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Atypical antipsychotics as noncompetitive inhibitors of $\alpha 4\beta 2$ and $\alpha 7$ neuronal nicotinic receptors

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Abstract

It has been suggested that the interaction of antipsychotic medications with neuronal nicotinic receptors may increase the cognitive dysfunction associated with schizophrenia and may explain why current therapies only partially address this core feature of the illness. In the present studies we compared the effects of the atypical antipsychotics quetiapine, clozapine and N-desmethylclozapine to those of the typical antipsychotics haloperidol and chlorpromazine on the $\alpha 4\beta 2$ and $\alpha 7$ nicotinic receptor subtypes. The binding of [³H]-nicotine to rat cortical $\alpha 4\beta 2$ receptors and [³H]-methyllycaconitine to rat hippocampal $\alpha 7$ receptors was not affected by any of the compounds tested. However, Rb⁺ efflux evoked either by nicotine or the selective $\alpha 4\beta 2$ agonist TC-1827 from $\alpha 4\beta 2$ receptors expressed in SH-EP1 cells and nicotine-evoked [³H]-dopamine release from rat striatal synaptosomes were non-competitively inhibited by all of the antipsychotics. Similarly, α -bungarotoxin-sensitive epibatidine-evoked [³H]-norepinephrine release from rat hippocampal slices and acetylcholine-activated currents of $\alpha 7$ nicotinic receptors expressed in oocytes were inhibited by haloperidol, chlorpromazine, clozapine and N-desmethylclozapine. The inhibitory effects on nicotinic receptor function produced by the antipsychotics tested occurred at concentrations similar to plasma levels achieved in schizophrenia patients, suggesting that they may lead to clinically relevant effects on cognition.

Keywords

Antipsychotics; Nicotinic receptors; Cholinergic; Schizophrenia

1. Introduction

The well established fact that nicotine improves cognitive performance in animals and in humans suggests a potential role for neuronal nicotinic receptors (NNRs) in the cognitive deficits associated with various diseases and disorders, including schizophrenia (Levin et al., 2006; Levin and Rezvani, 2007; Martin and Freedman, 2007; Sacco et al., 2004). The two main NNR subtypes in the brain ($\alpha 4\beta 2$ and $\alpha 7$) have both been implicated in cognitive processes (Bencherif and Schmitt, 2002), and a number of studies point to their involvement in schizophrenia. Abnormal expression of $\alpha 4\beta 2$ and $\alpha 7$ NNRs was found in post-mortem hippocampus and cortex of schizophrenia patients (Breese et al., 2000; Marutle et al., 2001). In addition, the human $\alpha 7$ receptor gene located on chromosome-15 has been linked to sensory inhibition in familial schizophrenia (Freedman et al., 2003). It has long been known

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that blockade of the $\alpha 7$ receptor induces sensory gating deficits similar to those seen in schizophrenia (Luntz-Leybman et al., 1992) and that $\alpha 7$ -selective agonists can improve sensory gating deficits in animal models such as the DBA/2 mouse (Martin and Freedman, 2007; Simosky et al., 2001). In clinical studies, nicotine ameliorates sensory gating deficits and smooth pursuit eye movement abnormalities in schizophrenics, providing a rationale for smoking as a form of self-medication (Kumari and Postma, 2005; Leonard et al., 2001).

The atypical antipsychotics exhibit advantages over the typical antipsychotics in treating schizophrenia due to their superior therapeutic outcome with respect to negative symptoms and to some extent cognition. For example, clozapine improves verbal fluency and attention, and risperidone has positive effects on working memory and attention (Meltzer and McGurk, 1999). There is some evidence that pro-cognitive effects of atypical antipsychotics such as clozapine and its active metabolite N-des-methylclozapine may occur through M_1 muscarinic cholinergic receptor-mediated mechanisms (Davies et al., 2005; Li et al., 2005; Weiner et al., 2004). However, few reports have addressed antipsychotic interactions with nicotinic cholinergic receptors. Clozapine and chlorpromazine are known to displace [3 H]-nicotine in bovine chromaffin cells (Park et al., 2001) as does chlorpromazine in rat pheochromocytoma cells (Lee et al., 1999). The typical antipsychotic chlorpromazine is known to interact with functional NNRs as a non-competitive inhibitor (Heidmann et al., 1983; Heidmann and Changeux, 1986). More recently the atypical antipsychotic clozapine was shown to inhibit the function of $\alpha 7$ NNRs in oocytes and in hippocampal slice preparations (Singhal et al., 2007).

To date there has been no systematic assessment of the effects of atypical antipsychotics on NNR function. The present studies were undertaken to define the interactions of both typical (haloperidol and chlorpromazine) and atypical (quetiapine, clozapine and N-desmethylclozapine) antipsychotics with $\alpha 4\beta 2$ and $\alpha 7$ NNRs. To do this we studied the effects of the compounds on receptor function in rat striatal synaptosomes (dopamine release), hippocampal slices (norepinephrine release), oocytes (changes in acetylcholine-evoked currents) and cell cultures (Rb^+ efflux). To further characterize the interaction of antipsychotics with $\alpha 4\beta 2$ NNRs, the selective $\alpha 4\beta 2$ agonist TC-1827 (Bohme et al., 2004) was used to mimic administration of antipsychotics to smokers with high nicotine plasma levels. The results suggest that non-competitive inhibition of NNRs by atypical antipsychotics may have a clinically significant impact on cognitive function since inhibitory effects occur at concentrations within the range of those achieved in the plasma of human patients.

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2. Materials and methods

2.1. Animals and materials

Female Sprague–Dawley rats weighing 150–200 g (Charles River Laboratories, Raleigh, NC) were housed individually (12/12-h light/dark cycle) with free access to food and water. All animal studies were carried out in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

3,4-[7-³H(N)]-Dihydroxyphenylethylamine (³H]-dopamine, 23.5 Ci/mmol), levo-[ring-2,5,6-³H]-norepinephrine (³H]-norepinephrine, 53.0 Ci/mmol), L-(–)-[N-methyl-³H]-nicotine (³H]-nicotine, 66.9 Ci/mmol) and ⁸⁶RbCl (6.6–17.5 Ci/mmol) were purchased from PerkinElmer Life Science (Boston, MA). N-desmethylclozapine, methyllycaconitine and [³H]-methyllycaconitine, (25.4 Ci/mmol) were purchased from Tocris, Inc. (Ellisville, MO). Dulbecco's phosphate-buffered saline (PBS) was purchased from Invitrogen Corporation, (Carlsbad, CA). Amphotericin B, α -bungarotoxin, chlorpromazine, clozapine, cytosine, haloperidol and S-(–)-nicotine were purchased from Sigma–Aldrich (St. Louis, MO). Quetiapine was purchased from Toronto Research Chemicals (North York, Canada). Remaining materials were purchased from Sigma–Aldrich or Fisher Scientific (Pittsburgh, PA) unless specified otherwise in the text. TC-1827 was synthesized by Targacept, Inc.

