h at 4 °C on a rotary mixer. After three

washes with 500 µl of lysis buffer, WGA-bound proteins were eluted with 40 µl of 1 × SDS-sample buffer, and whole eluates were resolved by SDS-PAGE. Rabbit polyclonal anti-5-HT_{2A} receptor antibody (Neuromics, Edina, MN) was used in a 1 : 500 dilution for 12 h at 4 °C for measurement of 5-HT_{2A} receptor protein. We have previously verified the specificity of the anti-5-HT_{2A} receptor antibody using 5-HT_{2A} WT and KO mice cortex tissue by immunoblotting (Magalhaes *et al*, 2010)

Head-Twitch Response

As previously detailed (Abbas *et al*, 2009), mice were injected i.p. with 0.5–5.0 mg/kg of DOI. Head twitches were counted simultaneously by two observers (live, not on recordings), one of whom was blinded to the genotype, and recorded in 5-min bins for a total period of 30 min immediately after injection.

Whole-Cell Recording in CA1 Pyramidal and Dorsal Raphe Neurons

Adult *pet1*$^{-/-}$ and *pet1*$^{+/+}$ littermate mice were decapitated, and their heads placed into a beaker containing ice-cold oxygenated artificial cerebrospinal fluid (aCSF: (in mM) sucrose 248, KCl 3.0, NaH_2PO_4 1.25, $MgSO_4$ 2.0, $CaCl_2$ 2.5, dextrose 10, and $NaHCO_3$ 26) for approximately 10 min. Following this, the brain was removed, 200 µm vibratome slices of the hippocampus or the midbrain were made and placed in a holding chamber in a water bath (36 °C) for 1 h, and then maintained at room temperature until used. Single slices were placed in a recording chamber (Warner Instruments, Hamden, CT), and continuously perfused with aCSF (NaCl 124 mM replaced sucrose) at 2 ml/min at 32 °C maintained by an in-line solution heater (SH-27B, Warner Instruments). Neurons were visualized using a Nikon E600 (Optical Apparatus, Ardmore, PA) upright microscope fitted with a × 60 water-immersion objective, differential interference contrast (DIC) optics, and an infrared filter (IR). Recording pipettes were fashioned on a P-97 micropipette puller (Sutter Instruments, Novato, CA) using borosilicate glass capillary tubing (1.2 mm OD, 0.69 mm ID; Warner Instruments). The electrolyte for recording cellular characteristics was (in mM): K gluconate 130, NaCl 5, $MgCl_2$ 1, Naphosphocreatinine 10, EGTA 0.02, HEPES 10, MgATP 2, Na_2GTP 0.5, and 0.1% Biocytin, pH 7.3. A visualized pyramidal cell in the CA1 subfield of the dorsal hippocampus or a raphe neuron from the ventro-medial subfield of the dorsal raphe was approached with the electrode, a giga Ω seal established, and the cell membrane ruptured to obtain a whole-cell recording using a multi-clamp 700B amplifier (Axon Instruments, Foster City, CA). Once the whole cell configuration was obtained, cell characteristics were recorded using current clamp techniques. The cellular characteristics were measured as previously described (Beck *et al*, 2004). Following the collection of cell characteristics, the pyramidal cell was returned to voltage clamp mode at −60 mV. After a stable baseline was established, 5-carboxyamidotryptamine (5-CT), a 5-HT_{1A} agonist, to a final concentration of 100 nM was added to the aCSF, and the change in current was measured. Once a steady-state response was established, the drug was removed from the aCSF and only one cell was recorded per slice. The access resistance and the input resistance were continuously monitored throughout the recording period to assure quality of the recordings. Recordings were abandoned if the access resistance was more than four times the resistance of the electrode, or if the input resistance changed by >15%. For the dorsal raphe neurons, current clamp recordings were obtained of the hyperpolarization induced by activation of the 5-HT_{1A} receptor by addition of 5-CT (100 nM) to the perfusion buffer. Unpaired *t*-tests were used to test for significance using the statistical package Prism.

Immunohistochemistry

Adult mice were anesthetized and transcardially perfused with 40 ml of phosphate-buffered saline (PBS), pH 7.4, followed by 4% paraformaldehyde (PFA) in PBS, pH 7.4. The brains were collected, postfixed overnight at 4 °C in 4% PFA, followed by dehydration in a 30% sucrose (w/v) solution in PBS overnight at 4 °C. Brain sections (30 µm coronal sections) were permeabilized for 1 h with 0.4% Triton X-100 in PBS, and were blocked for 2 h at room temperature in PBS containing 3% bovine serum albumin, 1% normal goat serum, and 0.4% Triton X-100. The slides were incubated with rabbit polyclonal anti-5-HT_{2A} antibody (1 : 250, Neuromics) in blocking buffer for 48 h at 4 °C, followed by addition of anti-MAP2 mouse monoclonal antibody (1 : 5000, Sigma), and further incubated overnight at 4 °C. Slides were washed five times with PBS containing 0.4% Triton X-100; after that, goat anti-rabbit AlexaFluor-488 and goat anti-mouse AlexaFluor-594 antisera (1 : 250, Invitrogen, Eugene, OR) and Hoechst 3342 (0.5 µg/ml) were applied to slides for 1 h at room temperature. Fluorescent images were collected on a Nikon 80i Research Upright Microscope (Nikon, Tokyo, Japan) equipped with Surveyor Software with TurboScan (Objective Imaging, Kansasville, WI). Tiled images were collected with a Qimaging Retiga-EXi camera (Qimaging, Surrey, BC, Canada). Separate images were collected for the 5-HT_{2A} receptor, nucleus (Hoechst), and MAP2 using either a × 10 or × 20 objective; these images were taken at 16-bit resolution (ie, 11 000 by 15 000 pixels). The relative distribution of the 5-HT_{2A} receptor was quantified using ImageJ* software (NIH). Raw images were corrected by subtracting the average intensity value of the background. Twelve regions of interest (ROIs), 200 by 200 pixels, were randomly selected for layer V and peripheral layers, the mean intensity was then measured for each ROI, and these were averaged for each brain section. The Hoechst and MAP2 staining were used as reference images.

Brain slice immunohistochemistry for biocytin and tryptophan hydroxylase was conducted to determine whether the neurons recorded from the *pet*$^{+/+}$ mice contained 5-HT as previously described (Beck *et al*, 2004). Slices from the *pet1*$^{-/-}$ mice were also processed to detect expression of the lacZ marker of 5-HT precursor neurons as previously described (Scott *et al*, 2005), but using chicken anti-β-galactosidase (1 : 1000, Abcam) and FITC-conjugated goat anti-chicken (1 : 250, Abcam) antibodies.

Prepulse Inhibition of Acoustic Startle

All prepulse inhibition (PPI) experiments were performed at the Mouse Behavioral Phenotyping Laboratory Core

Facility in the Neurodevelopmental Disorders Research Center of UNC using the SR-Lab equipment (San Diego Instruments) as previously described (Abbas *et al*, 2009). Briefly, mice were placed in a small Plexiglas cylinder housed within a large sound-proofed chamber (San Diego Instruments). Each session consisted of a 5-min habituation period followed by 74 trials of 3 types—(1) no stimulation, (2) 120 dB acoustic stimulus (AS50), and (3) four different prepulse stimuli ranging from 4 dB over background (PP68) to 16 dB over background (PP78). PPI of acoustic startle was measured in the presence of a 64-dB white-noise background. Mice were given three different types of trials. One trial type consisted of a 40 ms 120 dB white-noise startle stimulus. In the second, the startle stimulus was preceded by a 20-ms prepulse stimulus that was 4, 8, 12, or 16 dB above the white-noise background. In the third trial type, no auditory startle stimulus was presented, but only prepulse stimuli were presented, and these null trials served as controls for background movements by the animals. During testing, mice were acclimated to the apparatus for 5 min and then given 74 test trials. In 30 of the trials, the 120 dB stimulus was given alone (startle-only trials), whereas eight additional trials served as null trials. For the remaining 36 trials, the prepulse stimulus preceded the startle stimulus by 100 ms. The behavioral responses were measured as the peak startle response for each trial between 35 and 65 ms after the onset of the startle stimulus. Inhibition of the startle response was calculated for each intensity of prepulse as the ratio of the prepulse trials to the startle-only trials subtracted from 1 and expressed as a percentage inhibition of response, that is, % PPI = [1–(prepulse trials/startle-only trials)]*100. For pharmacological studies, *pet1*$^{-/-}$ mice and WT littermates were injected i.p. 30 min before PPI testing with vehicle (saline), clozapine (0.5 or 1.0 mg/kg), and olanzapine (0.5 mg/kg).

Locomotor and Stereotypic Activity

Locomotor activity was assessed in photocell-based activity chambers under standardized environmental conditions, using an AccuScan activity monitor (AccuScan Instruments, Columbus, OH) with a 25.8 × 25.8 cm Plexiglas chamber and a beam spacing of 1.52 cm as described (Abbas *et al*, 2009). Mice were injected i.p. with vehicle, haloperidol (0.1 mg/kg), and clozapine (0.5 or 1.0 mg/kg) 30 min before PCP (7.0 mg/kg), followed by placement in the activity chambers. Activity data were collected for each mouse over 60 min, beginning when the mouse was first placed in the testing chamber. Horizontal activity was measured as the total distance covered in centimeters as the total of all vectored X-Y coordinate changes. Stereotypy data were also collected in this automated fashion and calculated by these software packages based on contiguous breaks of the same single beam or a set of beams. Data are presented as total stereotypy counts, that is, number of repetitive beam breaks during 60 min of activity monitoring.

Ligand Sets for SEA

We extracted ligand sets from databases that annotate molecules by therapeutic or biological category (Keiser *et al*, 2009). For instance, the 2006.1 MDL Drug Data Report (MDDR) contains 518 molecules annotated as α1 adrenergic receptor blockers, which we grouped into a single 'α1 adrenergic blocker' set. Three chemical databases provided these ligand sets: the ChEMBL 2009 (available from EMBL-EBI, http://www.ebi.ac.uk/chembldb), the World of Molecular Bioactivity (WOMBAT) 2006.2 (Olah *et al*, 2004; Oprea *et al*, 2007), and the MDDR 2006.1 (MDL, now provided by Symyx) databases. For the ChEMBL and WOMBAT data sets, we organized ligands by their affinities, representing each protein target by three sets at 1 and 10 μM cutoffs (as well as an additional 100 μM cutoff for WOMBAT).

Similarity Ensemble Approach (SEA)

Clozapine, olanzapine, and haloperidol were computationally screened via SEA against a panel of 3725 protein targets. Each target was represented solely by its set of known ligands. We used both the 1024-bit folded ECFP4 (Hert *et al*, 2008) and 2048-bit daylight (James *et al*, 1992) fingerprints for SEA, as separate screens, and accepted the highest-scoring predictions arising from either fingerprint. Because of the small size of the query data set (three drugs), all SEA predictions are reported here by *P*-values instead of by *E*-values (Keiser *et al*, 2009).

Estimates of Number of Targets Screened

We extracted 3725 targets for the cheminformatics screen from three databases: MDDR (246 targets), ChEMBL (2010 targets), and WOMBAT (1469 targets). However, there was appreciable target overlap across databases; we estimate that only 2250 of these targets are unique. To do so, we extracted accession numbers and UniProt IDs for each target and merged redundant targets via a synonym lookup table using UniProtKB/SwissProt and UniProtKB/TrEMBL database downloads (from http://www.uniprot.org, accessed 27 January 2010). We found 511 targets in common between ChEMBL and WOMBAT, leaving 1499 targets only found in ChEMBL and 536 targets only found in WOMBAT. Of the 536 WOMBAT-only targets, 296 did not have an accession number or UniProt ID; we excluded these from our counts to arrive at the lower-bounds estimate of 2250 unique cheminformatics targets. We determined by manual inspection that 162 of the 307 targets from the physical screen overlapped with the 2250-target cheminformatics screen. This yielded a final estimate of 2395 unique targets screened (Supplementary Table 3).

Kinase Profiling and Checkpoint Kinase 2 Assay

We profiled clozapine (10 μM) activity on a set of 20 different kinases using Caliper Profiler Pro kinase assays and Labchip EZ reader according to the manufacturer's instruction (Caliper Life Sciences, Hopkinton, MA). The CHK2 activity assay was carried out in opaque 96-well plates using Omnia Kinase Assay Kit (Invitrogen, KNZ1031). The assay was set up in triplicate in a final reaction volume of 20 μl per well containing 10 μM of peptide substrate, 1 mM DTT, and varying concentrations of test drug or water to measure maximal kinase activity. Thereafter, 18 mU of CHK2 enzyme protein (Invitrogen no.

PV3367) was added to each well and the plate was loaded immediately into a FlexStation II reader (Molecular Diagnostics) at 30 °C, mixed for 5 s, and read (excitation at 360 nm and emission at 485 nm) every 30 s for the next 60 min. Plateau readings at the 30 min point were processed and analyzed to calculate IC_{50} of tested compounds using GraphPad Prism 5.0.

Statistical Analysis

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

RESULTS

Clozapine Interacts with a Defined Subset of Molecular Targets

Because the molecular target(s) responsible for clozapine's actions remain controversial, we initially utilized an unbiased physical screening approach in which clozapine and selected typical and atypical antipsychotic drugs were profiled against all known molecular targets implicated in antipsychotic drug actions. The targets interrogated included both validated antipsychotic drug targets (eg, D2, D3 dopamine; 5-HT_{2A} serotonin) as well as most of the currently investigated targets (eg, M1, M4 muscarinic; mGluR2/3 metabotropic glutamate; nicotinic acetylcholine) (Conn and Roth, 2008; Gray and Roth, 2007; Jensen *et al*, 2008). We then performed hierarchical clustering analysis to identify atypical antipsychotic drugs that were most similar to clozapine and typical antipsychotic drugs that were most dissimilar. As shown in Figure 1a, olanzapine was the most similar atypical antipsychotic drug to clozapine, whereas haloperidol was the most dissimilar typical antipsychotic drug. We then chose clozapine, olanzapine, and haloperidol for further study.

The 'druggable genome,' of which the receptorome (Armbruster and Roth, 2005), the kinome (Manning *et al*, 2002), enzymes, and other 'druggable' targets (Hopkins and Groom, 2002) are part, is estimated to represent perhaps 2300 distinct molecular targets (Hopkins and Groom, 2002). Although it is not currently technically feasible to obtain the activity profile of clozapine at every conceivable druggable target, we have been able to achieve satisfactory coverage of these druggable targets falling into the GPCR, kinase, transporter, ion channel, and enzyme classes. To investigate whether there might be other likely targets, not available among these readily screenable targets, we used our newly validated computational SEA (Keiser *et al*, 2007; Keiser *et al*, 2009) to screen all three drugs against a panel of $\sim$2250 molecular targets, all of which have annotated ligands. These calculations also allowed us to investigate the similarities and differences in target association among the three drugs; we were particularly interested to see whether clozapine and olanzapine associated with similar targets, and whether these differed substantially from the targets with which SEA predicted haloperidol to associate (Supplementary Table 1). SEA predicted only one new target for clozapine, farnesyl protein transferase (PFTase, Supplementary Table 1). The SEA predictions suggested new targets for olanzapine, such as the 5-HT_{3A} serotonin receptor (HTR3A; likelihood of random association, *P*-value, 1.09×10^{-10}), the 5-HT_{3B} serotonin receptor (HatAR3B; *P*-value 1.18×10^{-8}), the H4 histamine receptor (HRH4; *P*-value 3.69×10^{-8}), and UDP-glucuronosyltransferase 1A4 (UGT1A4, *P*-value 1.63×10^{-18}; Supplementary Table 1). In each of these cases, the olanzapine-predicted target is a known target of clozapine that is neither predicted for nor known to interact with haloperidol. However, upon inspection, each of these targets is already reported to be a target for olanzapine in KiDB, whereas UGT1A4 is known to directly glucuronidate olanzapine (Linnet, 2002). Except for PFTase (*P*-value 2.46×10^{-5}), none of the predicted targets differs greatly from those that both drugs were already known to modulate.