2.2. Binding assays

The $\alpha 4\beta 2$ /SH-EP1 cells were harvested in ice-cold PBS (pH 7.4), centrifuged, and cell sediments were covered with 4 mL PBS and kept at –20 °C. After thawing, PBS was decanted and the cells were homogenized in 8 mL PBS with a Polytron (Brinkmann Instruments, Westbury, NY) at setting 6 for 15 s and centrifuged at 40,000 $\times g$ for 20 min (4 °C). The pellet was resuspended in 6 mL of ice-cold PBS and centrifuged again. The final pellet was resuspended in 6 mL of PBS for immediate use. Final preparations contained 0.16–0.20 mg/mL of total membrane protein.

[³H]-Nicotine and [³H]-methyllycaconitine were used to probe $\alpha 4\beta 2$ and $\alpha 7$ nicotinic receptor binding sites, respectively, at final radioligand concentrations of 0.1–40 nM for saturation assays or at 5 nM for competition binding assays, as previously described (Grinevich et al., 2005). Binding was assayed in PBS buffer containing: 0.9 mM CaCl₂; 2.67 mM KCl; 1.47 mM KH₂PO₄; 0.49 mM MgCl₂; 137.93 mM NaCl and 4.29 mM Na₂HPO₄, pH 7.4 in 48-well plates. Each sample (assayed in duplicate or triplicate) contained 50 μ L of test compound in solution at the desired concentration, 50 μ L of buffer (total binding) or cytosine/methyllycaconitine solution (cytosine or methyllycaconitine were used at 10 μ M final concentration to measure [³H]-nicotine or [³H]-methyllycaconitine nonspecific binding, respectively), 50 μ L of 5 \times radioligand stock solution, and 100 μ L of membrane suspension. Incubations were conducted for 2 h at 4 °C for [³H]-nicotine or at room temperature for [³H]-methyllycaconitine. Binding was terminated by dilution with cold PBS and immediate filtration onto GF/B filters (presoaked in 0.3% PEI) using a 48-sample, semi-auto harvester (Brandel, Gaithersburg, MD). After washing 3 times with ~1 mL of buffer, filters were transferred into scintillation vials filled with 3 mL of scintillation cocktail. Radioactivity was measured after 8–12 h using a liquid scintillation analyzer (model Tri-Carb 2200CA, PerkinElmer Life Sciences Inc., Boston, MA). Data expressed in dpm were transformed to fmol of bound radioligand per mg of total protein or as a percent of control [³H]-EPI binding (i.e., total – nonspecific). Competition assays using [³H]-nicotine binding to rat cortical membranes or [³H]-methyllycaconitine binding to rat hippocampal membranes were performed at 5 nM radioligand concentration. The procedure was identical to that described above for [³H]-EPI binding except that in these assays nonspecific binding was determined in the presence of 10 μ M nicotine or 10 μ M methyllycaconitine, respectively.

2.3. ⁸⁶Rb⁺ efflux in SH-EP1 cells

Cells of the SH-EP1 human epithelial line stably transfected with $\alpha 4\beta 2$ nicotinic receptors ($\alpha 4\beta 2$ /SH-EP1, kindly provided by Dr. R. Lukas, Barrow Neurological Institute, Phoenix, Arizona) were grown in Dulbecco's modified Eagle's medium (DMEM) with high glucose and L-glutamine supplemented with 10% horse serum, 5% fetal bovine serum, 100 U/mL

penicillin, 100 µg/mL streptomycin (all from Invitrogen, Chicago, IL) and 0.25 µg/mL amphotericin B at 37 °C in 5% CO₂/humidified air.

α4β2/SH-EP1 cells plated in 12-well clusters (Corning Inc., NY) and maintained to achieve confluency were loaded with ⁸⁶Rb⁺ (3 h; 37 °C) in identical growth medium supplemented with 2 µCi/mL isotope, then rinsed twice with PBS, pH 7.4. After a 4 min exposure to cholinergic agonist or test compound, efflux medium samples were collected into scintillation vials, added to scintillation cocktail, and radioactivity (cpm) was measured using a liquid scintillation analyzer (model Tri-Carb 2200CA, PerkinElmer Life Sciences Inc., Boston, MA). Basal ion efflux was measured using samples containing no agonist and specific nicotinic receptor function was defined as evoked over basal level ion efflux in the presence of agonist (100% control, at maximal response). Responses elicited by test compounds at different concentrations were normalized (%) to control.

2.4. Neurotransmitter release assays

[³H]-dopamine release studies were performed using rat striatal synaptosomes as previously described (Bencherif et al., 1998). Briefly, rats (150–200 g) were killed by decapitation after anesthesia with 70% CO₂. For synaptosomal preparations, striata from 2 animals were rapidly dissected and homogenized in 0.32 M sucrose containing 5 mM HEPES, pH 7.4 (7.5 mL per striatum). The tissue was then centrifuged at 1000 × g for 10 min and the pellet discarded. The supernatant was centrifuged at 12,000 × g for 20 min. The resultant pellet was resuspended in perfusion buffer (128 mM NaCl, 1.2 mM KH₂PO₄, 2.4 mM KCl, 3.2 mM CaCl₂, 1.2 mM MgSO₄, 25 mM HEPES, 1 mM Ascorbic acid, 0.01 mM pargyline-HCl and 10 mM glucose, pH 7.4) and centrifuged at 25,000 × g for 15 min. The final pellet was resuspended in perfusion buffer (2 mL) oxygenated with 95% O₂/5% CO₂ (oxygenated buffer was used throughout all subsequent steps).

The synaptosomal preparation was incubated in perfusion buffer for 10 min at 37 °C in order to restore metabolic activity. The medium was then supplemented with 0.1 µM [³H]-dopamine for 10 min to promote neurotransmitter uptake into synaptosomes. Aliquots of [³H]-dopamine-loaded synaptosomes (100 µL) were transferred onto GF/B filters mounted into chambers of an 18-channel Brandel superfusion system (Brandel, Gaithersburg, MD). The tissues were washed for 8 min with the buffer (room temperature, flow rate of 1.2 mL/min), then perfused for another 8 min period with antipsychotic solutions (0.01–100 µM, each solution in separate flow channel) to equilibrate their interaction with nicotinic receptors. Control channels (nicotine-evoked full response) were perfused without antipsychotics. Then nicotine (10 µM) alone or in combination with antipsychotics was applied in the perfusion stream of all channels for 48 s. Fractions (24 s) were continuously collected from each channel throughout the experiment to capture basal outflow, agonist-induced [³H]-dopamine release, and to re-establish the baseline following the agonist application (up to 6 min). The perfusate was collected directly into scintillation vials, scintillation cocktail was added and radioactivity was measured by liquid scintillation counting (LS6000, Beckman Coulter, Inc., Fullerton, CA). Nicotine-evoked release of [³H]-dopamine was assessed by summing the radioactivity of fractional dopamine releases over the basal level of [³H] outflow, and was normalized to the basal level of [³H] outflow. Nicotine-evoked release of [³H]-dopamine in samples containing antipsychotics was expressed as a percentage of the effect of a maximally active concentration of 10 µM nicotine.

[³H]-Norepinephrine release assays were conducted using rat hippocampal slices and the same protocol as for [³H]-dopamine. Hippocampi from 2 rats were cross-chopped (McIlwain tissue chopper, Mickle Laboratory Engineering, Surrey, UK) at approximately 250 µm thickness, rinsed twice and immediately immersed in oxygenated perfusion buffer

(see above, 2 mL). Then medium was supplemented with 50 nM [³H]-norepinephrine for 30 min at 37 °C for neurotransmitter uptake into slices. Epibatidine-evoked responses were used to measure the nicotinic receptor-mediated hippocampal norepinephrine release. Due to slow association kinetics, α -bungarotoxin (10 μ M) was added during the probe-loading procedure for 60 min.

2.5. Electrophysiological studies

The antipsychotics (10 mM stock solutions in DMSO) were diluted in Ringer's solution to the concentrations used in the experiments. The response to control applications of acetylcholine in the presence 1% DMSO (maximal final concentration) was not different from the response to acetylcholine alone (data not shown). Experiments were conducted using an OpusXpress 6000A (Axon Instruments, Union City, CA, USA). OpusXpress is an integrated system that provides automated impalement and voltage clamp of up to eight oocytes in parallel. Both the voltage and current electrodes were filled with 3 M KCl. Cells were voltage-clamped at a holding potential of -60 mV. Data were collected at 50 Hz and filtered at 20 Hz. Cells were bath-perfused with Ringer's solution, and agonist solutions were delivered from a 96-well plate via disposable tips, which eliminated any possibility of cross-contamination. Flow rates were set at 2 mL/min. Drug applications alternated between acetylcholine controls and experimental agonists. Applications were 12 s in duration followed by 181-s washout periods. Responses were calculated as net charge (Papke and Porter Papke, 2002). For the determination of IC_{50} , each oocyte received an initial control application of 60 μ M acetylcholine, then co-application of 60 μ M acetylcholine and antipsychotic at the desired concentration, and a follow-up control application of 60 μ M acetylcholine. A control concentration of 60 μ M was used since it was sufficient to evoke a large net charge response, yet exhibited responses slow enough to achieve even mixing of agonist and antagonist in a co-application protocol (Papke and Porter Papke, 2002). Responses to drug applications were normalized to the preceding acetylcholine control response, compensating for the varying levels of channel expression among the oocytes. Mean and S.E.M. were calculated from normalized responses of at least four oocytes for each experimental condition. The inhibition study was conducted by comparing acetylcholine net charge values across acetylcholine concentrations in the absence and in the presence of N-desmethylclozapine, at its IC_{50} level of 6 μ M. Under both conditions (i.e. with and without N-desmethylclozapine) responses were normalized to the control response of 300 μ M acetylcholine recorded from the same oocyte.

2.6. Calculations and statistics

Results calculated from observations performed in duplicate or triplicate were expressed as arithmetic means \pm S.E.M., except for effective doses (EC_{50}) and inhibition constants (IC_{50}), which were expressed as geometric means (with 95% CI, confidence intervals). Maximal responses (E_{max} values) were obtained by averaging values generated from sigmoid nonlinear regression analyses of individual concentration–response curves. IC_{50} values and pseudo-Hill coefficients (n_H) were determined from fixed and variable slope sigmoid fits, respectively. All data analyses were performed using GraphPAD Prism (GraphPAD, San Diego, CA).

3. Results

3.1. Lack of orthosteric interactions with $\alpha 4\beta 2$ and $\alpha 7$ nicotinic receptors

The typical antipsychotics haloperidol and chlorpromazine, and the atypical antipsychotic clozapine and its major metabolite N-desmethylclozapine, were selected to examine whether antipsychotics interact directly with the orthosteric (i.e., agonist and competitive antagonist) binding sites of nicotinic receptors. [³H]-Nicotine was used to probe $\alpha 4\beta 2$ NNR high

affinity binding sites in isolated rat cortical tissue and in recombinant $\alpha 4\beta 2$ NNRs expressed in SH-EP1 cells. [^3H]-methyllycaconitine was used to probe $\alpha 7$ NNR binding sites in rat hippocampal preparations. None of the compounds tested inhibited binding of the high affinity orthosteric ligands at concentrations up to 100 μM . No displacement of 5 nM [^3H]-nicotine or 5 nM [^3H]-methyllycaconitine bound to nicotinic receptors was observed. Neither the dissociation constant (K_d) nor the maximal number of binding sites (B_{max}) for saturation binding of [^3H]-nicotine or [^3H]-methyllycaconitine at 0.5–15 nM were affected (data not presented). Since nicotinic acetylcholine receptors have long been known to undergo allosteric modulation by non-competitive inhibitors (Heidmann et al., 1983) we conducted a series of studies (described below) to determine if the antipsychotics exhibited allosteric effects on $\alpha 4\beta 2$ or $\alpha 7$ NNR function.