We next physically interrogated a large portion of the druggable genome to determine if there could be additional targets for clozapine that were not predicted by SEA. Figure 1b shows the combined results obtained from our queries of internally derived physical screening campaigns from the NIMH-PDSP as well as the publicly available physical screening campaigns extracted from the PubChem database (see Supplementary Table 2 for the complete data set) at >300 target-based assays. As shown in Figure 1b and Supplementary Table 2, clozapine and olanzapine differed from haloperidol by virtue of their high-affinity interactions with most of the 14 human serotonin GPCRs and relatively lower affinity interactions with D2, D3, and D4 dopamine receptors. Furthermore, Figure 2a shows the lack of activity of clozapine at various protein kinases implicated in psychotherapeutic drug actions (eg, GSK3β, Erk1, Erk2, and Akt1) (Beaulieu *et al*, 2009; Beaulieu *et al*, 2008; Beaulieu *et al*, 2005). In passing, we note that clozapine was a moderately potent inhibitor of CHK2 (Figure 2b)—a kinase involved in cell cycle regulation (Matsuoka *et al*, 1998).

The Presynaptic Component of Serotonin Neurons Is Essential for Clozapine's Ability to Normalize the NMDA-Receptor Hypofunction Characteristic of Schizophrenia

The results of Figure 1 suggested to us that the most likely locus of clozapine's actions was engagement of the serotonergic neuronal system. Because many serotonin receptors are both pre- and post-synaptic with respect to serotonin neurons, it was not clear whether the requirement for serotonergic engagement would be pre- or post-synaptic, although current hypotheses posit a selective effect of clozapine on postsynaptic serotonin receptors (eg, HTR2A, HTR2C, HTR6, and HTR7; Conn and Roth, 2008; Roth *et al*, 2004). These hypotheses predict that deletion of the presynaptic component of the serotonin neuronal system would not affect clozapine's therapeutic actions. To test these hypotheses, we took advantage of the observation that the ETS-domain transcription factor *pet1* is essential for normal expression of the serotonergic phenotype in neurons, and that its deletion greatly

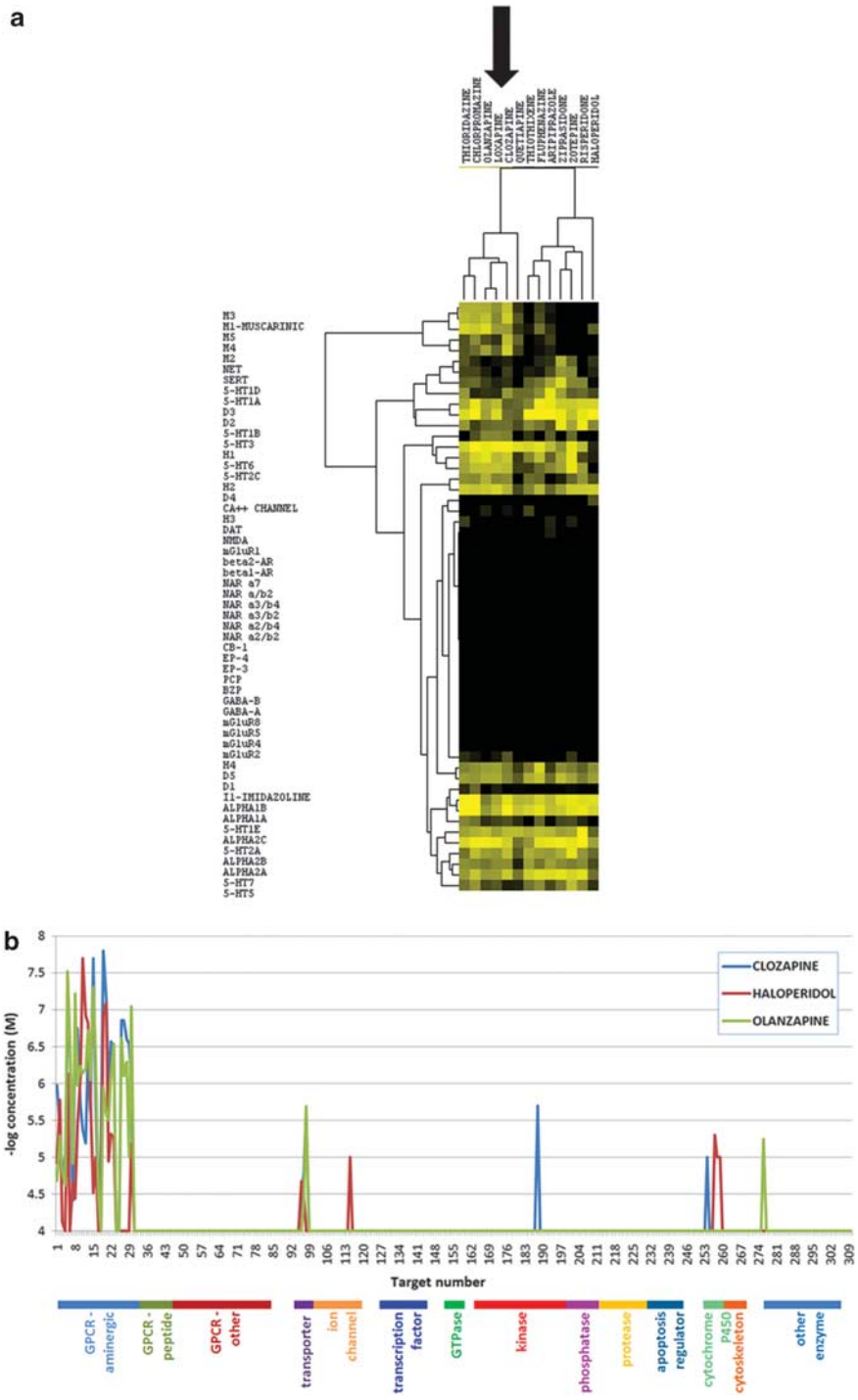


Figure 1 Mining the druggable genome identifies molecular targets for clozapine action. (a) Results from hierarchical clustering analysis of affinities of approved typical and atypical antipsychotic drugs at molecular targets implicated in antipsychotic drug action (Gray and Roth, 2007; Roth *et al*, 2004). See Supplementary Table 2 for complete data set. Arrow indicates location of node specifying clozapine and olanzapine. (b) Summary results from an interrogation of clozapine, olanzapine, and haloperidol at >300 distinct molecular targets (see Supplementary Table 2 for complete data set).

diminishes expression of presynaptic serotonergic markers (Hendricks *et al*, 2003; Jensen *et al*, 2008; Scott *et al*, 2005).