3.2. Inhibition of ion flux through $\alpha 4\beta 2$ NNRs by antipsychotics

$^{86}\text{Rb}^+$ efflux was measured in SH-EP1 cells stably transfected with $\alpha 4\beta 2$ nicotinic receptors ($\alpha 4\beta 2$ /SH-EP1 cells). The atypical antipsychotics quetiapine, clozapine and the clozapine metabolite N-desmethylclozapine were selected to examine whether members of this class of antipsychotics directly inhibit the channel permeability of $\alpha 4\beta 2$ NNRs. The effects of the typical antipsychotics haloperidol and chlorpromazine were also assessed for comparison. Activation of $^{86}\text{Rb}^+$ efflux by a sub-maximal concentration of nicotine (10 μM) was used to determine the relative effects of the antipsychotic drugs on agonist-induced ion flux. None of these compounds affected the basal $^{86}\text{Rb}^+$ efflux by themselves (data not shown) but all of them completely inhibited nicotine-evoked $^{86}\text{Rb}^+$ efflux. The inhibition potencies [IC_{50} , mean] were as follows: clozapine, 5.2 μM , N-desmethylclozapine, 2.1 μM , quetiapine, 13.1 μM , haloperidol, 8.6 μM and chlorpromazine, 7.6 μM (Fig. 1A and Table 1). The mechanism of antipsychotic-induced inhibition of nicotinic receptor-channel permeability was further assessed by testing various concentrations of nicotine up to 100 μM , in the presence of antipsychotics at their respective IC_{50} concentrations (Fig. 1B). At concentrations above 1 μM nicotine activated $^{86}\text{Rb}^+$ efflux in a dose-dependent manner, reaching a plateau in the concentration range 10–100 μM . All antipsychotics tested insurmountably inhibited nicotine-evoked $^{86}\text{Rb}^+$ efflux, decreasing the maximal response (E_{max}) but not significantly affecting the potency (EC_{50} range 0.7–2.0 μM). These results support a non-competitive mechanism of inhibition of $\alpha 4\beta 2$ NNRs by all compounds tested, in agreement with previous reports showing similar effects of chlorpromazine on *Torpedo marmorata* acetylcholine receptor preparations (Heidmann et al., 1983) and clozapine on $\alpha 3\beta 4$ ganglionic nicotinic receptors (Heidmann et al., 1983; Jozwiak et al., 2004).

Since nicotine is a relatively non-selective NNR agonist, we evaluated the effects of the antipsychotics on $\alpha 4\beta 2$ /SH-EP1 cells activated by the pro-cognitive $\alpha 4\beta 2$ -selective compound TC-1827 (Bohme et al., 2004). We found that TC-1827 dose-dependently activates $^{86}\text{Rb}^+$ efflux from $\alpha 4\beta 2$ /SH-EP1 cells, achieving maximal responses at concentrations of 100–300 μM with a potency [EC_{50} , mean (95% CI) = 2.1 μM (1.5–3.5)] nearly an order of magnitude less than that of nicotine [18.9 μM (13.1–27.5)] (compare Fig. 1B and D). Activation of $^{86}\text{Rb}^+$ efflux by 100 μM TC-1827 was fully inhibited by clozapine and N-desmethylclozapine with IC_{50} values of 20.3 μM (15.1–28.7) and 6.4 μM (3.2–7.7), respectively (Fig. 1C). This inhibition was also insurmountable by TC-1827 at concentrations as high as 300 μM , suggesting a non-competitive mechanism. This conclusion is further supported by the findings that EC_{50} values for TC-1827, when applied alone [18.9 μM (13.1–27.5)] or in the presence of 15 μM clozapine [17.6 μM (9.3–33.0)] or 5 μM N-desmethylclozapine [22.2 μM (12.0–41.0)], were not significantly different. However, these values were slightly higher in the presence of 40 μM clozapine or 20 μM N-desmethylclozapine, reflecting a typical rightward shift at high concentrations of the non-competitive inhibitor (Fig. 1D). In order to examine the dose dependency of the non-

competitive mechanism and to exclude a possible ceiling effect, the actions of clozapine or N-desmethylclozapine on TC-1827- and nicotine-evoked responses were compared at moderate (15 and 1.5 μM , respectively) and at high (100 and 10 μM , respectively) concentrations of agonists. No significant differences in the potencies [IC_{50} values, mean (95% CI)] of clozapine [19.8 μM (10.2–38.6) and 20.8 μM (15.1–28.7)] or N-desmethylclozapine [8.2 μM (5.2–12.8) and 5.0 μM (3.2–7.7)] were found between these two conditions. Together these data confirm that clozapine and N-desmethylclozapine-induced inhibition is mediated entirely through a non-competitive mechanism.

3.3. Inhibition of nicotinic receptor electrophysiological responses by antipsychotics

Acetylcholine-stimulated electrophysiological responses of *Xenopus laevis* oocytes expressing rat $\alpha 7$ NNRs were employed to study the effect of selected antipsychotics on receptor function. All antipsychotics tested (haloperidol, chlorpromazine, clozapine, N-desmethylclozapine), when co-applied with 60 μM acetylcholine produced reversible inhibition of ACh-evoked currents. Responses to consecutive applications of 30 and 100 μM ACh following drug applications recovered to $100 \pm 6\%$ and $87 \pm 5\%$ for clozapine, $90 \pm 3\%$ and $107 \pm 6\%$ for N-desmethylclozapine, $87 \pm 6\%$ and $97 \pm 10\%$ for haloperidol, and $113 \pm 17\%$ and $59 \pm 6\%$ for chlorpromazine. The potencies [IC_{50} , mean] determined for inhibition of ACh-evoked currents were: clozapine, 8.4 μM , N-desmethylclozapine, 3.2 μM , haloperidol, 10.5 μM and chlorpromazine, 9.1 μM (Fig. 2A and Table 1). Variable slope sigmoid fits of the data revealed no visible cooperativity for interactions of the antipsychotics with $\alpha 7$ NNRs. The pseudo-Hill slopes (n_{H}) were near unity for clozapine (–0.91), N-desmethylclozapine (–1.3), haloperidol (–1.1) and chlorpromazine (–0.84), consistent with a one-site model for their interactions. Co-application of 6 μM N-desmethylclozapine with increasing concentrations of ACh (1 μM –1 mM) resulted in insurmountable inhibition of net charge responses, reducing the maximal response up to $37.5 \pm 5\%$ (Fig. 2B). This effect of N-desmethylclozapine implies a non-competitive mechanism of action.