For initial studies, we examined the ability of clozapine to normalize the disruption of PPI induced by the noncompetitive NMDA-receptor antagonist phencyclidine (PCP). We chose PCP because it is known to induce a psychotic state that reliably mimics both the positive and negative symptoms of schizophrenia in humans (Javitt and Zukin, 1991). Disrupted PPI is also well established and validated as a measure of the impaired sensory-motor gating characteristic of schizophrenia (Geyer and Braff, 1987; Mansbach and Geyer, 1989). Importantly, atypical antipsychotic drugs like clozapine are unique in their ability to normalize PCP-induced disruption of PPI (Bakshi

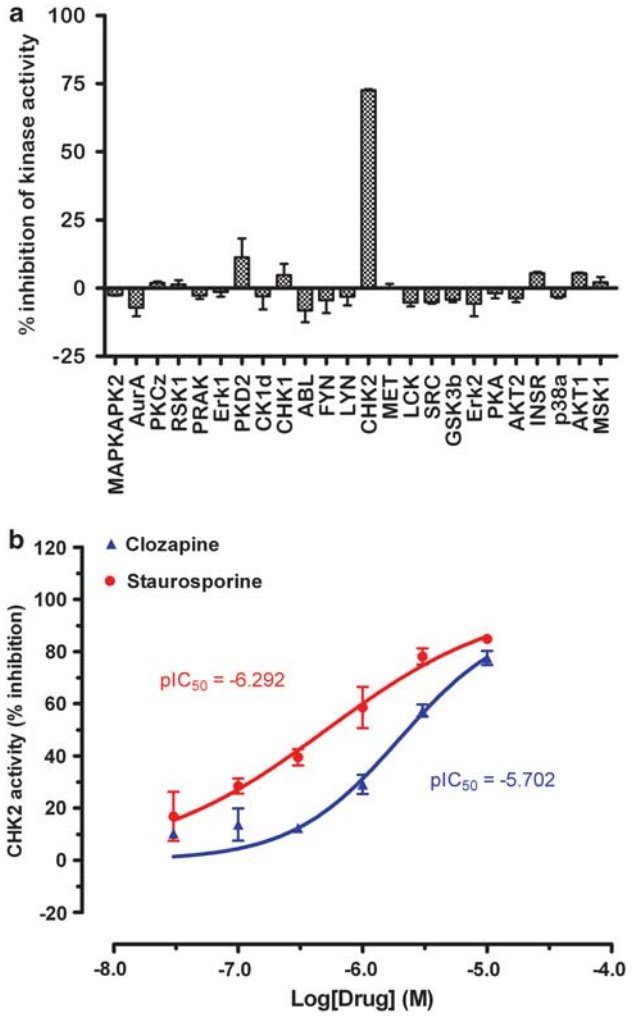


Figure 2 Kinase profiling reveals activity of clozapine only at checkpoint kinase 2 (ChK2). (a) Shown are results from a kinase profile performed with a Caliper microfluidics-based screening platform (part no. 760373) that comprises 20 different purified kinases. Clozapine (10 μM) was tested against all the kinases shown above and data are presented as % inhibition of kinase activity. (b) Dose-response studies with clozapine and the nonspecific kinase inhibitor staurosporine at Chk2. Data represent mean ± error of $N = 3$ separate determinations. EC_{50} values determined using GraphPad Prizm.

et al, 1994). As is shown in Figure 3a, acute administration of PCP induced a marked disruption of PPI in both *pet1*$^{-/-}$ and *pet1*$^{+/+}$ littermate control mice. Significantly, clozapine normalized PCP-disrupted PPI in *pet1*$^{+/+}$ but not in *pet1*$^{-/-}$ mice (Figure 3a). To determine if these results were unique to clozapine, we tested olanzapine as well, because it most closely mimics the polypharmacological profile of clozapine. As shown in Figure 3b, olanzapine normalized PCP-disrupted PPI in *pet1*$^{+/+}$ but not in *pet1*$^{-/-}$ mice. These results indicate that genetic deletion of *pet1* abolishes the ability of both clozapine and olanzapine to normalize NMDA-receptor hypofunctioning. Therefore, the actions of clozapine and olanzapine depend on the presynaptic component of the serotonergic neuronal system. We also examined the ability of clozapine to normalize the PCP-induced enhancement of locomotor activity and stereotypy. As shown in Figures 3c and d and 4, PCP induced a significant enhancement of locomotor behavior and stereotypical movements, which were normalized by clozapine in *pet1*$^{+/+}$ (Figure 3c) but not in *pet1*$^{-/-}$ (Figure 3d) mice. As an essential control, we show that the typical antipsychotic haloperidol normalized locomotor behavior and stereotypy in both *pet1*$^{+/+}$ and *pet1*$^{-/-}$ mice (Figures 3e, f and 4a)—a result predicted based on its relatively selectively potent interaction with D2-family dopamine receptors, in contrast to the profiles of clozapine or olanzapine, which more preferentially target 5-HT receptors.

Given these results, we wondered if genetic deletion of 5-HT_{2A} serotonin receptors, which have been proposed to mediate many of clozapine's unique actions (Meltzer *et al*, 1989), might phenocopy *pet1* deletion, at least with respect to clozapine's antipsychotic-like actions. Accordingly, we examined the ability of clozapine to normalize PCP-induced disruption of PPI in 5-$HT_{2A}^{+/+}$ and 5-$HT_{2A}^{-/-}$ mice. As shown in Figure 4b, clozapine normalized PCP-induced PPI disruption in both 5-$HT_{2A}^{-/-}$ and WT littermate control mice. We also noted that 5-$HT_{2A}^{+/+}$ and 5-$HT_{2A}^{-/-}$ mice were generated on the 129S background strain and displayed a diminished sensitivity to clozapine. We performed dose response with clozapine (data not presented) in these mice, and the data with the lowest effective doses of clozapine in 5-$HT_{2A}^{-/-}$ and WT littermates are shown in Figure 4b. These results indicate that postsynaptic 5-HT_{2A} receptors are not essential for mediating clozapine's ability to normalize PCP-induced disruption of sensory-motor gating *in vivo*.

Given these results, we wondered if one possible explanation for the inability of clozapine to normalize behavioral measures of NMDA-receptor hypofunction could be that genetic disruption of *pet1* has altered the cellular expression and/or sensitivity of postsynaptic 5-HT_{2A} serotonin receptors. To address this possibility, we measured cortical 5-HT_{2A} receptor protein levels via western blot, ^{3}H-ketanserin radioligand binding, and immunofluorescence analysis. We found equivalent amounts of 5-HT_{2A} receptor protein, radioligand binding (Figure 5a–c), and a similar cellular and subcellular distribution of 5-HT_{2A} receptors in *pet1*$^{+/+}$ and *pet1*$^{-/-}$ mice (Figure 5d and e). To determine if a change in postsynaptic 5-HT_{2A} receptor sensitivity was present in *pet1*$^{-/-}$ mice, we evaluated DOI-induced head-twitch and pERK responses in *pet1*$^{+/+}$ and *pet1*$^{-/-}$ mice, as previously described (Abbas *et al*, 2009). As can be seen in Figure 5f–h, DOI-induced responses were augmented in *pet1*$^{-/-}$ mice, as might be expected given the loss of presynaptic 5-HT innervations seen in *pet1*$^{-/-}$ mice. We also determined the 5-HT_{2C} expression level in hippocampus, as clozapine has affinity for this receptor, and found significantly increased 5-HT_{2C} expression in hippocampus of *pet1*$^{-/-}$ mice in comparison with WT littermate controls (Supplementary Figure 1). Furthermore, the antipsychotic-like properties of M100907 (5-HT_{2A} selective antagonist) and MK212 (5-HT_{2C} selective agonist) for normalizing the PCP-induced disruption of PPI is selectively blunted in *pet1*$^{-/-}$ mice (Supplementary Figures 2 and 3). These results indicate that the inability of atypical antipsychotic drugs to normalize NMDA-receptor hypofunction in *pet1*$^{-/-}$ mice is not because of 5-HT_{2A} or 5-HT_{2C} postsynaptic receptor insensitivity.

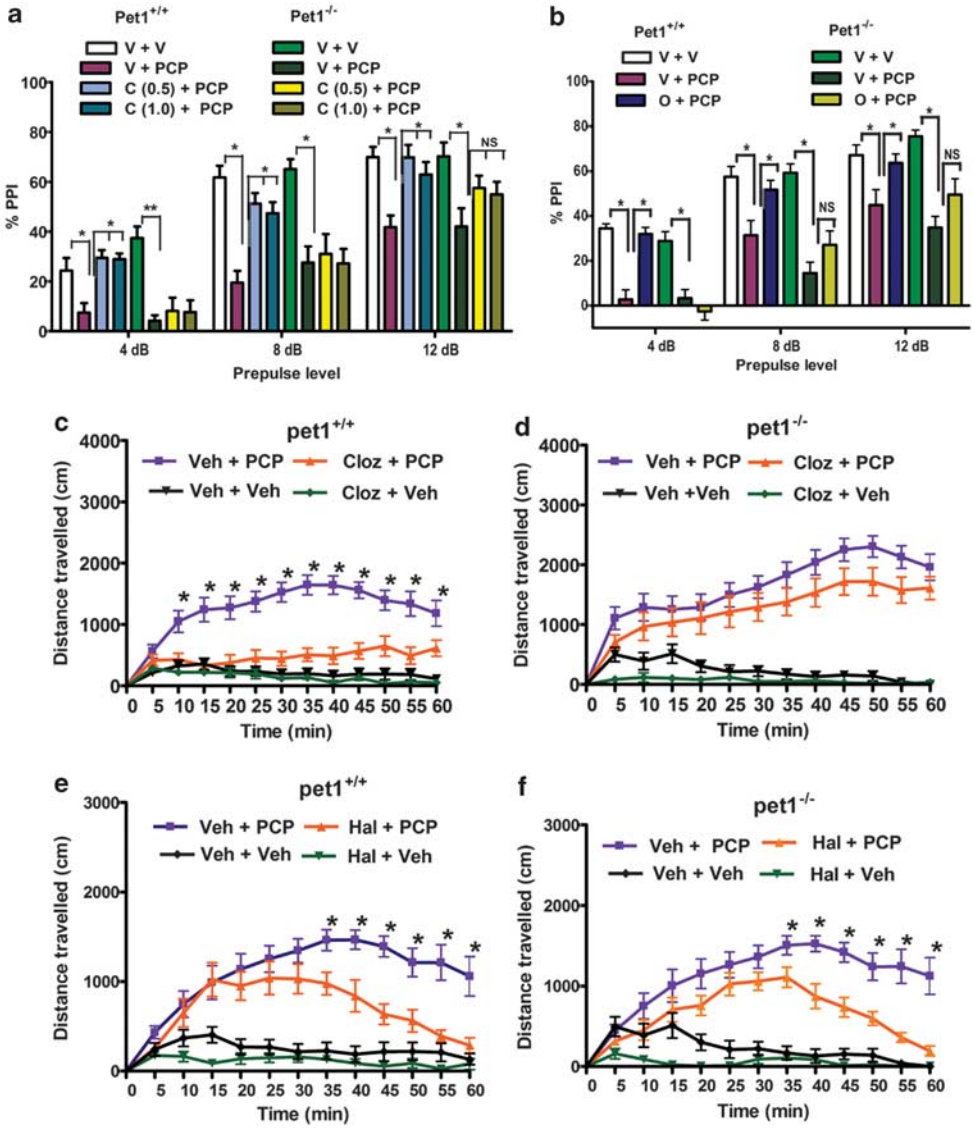


Figure 3 The presynaptic component of serotonin neuronal system is required for clozapine's therapeutic actions *in vivo*. (a) Effect of clozapine (C, 0.5 mg/kg or 1.0 mg/kg) on PCP (7.0 mg/kg)-induced disruption of prepulse inhibition (PPI) was measured. In *pet1*$^{+/+}$ mice, clozapine pretreatment completely normalized disruption of PPI by PCP at all prepulse levels, whereas it had no effect on *pet1*$^{-/-}$ mice ($N = 16$/group). (b) Effect of olanzapine (O, 0.5 mg/kg) on PCP (7.0 mg/kg)-induced disruption of PPI was measured. Similar to clozapine, olanzapine also did not exhibit any significant effect on PCP-induced PPI deficit in *pet1*$^{-/-}$ mice. (c, d) Pretreatment (30 min) with clozapine (0.5 mg/kg) significantly blocked PCP (7.0 mg/kg)-induced hyperactivity in pet1$^{+/+}$ mice, but had no significant effect on *pet1*$^{-/-}$ mice ($N = 14$ per group). Data are expressed as mean total horizontal distance traveled in 5-min bins over 60 min after PCP administration (±SEM). (e, f) Haloperidol (Hal, 0.1 mg/kg) significantly attenuated PCP-induced hyperactivity in both genotypes ($N = 12$ per group). Data are expressed as mean total horizontal distance traveled over 60 min in 5-min bins after PCP administration (±SEM). *$p < 0.05$, two-way ANOVA followed by Bonferroni post tests for multiple comparisons.

It has also been suggested that clozapine-like drugs exert their effects principally via direct (or indirect) activation of 5-HT$_{1A}$ serotonin receptors (Newman-Tancredi *et al*, 1998; Rollema *et al*, 1997). It is conceivable, therefore, that a diminution of 5-HT$_{1A}$ receptor number and/or sensitivity could exist in mice with a genetic deletion of *pet1*. To address this possibility, we quantified cortical 5-HT$_{1A}$ receptors via ^{3}H-WAY100635 radioligand binding and found that *pet1*$^{-/-}$ mice showed more (not less) ^{3}H-WAY100635 binding than *pet1*$^{+/+}$ mice in the cortex and hippocampus (Figure 6a). We also examined the electrophysiological properties of 5-HT$_{1A}$ receptors in hippocampal slices from *pet1*$^{+/+}$ and *pet1*$^{-/-}$ mice and found, as would be expected from the binding data, a significantly exaggerated response in *pet1*$^{-/-}$ when compared with *pet*$^{+/+}$ mice (Figure 6b and c). Taken together, these results indicate that clozapine's inability to rescue NMDA-receptor hypofunctioning is not because of diminished forebrain 5-HT$_{1A}$ receptor expression or activity.

Finally, and most critically, we interrogated the physiological function of the presynaptic component of the serotonin neuronal system to verify its relative ablation in *pet1*$^{-/-}$ mice. We first utilized the psychotomimetic agent methylene-dioxy-methamphetamine (MDMA) that induces locomotion via a presynaptic release of 5-HT. As shown in Figure 7a and b, the locomotor effects of MDMA were

abolished in *pet1*$^{-/-}$ mice. We next tested the presynaptic 5-HT$_{1A}$ receptor-mediated response electrophysiologically in midbrain raphe slices from *pet1*$^{+/+}$ and *pet1*$^{-/-}$ mice. The magnitude of the 5-HT$_{1A}$ hyperpolarization recorded from the *pet1* knockout mice was significantly less, and 14 out of the 24 neurons recorded demonstrated no response at all, as shown in Figure 7c and d. In contrast, the responses recorded from the *pet1* wild-type mice were normal in magnitude (Figure 7c and d). These results confirm a functional ablation of the presynaptic component of the serotonergic neuronal system in *pet1*$^{-/-}$ mice. Taken together, these results demonstrate that an intact presynaptic component of the 5-HT neuronal system is essential for clozapine's actions.