3.4. Inhibitory effects of antipsychotics on neurotransmitter release

Nicotine-evoked [^3H]-dopamine release from rat striatal synaptosomes was used to probe the function of presynaptic naturally-expressed $\alpha 4\beta 2$ -containing nicotinic receptors (Champtiaux et al., 2003). The present studies did not attempt to differentiate $\alpha 6^*$ NNR contributions to release. Nicotine application (10 μM for 48 s) rapidly produced a [^3H]-dopamine release peak above that of basal release from perfused [^3H]-dopamine-loaded synaptosomes (Fig. 3A). Nicotine-activated dopamine release returned to basal levels within 5 min after nicotine exposure. Pre-application of antipsychotics for 6 min followed by their co-application with 10 μM nicotine completely abolished the nicotine-evoked response. Representative data are shown for 30 μM clozapine and N-desmethylclozapine in Fig. 3A (filled symbols). The ratio of total agonist-evoked [^3H]-dopamine release to the basal level of [^3H]-dopamine release was used to quantify the effects of the antipsychotics. It was found that all drugs tested inhibited dopamine release in a concentration-dependent manner with potencies [IC_{50} , mean]: clozapine, 5.3 μM , N-desmethylclozapine, 2.1 μM , quetiapine, 5.9 μM , haloperidol, 4.8 μM and chlorpromazine, 2.8 μM (Fig. 3B and Table 1).

In additional studies, α -Bungarotoxin-sensitive epibatidine-evoked [^3H]-NE release from rat hippocampal slices was used to evaluate the effects of the antipsychotics on $\alpha 7$ NNRs, a subtype known to be involved in memory function in this area of the brain. All of the antipsychotics tested, both atypical and typical, were found to completely inhibit responses to epibatidine with IC_{50} values in the range of 1–2 μM (Fig. 4). Quetiapine was not tested in the [^3H]-NE release studies.

4. Discussion

The present results demonstrate that the atypical antipsychotics quetiapine, clozapine, and the clozapine active metabolite N-desmethylclozapine, as well as the typical antipsychotics haloperidol and chlorpromazine non-competitively inhibit the function of mammalian neuronal $\alpha 4\beta 2$ and $\alpha 7$ NNRs with potencies comparable to that of the classical nicotinic receptor antagonist mecamylamine. This was observed for NNRs expressed both in recombinant systems (human SH-EP1 cells and frog oocytes) and in mammalian brain preparations (rat brain synaptosomes and hippocampal slices). With respect to functional inhibition, differences were noted in the potencies of individual antipsychotics but there did not appear to be substantial differences between the typical and atypical antipsychotic classes. Because the antipsychotics tested did not displace [^3H]-nicotine or [^3H]-methyllycaconitine binding to $\alpha 4\beta 2$ and $\alpha 7$ NNRs, our data do not support a direct interaction with orthosteric binding sites. These results contrast with previous reports that the atypical antipsychotic clozapine inhibited [^3H]-nicotine binding in bovine chromaffin cells (Park et al., 2001) and that the typical antipsychotic chlorpromazine inhibited [^3H]-nicotine binding both in bovine chromaffin cells (Park et al., 2001) and in rat pheochromocytoma (PC-12) cells (Lee et al., 1999). The presence in those cell types of other non- $\alpha 4\beta 2$ and non- $\alpha 7$ NNR subtypes with different binding characteristics may account in part for the discrepant findings. A number of studies have suggested that functional interactions of antipsychotics with cholinergic receptors are mediated through M_1 muscarinic mechanisms (Davies et al., 2005; Friedman, 2004; Weiner et al., 2004). However, the typical antipsychotic chlorpromazine has long been known to interact with non-neuronal nicotinic cholinergic receptors as a non-competitive inhibitor (Heidmann et al., 1983; Heidmann and Changeux, 1986; Lee et al., 1999). A few reports have described functional inhibition of nicotinic receptors by atypical antipsychotics as well, including non-neuronal subtypes in bovine adrenal chromaffin cells (Park et al., 2001) and the vertebrate neuromuscular junction (Nguyen et al., 2002) and more recently the neuronal $\alpha 7$ subtype (Singhal et al., 2007).

Nicotinic receptor-mediated cognitive effects have been demonstrated in numerous studies, both in animals and in humans (Levin et al., 2006) and the interaction of nicotine with antipsychotic medications is known to impact cognitive processes (Addy and Levin, 2002; Levin and Rezvani, 2007). For example, clinical studies have demonstrated that nicotine causes a dose-related reversal of haloperidol-induced impairments of memory performance in schizophrenics (Levin et al., 1996). By comparison, working memory deficits caused by the nicotinic antagonist mecamylamine are potentiated by haloperidol (Levin and Rose, 1995). Our data indicate that both typical and atypical antipsychotics inhibit NNR function with potencies in the low micromolar range, as judged by agonist-stimulated ion flux and neurotransmitter release. Importantly, the IC_{50} values for all of the antipsychotics tested, with the exception of haloperidol, fall within or near the maximum plasma concentration ranges of antipsychotics observed in human patients (see Table 1 and Fig. 5). The net impact on cognition in schizophrenia, whether it is enhancement or impairment, cannot be predicted based on the present evidence but the potency of the inhibitory effects on $\alpha 4\beta 2$ and $\alpha 7$ NNRs suggests that clinically relevant effects are possible.

In schizophrenia patients, cognitive impairment may be the result of disease evolution but an iatrogenic component has been documented with haloperidol and other antipsychotics (Stip, 2006). These observations, together with the present data, suggest that a component of this cognitive impairment could be associated with their non-competitive inhibition of NNRs. For example, it is well established that chemical blockade with compounds such as mecamylamine (Newhouse et al., 1994) or MK-801 (Wang et al., 2007) or biological interference using animal knock-out models of either $\alpha 4\beta 2$ (Hogg et al., 2003) or $\alpha 7$ NNRs