DISCUSSION

In this paper, we identify the presynaptic component of the serotonin neuronal system as an essential mediator of atypical antipsychotic drug action *in vivo*. Although literally thousands of studies have been published regarding clozapine's actions, the molecular substrates essential for its unique effects remain intensely controversial. Indeed, previous studies have demonstrated that clozapine and related atypical antipsychotic drugs have preferentially high affinity for postsynaptic 5-HT$_{2A}$ serotonin (Meltzer *et al*, 1989), D4 dopamine (Van Tol *et al*, 1991), muscarinic acetylcholine (Miller and Hiley, 1974), as well as dozens of other GPCRs (Roth *et al*, 2004). Indeed, each of these

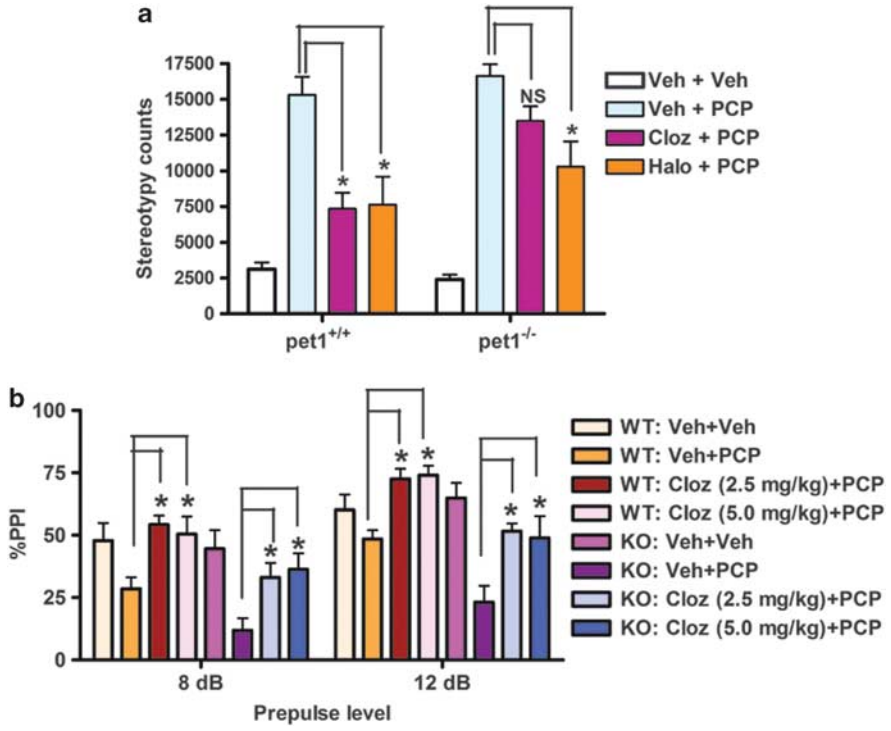
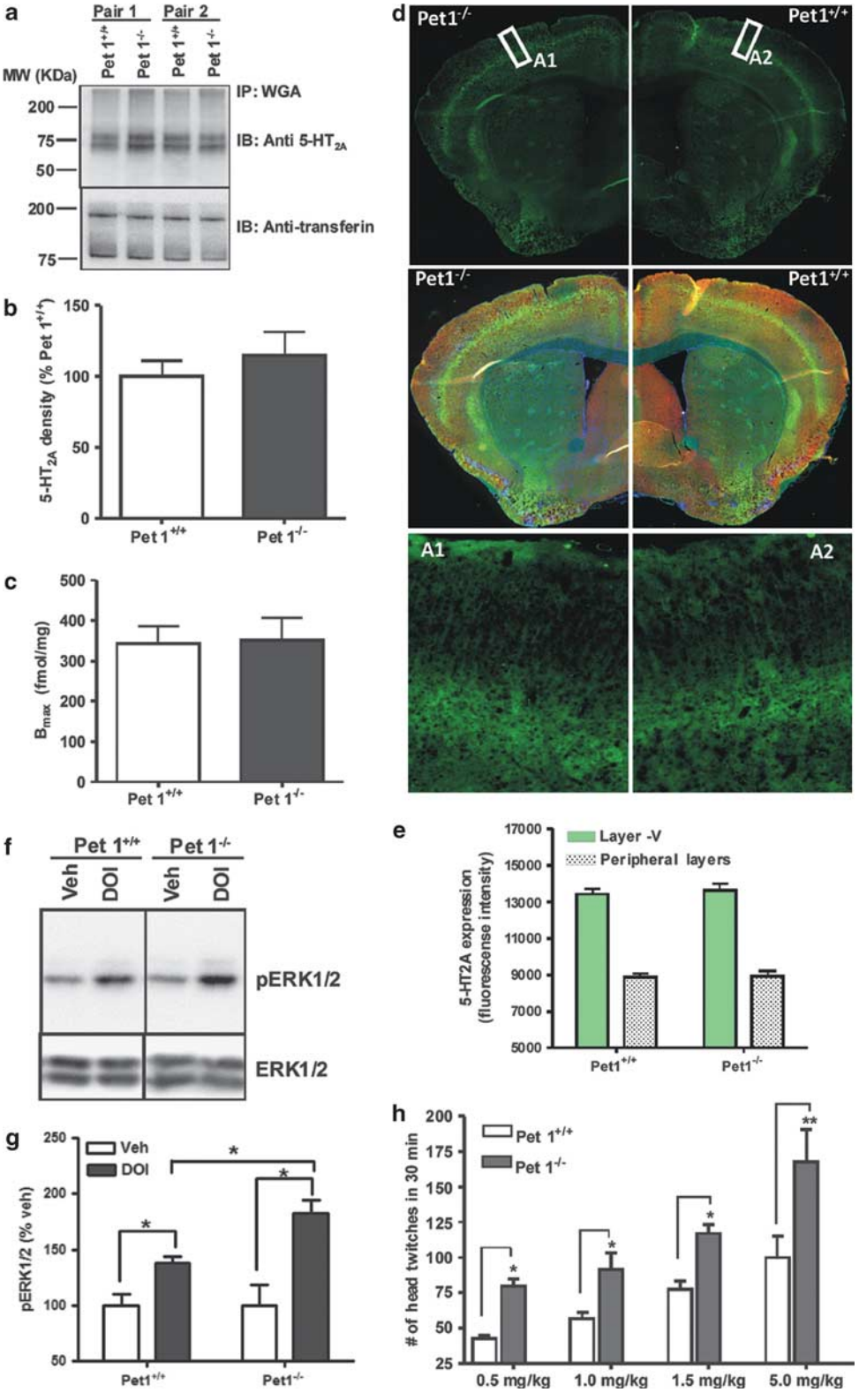


Figure 4 5-HT$_{2A}$ receptor is not essential for clozapine's antipsychotic drug actions. (a) Pretreatment (30 min) with clozapine (0.5 mg/kg) significantly blocked PCP (7.0 mg/kg)-induced stereotypy in *pet1*$^{+/+}$ mice, but had no significant effect on *pet1*$^{-/-}$ mice ($N=8$ per group). However, haloperidol (0.1 mg/kg) significantly attenuated PCP-induced stereotypy in both group of mice. Data are expressed as mean total stereotypy counts in 60 min after PCP administration (± SEM). *$p<0.01$, two-way ANOVA followed by Bonferroni post test for multiple comparisons. NS, not significant. (b) Clozapine normalizes the PCP induced PPI deficit in 5-HT2A KO mice. Effect of clozapine on PCP (7.0 mg/kg)-induced disruption of prepulse inhibition (PPI) was measured. Clozapine pretreatment (30 min) completely normalized disruption of PPI by PCP in both WT and KO mice ($N=8$ per group). *$p<0.01$, two-way ANOVA followed by Bonferroni post test for multiple comparisons.