(Young et al., 2007) can produce cognitive dysfunction. However the fact that the atypical antipsychotics are generally less likely to induce cognitive impairment suggests a more complicated relationship. Differential effects noted among some of the typical and atypical antipsychotic drugs are evidenced by studies showing that $\alpha 7$ NNRs contribute to the action of clozapine, but not haloperidol, in reversing auditory gating deficits in animal models (Simosky et al., 2003) and that the atypical neuroleptic risperidone is associated with time dependent and persistent negative effects on $\alpha 7$ NNRs (Terry et al., 2005). In addition, the effects of antipsychotics on cognition can differ, depending on which NNR subtypes are involved and where they are located. For example, blockade of $\alpha 7$ NNRs in the hippocampus with methyllycaconitine has been shown to enhance clozapine-induced memory impairment in rats while blockade of $\alpha 4\beta 2$ NNRs with dihydro- β -erythroidine leads to clozapine-induced improvement in memory (Pocivavsek et al., 2006). The latter observation may suggest the possibility that for some NNR subtypes inhibition of receptor function is a feature, rather than a side effect, of the clinical efficacy of atypical antipsychotics and that inhibition is required for efficacy. However, underactivity of medial frontal cortical $\beta 2$ -containing NNRs increases clozapine-induced working memory impairment in rats (Levin et al., 2009). The dissociation of iatrogenic and disease-induced cognitive impairment, the differential effects of antipsychotic drugs on nicotinic receptors, and the observation that ligand-activated nicotinic receptor-mediated neurotransmitter release is influenced by antipsychotic drugs converge to suggest multiple mechanisms of action for antipsychotics. If NNR blockade is operative *in vivo*, the true potential of antipsychotics to alleviate cognitive impairment may be diminished to some extent by inhibition of key NNR subtypes. Adjunctive therapies based on nicotinic pharmacologies may either counteract this blockade or may be futile if the inhibition is insurmountable, although it is at least theoretically possible that non-competitive inhibition by the antipsychotics could be competitively antagonized by a ligand that affects their allosteric binding sites. The identification of atypical antipsychotics that enhance cognition but do not block NNR function would help to further clarify this issue. Clearly the final relative concentrations of these drugs in the synapse will define the overall outcome on cholinergic tone. In this regard, future studies involving a more in depth characterization of structure–activity relationships and pharmacokinetic/pharmacodynamic properties of the typical and atypical antipsychotics vis-à-vis NNR interactions would be instructive.

The heavy smoking behavior of schizophrenia patients will also certainly influence receptor interactions and neurotransmitter tone. This is consistent with previous proposals that their smoking behavior is at least partly motivated by the need to overcome undesirable side effects associated with antipsychotic agents (Barnes et al., 2006). However, there is also literature suggesting that both neuroleptic-naïve schizophrenics as well as in-patient schizophrenics display heavy smoking behavior and that this feature may be independent of side effects (McEvoy and Brown, 1999). The present results with the pro-cognitive $\alpha 4\beta 2$ -selective NNR agonist TC-1827 suggest that this NNR subtype may mediate complex interactions of nicotinic agonists with antipsychotics that affect cognitive tone, possibly providing additional motivation to smoke. Thus in the development of cognition-enhancing drugs for schizophrenia that target this NNR subtype, it should be considered that the presence of nicotine may prevent optimal activation of the receptors. The picture is further complicated by our emerging understanding of the structural and functional diversity of NNR subtypes. For example, the stoichiometry of $\alpha 4\beta 2$ NNRs [$(\alpha 4)_2(\beta 2)_3$ or $(\alpha 4)_3(\beta 2)_2$] can impact sensitivity to agonist activation (Moroni et al., 2006). Similarly, $\alpha 7$ NNRs can exist both as homomeric [$(\alpha 7)_5$] and heteromeric ($\alpha 7\beta 2^*$) subtypes (Liu et al., 2009). The present approaches have not probed these differences. Therefore additional studies are needed to fully understand the influence of NNR subforms. Hopefully a more complete understanding of drug–drug interactions between antipsychotics and NNR-targeted drugs

will unlock the potential of NNR-based therapies in the management of positive and negative symptoms as well as cognitive deficits in neuropsychiatric disorders.

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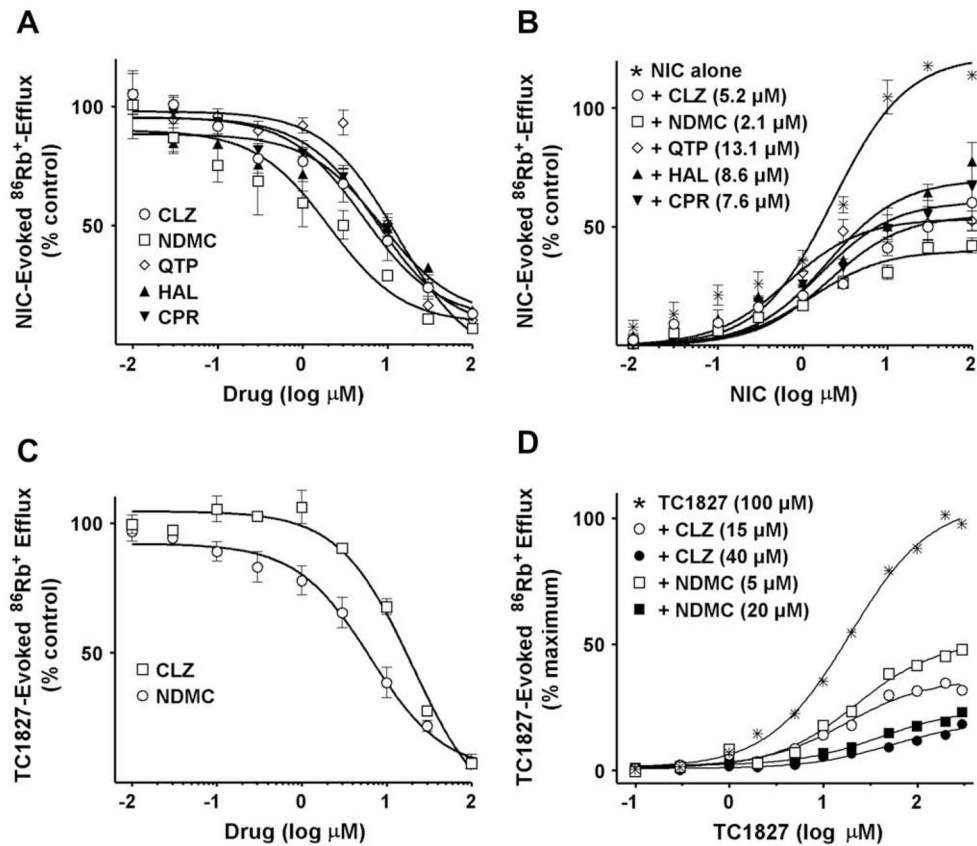


Fig. 1. (A) Inhibition of nicotine (NIC)-evoked $^{86}\text{Rb}^+$ -efflux from $\alpha 4\beta 2/\text{SH-EP1}$ cells by antipsychotics. Data are expressed as percent of control response evoked by 10 μM nicotine (Mean \pm S.E.M. for 3–4 independent experiments). Curves were generated using nonlinear regression (individual data were best fitted to a one-site model) for clozapine (CLZ) (○), quetiapine (QTP) (◇), the clozapine metabolite N-desmethylclozapine (NDMC) (□), haloperidol (HAL) (▲) and chlorpromazine (CPR) (▼). (B) Insurmountable effect of antipsychotics at their IC_{50} concentrations on concentration–response relationship of nicotine for stimulation of $^{86}\text{Rb}^+$ -efflux from $\alpha 4\beta 2/\text{SH-EP1}$ cells. Data are expressed as a percent of control response evoked by 10 μM nicotine (Mean \pm S.E.M. of 3–4 independent experiments). Curves were generated using nonlinear regression (individual data were best fitted to a one-site model). (C) Inhibition of TC-1827-evoked $^{86}\text{Rb}^+$ -efflux from $\alpha 4\beta 2/\text{SH-EP1}$ cells by clozapine (□) or N-desmethylclozapine (○). Data are expressed as percent of control response evoked by 100 μM TC-1827 (Means \pm S.E.M. of 4 independent experiments). Curves were generated using nonlinear regression (individual data were best fitted to a one-site model). (D) Insurmountable effect of 15 μM (○) and 40 μM (●) clozapine, or 5 μM (□) and 20 μM (■) N-desmethylclozapine on the concentration–response relationship of TC-1827 for stimulation of $^{86}\text{Rb}^+$ -efflux from $\alpha 4\beta 2/\text{SH-EP1}$ cells. Data expressed as a percent of control response evoked by 100 μM TC-1827 (Mean \pm S.E.M. of 3 independent experiments). Curves were generated using nonlinear regression (individual data were best fitted to a one-site model).

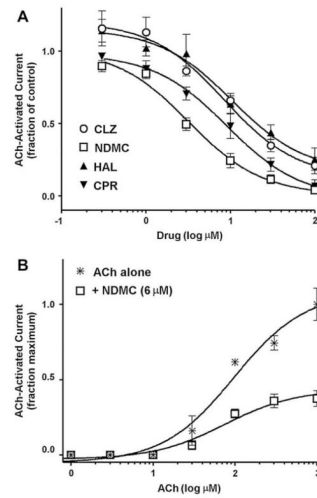


Fig. 2.

(A) Inhibition of acetylcholine (ACh, 60 μM)-stimulated electrophysiological (net charge) responses of *Xenopus laevis* oocytes expressing rat $\alpha 7$ nicotinic receptors by the antipsychotics clozapine (CLZ) (\circ), the clozapine metabolite N-desmethylclozapine (NDMC) (\square), haloperidol (HAL) (\blacktriangle) and chlorpromazine (CPR) (\blacktriangledown). Oocytes were voltage-clamped at -60 mV, and data were collected 50 Hz and filtered at 20 Hz. Acetylcholine and drugs were applied (12 s) in the perfusion stream of Ringer's solution used to bathe (22 $^{\circ}\text{C}$) the cells. Applications were with ACh alone, then with combination of ACh and antipsychotic, followed by ACh alone for a recovery control. Data are normalized to ACh-alone responses (Means \pm S.E.M. for responses of at least 4 oocytes). Curves were generated using nonlinear regression (individual data were best fitted to a one-site model). (B) Insurmountable effect of N-desmethylclozapine (6 μM) on concentration-response relationship of ACh in evoking net charge responses in *X. laevis* oocytes expressing rat $\alpha 7$ nicotinic receptors. The experimental protocol and data analysis are the same as described for Fig. 2(A) above except that cells were pre-equilibrated with 6 μM N-desmethylclozapine alone for 10 s before the co-application of 6 μM N-desmethylclozapine and varying concentrations of ACh. Data are normalized to ACh-alone maximal response (Means \pm S.E.M. for responses of at least 4 oocytes).

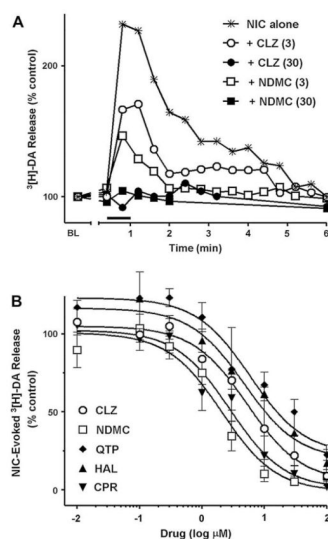


Fig. 3.

(A) Representative fractional [^3H]-dopamine release from rat striatal synaptosomes evoked by 10 μM nicotine alone (*) or inhibited by 3 μM clozapine (CLZ) (\circ), 3 μM N-desmethylclozapine (NDMC) (\square), 30 μM CLZ (\bullet), and 30 μM NDMC (\blacksquare). Data are expressed as % of basal release (BL = mean release of 3 preceding fractions). Each fraction was collected for 24 s. The bar above X axis shows the duration of nicotine application (48 s). Drugs were applied in perfusion stream for 6 min before nicotine and discontinued immediately after co-application with nicotine. (B) Inhibition of 10 μM nicotine-evoked total [^3H]-dopamine release (sum of fractional releases) from rat striatal synaptosomes by CLZ (\circ), NDMC (\square), quetiapine (QTP) (\diamond), haloperidol (HAL) (\blacktriangle) and chlorpromazine (CPR) (\blacktriangledown). Experimental protocol was the same as described for Fig. 3(A). Data are expressed as % of control (nicotine alone) and are Means \pm S.E.M. for 3–4 independent experiments. Curves were generated using nonlinear regression (individual data were best fitted to a one-site model).