Figure 5 Postsynaptic supersensitivity of 5-HT$_{2A}$ receptors following deletion of *pet1*. (a) Immunoblot of WGA immunoprecipitates from cortex membrane lysate of two representative *pet-1*$^{-/-}$ and *pet1*$^{+/+}$ littermate pairs. The same blots were stripped and probed for transferrin receptor as loading controls. (b) Densitometry of immunoblots of WGA IP from five pairs of WT and *pet1*$^{-/-}$ mice. (c) B_{max} estimates were obtained by performing [^{3}H]-ketanserin saturation binding on DLFC membrane homogenates. Data are presented as mean ± SEM ($N=5$ per group). (d) 5-HT$_{2A}$ receptor (green, top and bottom panels) and MAP2 (red) immunochemistry in coronal sections of a *pet1*$^{-/-}$ and *pet*$^{+/+}$ littermate pair. Middle panel is overlay of 5-HT$_{2A}$ (green), MAP2 (red), and Hoechst (blue) immunostaining to compare the distribution of 5-HT$_{2A}$ receptors between dendrites and cell bodies. (e) Estimates of 5-HT$_{2A}$ receptor abundance by immunofluorescence in layer V and in peripheral layers. 12 regions of interest (ROIs), 200 by 200 pixels, were randomly selected for both layer V and the peripheral layers. The mean intensity was then measured for each ROI and these were averaged for each brain section ($N=3$ per genotype). (f) 5-HT$_{2A}$-mediated signaling is potentiated in *pet1*$^{-/-}$ mice. Representative immunoblot of DOI (5.0 mg/kg, i.p. 15 min)-induced pERK1/2 and total ERK1/2 (as loading control) in microdissected cortical lysates. (g) Densitometry of DOI-induced pERK1/2 immunoblots; data are expressed as % vehicle (veh), $N=5$ to 6 per group, *$p<0.05$, one-way ANOVA followed by Neuman–Keuls *post hoc* analysis for multiple comparisons. (h) DOI (0.5–5.0 mg/kg)-induced head twitches were counted as measure of hallucinogenic activity; data are expressed as mean ± SEM ($N=6$–8 per group, *$p<0.05$, **$p<0.01$; unpaired *t*-test).

individual molecular targets to which clozapine binds with high affinity has been subsequently exploited for drug discovery purposes. Drugs that have been developed that attempt to mimic clozapine's actions by selectively targeting these 'clozapine receptors' have all failed to be approved as efficacious treatments of schizophrenia. This spectacular drug discovery failure prompted us to re-examine the molecular and neuronal substrates responsible for clozapine's actions *in vivo*.

To comprehensively and in an unbiased fashion identify all the potential site(s) of action of clozapine, we initially screened a representative sample of known typical and



atypical antipsychotic drugs against a panel of known molecular targets implicated in antipsychotic drug actions. Hierarchical clustering revealed that clozapine and olanzapine were most similar to each other among approved atypical antipsychotic drugs, and were very distinct from haloperidol. We then evaluated these three drugs against >300 distinct molecular targets to gain satisfactory physical screening coverage of the 'druggable genome' (Hopkins and Groom, 2002). We discovered that clozapine and olanzapine differed from haloperidol mainly by virtue of high affinities for nearly all known 5-HT receptors. In passing, we noted that clozapine and olanzapine were weak inhibitors of Chk2—a checkpoint kinase—although the potencies of clozapine and olanzapine for Chk2 (~10 μM) are probably too low to be of therapeutic relevance. One unique class of druggable targets represented in our screen are the orphan GPCRs. Despite screening 26 of these orphans, we have failed to identify unique clozapine targets among them (data not shown). Efforts are ongoing to screen all 160+ orphan GPCRs as potential clozapine targets (WK Kroeze and BL Roth, unpublished).

To cast an even wider net, we also employed a new and unbiased computational method, the SEA, to identify possible targets for these three drugs beyond the panel of targets explicitly screened (Keiser *et al*, 2007; Keiser *et al*, 2009). SEA predictions predominantly recapitulated the known targets of these drugs, and even where new and previously uncharacterized targets were suggested for olanzapine, these had already been characterized for clozapine. Indeed, clozapine and olanzapine often shared predicted targets by SEA—primarily aminergic GPCRs—with which haloperidol was not predicted to associate. Combining the 307 targets interrogated from the physical screening campaign and the 2250 targets from the SEA screen, we have interrogated over 2350 targets by a combination of these two methods. Taken together, these results indicate that the three drugs evaluated interact mainly with known biogenic amine receptors, and that the probability is low that any additional druggable targets exist that could account for the unique actions of clozapine.

Given the preferentially high affinity of clozapine and olanzapine for most known 5-HT receptors, we postulated that the serotonergic neuronal system is required for clozapine's action. Indeed, for decades, it has been suggested, without confirmatory studies using knockout mice, that 5-HT and its receptors are key mediators of clozapine's actions (Fink *et al*, 1984; Carlsson *et al*, 1999). Here, we took advantage of the observation that genetic deletion of *pet1* results in a generalized loss of 5-HT and the presynaptic component of the serotonin neuronal system (Hendricks *et al*, 2003). This diminution of brain serotonin led to an enhancement in 5-HT_{2A}, 5-HT_{2C}, and 5-HT_{1A} responses. However, despite this apparent postsynaptic supersensitivity, we found that deletion of *pet1* abolished clozapine's efficacy. Importantly, we also found that genetic deletion of 5-HT_{2A} receptors—heretofore considered the main target implicated in clozapine's actions—only marginally attenuated clozapine's antipsychotic-like actions. Instead, these present findings implicate the presynaptic component of the 5-HT neuronal system as being essential for clozapine's unique actions.

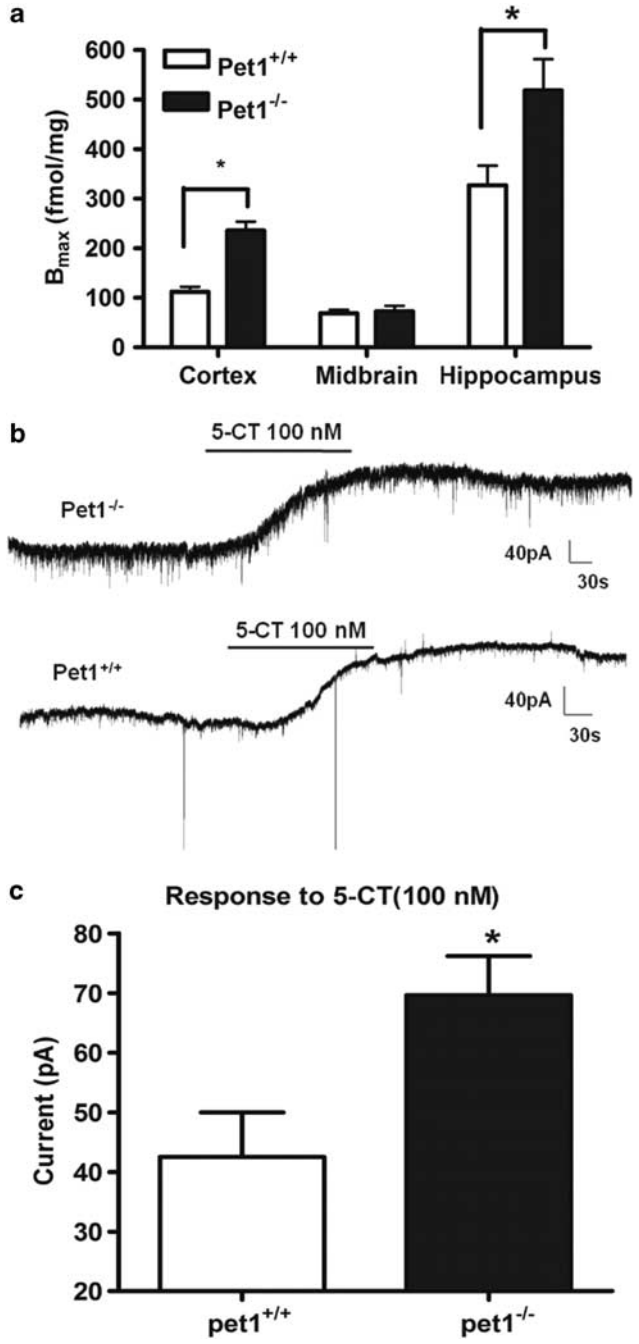


Figure 6 Upregulation and supersensitization of postsynaptic 5-HT_{1A} receptors in *pet1*$^{-/-}$. (a) Comparison of 5-HT_{1A} expression (B_{max} estimates) in cortex, midbrain, and hippocampus. B_{max} values were obtained by [^{3}H]-WAY100635 saturation binding, and data are presented as mean ± SEM ($N = 5$ to 6 per genotype). *$p < 0.05$, unpaired *t*-test. (b, c) 5-CT-induced changes in whole-cell current in CA1 pyramidal neurons. (a) Representative chart recordings of current elicited by bath application of 5-CT. All cells were recorded in voltage clamp mode with cells held at −60 mV; length of line above each chart depicts the amount of time the drug was in chamber. (b) Graphical presentation of whole-cell current index are presented from 12 cells per group derived from 6 mice per genotype. (*$p < 0.05$, unpaired *t*-test.)