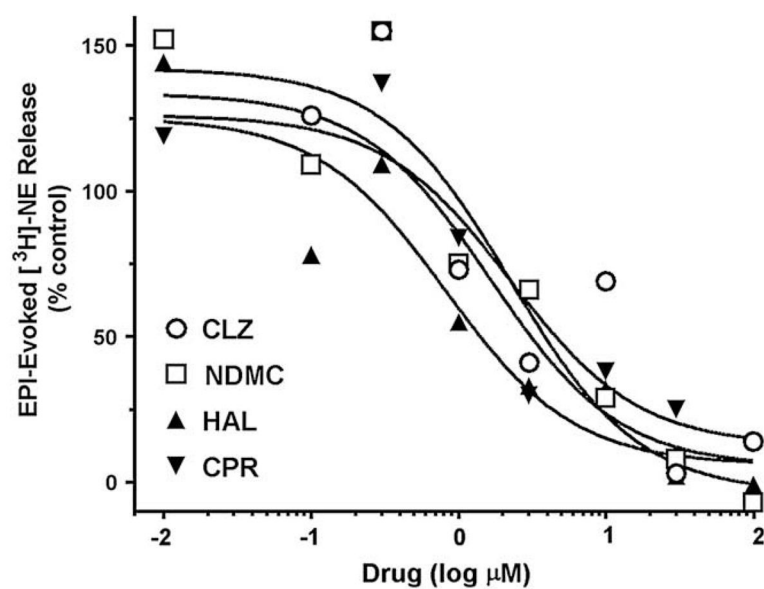


Fig. 4. Inhibition of 100 nM epibatidine (EPI)-evoked total [^3H]-Norepinephrine (NE) release (sum of fractional releases) from rat hippocampal slices by clozapine (CLZ) (\circ), N-desmethylclozapine (NDMC) (\square), haloperidol (HAL) (\blacktriangle) and chlorpromazine (CPR) (\blacktriangledown). Experimental protocol was the same as described for Fig. 3(A). Data are expressed as % of control (NE alone) and represent Means from representative experiments. Curves were generated using nonlinear regression (individual data were best fitted to a one-site model).

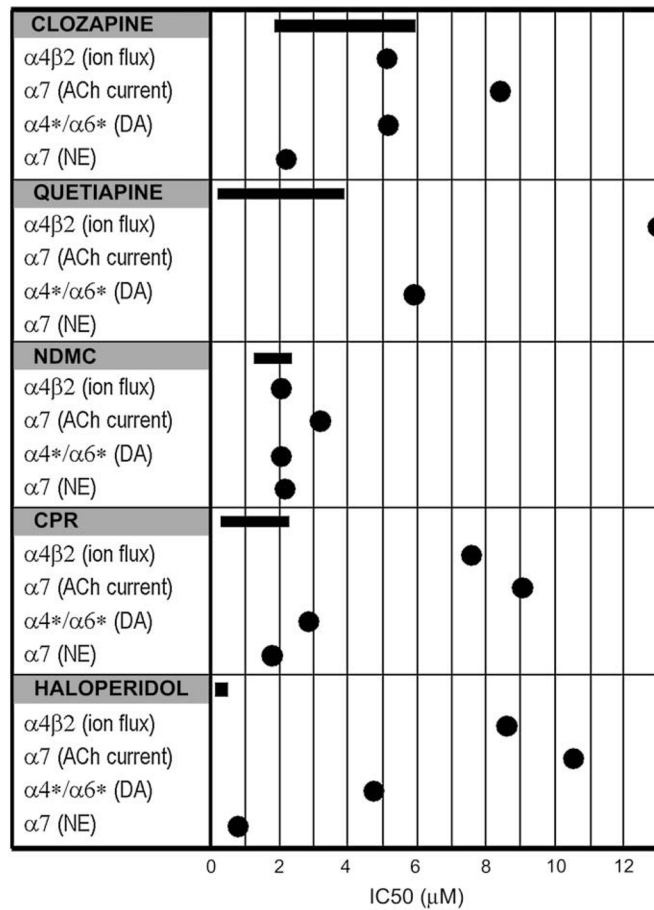


Fig. 5. Comparison of Antipsychotic Inhibitory Potencies to Clinical Plasma Levels The IC₅₀ values for inhibition of receptor function by each of the antipsychotics were plotted for each of the NNR subtypes (●) and compared to the maximum plasma ranges achieved in clinical studies (–). Plasma ranges were derived from literature values shown in Table 1.

Table 1

Comparison of In Vitro Potencies of Antipsychotic Effects with Clinical Plasma Levels The IC₅₀ values are shown for antipsychotic-mediated inhibition of nicotine-evoked Rb + efflux from $\alpha 4\beta 2$ /SH-EP1 cells, ACh-induced $\alpha 7$ current in oocytes, nicotine-evoked dopamine (DA) release from striatal synaptosomes and epibatidine-evoked norepinephrine (NE) release from hippocampal slices. Numbers in parentheses represent 95% confidence intervals. Maximum plasma concentrations (ng/mL) of antipsychotics represent values achieved in clinical studies, reported in the literature (supporting references at right). Maximum plasma levels (μ M) were calculated from reported concentrations and known molecular weights of the individual compounds (left column).

Anti-psychotic	Rb + Flux IC ₅₀ (μ M)	$\alpha 7$ Current IC ₅₀ (μ M)	DA Release IC ₅₀ (μ M)	NE Release IC ₅₀ (μ M)	Max. Plasma Levels (ng/mL)	Max. Plasma Levels (μ M)	References
Clozapine MW = 326.8	5.2 (2.8–9.9)	8.4 (3.8–18.8)	5.3 (2.4–11.6)	2.2	574	1.8	Diaz et al., 2005
					880	2.7	Freudenreich et al., 1996
					1088	3.3	Perry et al., 1991
					1310	4.0	Dumortier et al., 1998
					1920	5.9	Wong et al., 2006
N-desmethyl-clozapine MW = 312.8	2.1 (0.9–4.7)	3.2 (1.3–7.7)	2.1 (1.1–3.9)	2.2	420	1.3	Flanagan et al., 2003
					580	1.9	Dumortier et al., 1998
Quetiapine MW = 383.5	13.1 (9.1–18.9)	ND	5.9 (1.1–20.9)	ND	720	2.3	Wong et al., 2006
					91	0.24	Lin et al., 2004
					491	1.3	Aichhorn et al., 2006
					1042	2.7	Grimm et al., 2006
					1454	3.8	Tauscher-Wisniewski et al., 2002
Chlorpromazine MW = 355.3	7.6 (4.5–12.7)	9.1 (6.3–13.2)	2.8 (1.8–19.6)	1.7	124	0.4	Della Corte et al., 1993
					422	1.2	Chetty et al., 1996
					839	2.4	Chetty and Miller, 2001
Haloperidol MW = 375.9	8.6 (4.2–17.6)	10.5 (4.5–24.7)	4.8 (1.2–6.7)	0.8	86	0.23	Roh et al., 2001

ND = not done.