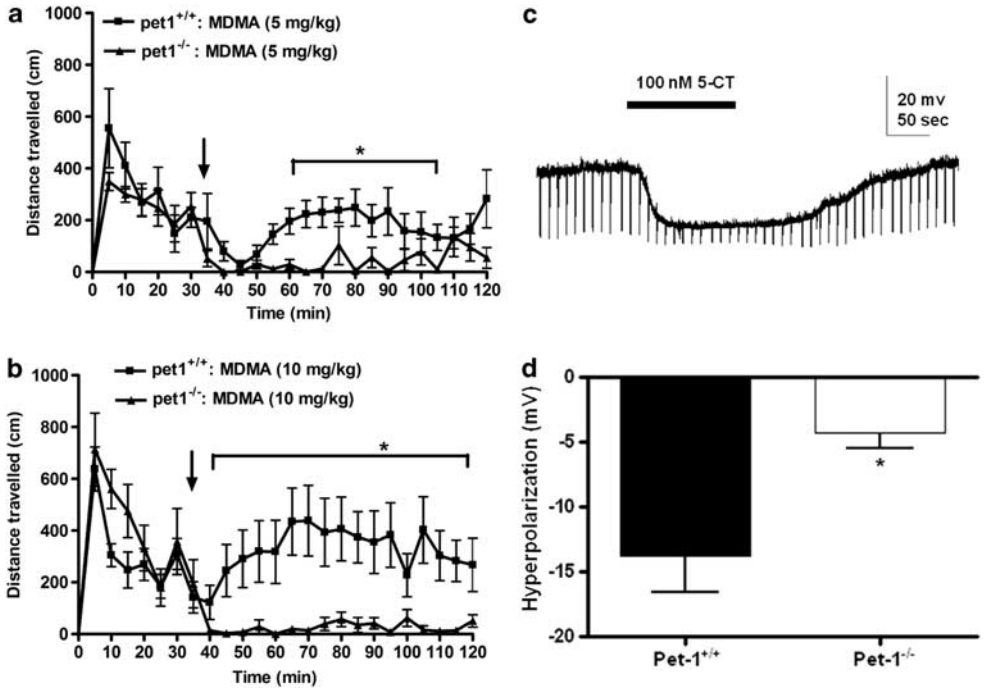


Figure 7 Serotonin neurons are silent in *pet1*$^{-/-}$ mice. (a, b) MDMA-induced locomotor response is abolished in *pet1*$^{-/-}$ mice. *pet1*$^{-/-}$ and *pet1*$^{+/+}$ littermate controls were injected with 5 mg/kg (a) or 10 mg/kg (b) MDMA after a 30-min acclimatization and then locomotor activity was measured for the next 90 min. *$p<0.05$, two-way ANOVA followed by Bonferroni correction for multiple comparisons. (c, d) 5-CT-induced changes in membrane potential recorded from the ventromedial subfield of the dorsal raphe. (c) Representative chart recording (in current clamp mode) of a neuron from *pet1*$^{+/+}$ mice showing the hyperpolarization induced by 5-CT (100 nM) bath application. (d) The graph summarizes the findings from all of the recorded cells identified as 5-HT containing in the *pet1*$^{+/+}$ mice or as 5-HT precursor neurons by β-galactosidase staining in the slices from *pet1*$^{-/-}$ mice ($N=24$ per genotype, *$p<0.001$, unpaired *t*-test).

Implications of These Findings for Psychiatric Drug Discovery

Although there is little debate regarding clozapine's unique efficacy in treating schizophrenia, the molecular and neuronal determinant(s) of clozapine's actions remain highly controversial. Some groups have proposed that clozapine is uniquely efficacious because of a combination of relatively low D2 receptor affinity (and associated fast dissociation rate) and/or high D4 dopamine receptor affinity (Kapur and Seeman, 2001; Seeman *et al*, 1997). This approach has led to the successful development of drugs like amisulpride that are relatively selective D2/D3 dopamine receptor antagonists (Schoemaker *et al*, 1997).

Others have suggested that clozapine via its principal metabolite *N*-desmethylclozapine potentiates NMDA-receptor function by virtue of M1 muscarinic receptor agonism (Sur *et al*, 2003; Wittmann *et al*, 2005). This hypothesis has led to a new crop of muscarinic subtype-selective allosteric potentiators currently being developed (Conn and Roth, 2008; Gray and Roth, 2007). Furthermore, it has also been shown that clozapine can potentiate NMDA activity by directly binding to glycine B site (Bressan *et al*, 2005; Javitt *et al*, 2005; Schwieler *et al*, 2008), thus contributing to its unique clinical efficacy. Clearly, however, the PCP-induced PPI disruption and hyperlocomoter response in mice do not model all aspects of schizophrenia and, therefore, other potential actions of clozapine were not addressed here.

Others, most prominently Meltzer and colleagues (Nash *et al*, 1988), who discovered that clozapine and related atypical antipsychotic drugs were characterized by relatively higher affinities for postsynaptic 5-HT_{2A} serotonin receptors (HTR2A) when compared with D2 receptors, suggested that a balanced antagonism of 5-HT_{2A}/D2 was required. This hypothesis led to the successful development of many currently prescribed atypical antipsychotic drugs, although none of them recapitulates the unique efficacy of clozapine (Lieberman *et al*, 2005). Meltzer and colleagues (Li *et al*, 2009) have also reported a requirement for cortical postsynaptic 5-HT_{1A} receptors in mediating certain actions of clozapine and related atypical antipsychotic drugs. Based on the relatively high affinity of clozapine for various 5-HT receptors, a number of attempts have been made to develop clozapine-like atypical antipsychotic drugs that function as *inverse agonists* for various postsynaptic 5-HT_{2A}, 5-HT_{2C}, 5-HT_6, and 5-HT_7 receptors (Gray and Roth, 2007), although, to date, these efforts have not yielded approved atypical antipsychotic drugs.

Many other molecular target-based approaches for developing atypical antipsychotic drugs are currently underway. These include targeting various components of the glutamate synapse (eg, mGluR2/3 allosteric potentiators, mGluR5 potentiators, GlyT1 glycine transporter inhibitors, and AMPA receptor potentiators), nicotinic acetylcholine subtype-selective agents, phosphodiesterase 10A inhibitors, NK3-neurokinin receptor antagonists, and others (Conn

and Roth, 2008; Gray and Roth, 2007; Karam *et al*, 2010). At present, it is unknown which, if any, of the above mechanistically designed (or single-target-based) approaches to treat schizophrenia require an intact presynaptic serotonergic neuronal system, although we are actively engaged in evaluating many of these drugs in *pet1*$^{-/-}$ mice (PN Yadav *et al*, unpublished).

By way of contrast, our results indicate that the presynaptic component of the serotonin neuronal system is a key determinant of clozapine's antipsychotic-like actions in mice *in vivo*. Therefore, a key feature for new antipsychotics would be a requirement for activity at the presynaptic component of the serotonergic neuronal system while simultaneously targeting various postsynaptic receptors to yield maximum efficacy. Clearly, designing such drugs with a focused serotonergic action, while minimizing side effects, will be the challenge for the next revolution in treating schizophrenia.

DISCLOSURE

Dr Roth in the past 24 months has been a consultant for Bristol Myers Squibb, Otsuka Pharmaceuticals, Merck, Medivation, Galenea Pharmaceuticals, the National Institutes of Health, Invitrogen, and Albany Molecular Researech, and has received royalty payments from licensing of 5-HT receptor patents (which are owned by UNC and Case Western Reserve University) to Galena Pharmaceuticals. Drs Shoichet and Irwin are co-founders of SeaChange, and Dr Keiser is an employee of SeaChange. The other authors declare no conflict of interest.

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