Typical and atypical antipsychotics do not differ markedly in their reversibility of antagonism of the dopamine D₂ receptor

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Abstract
It has been suggested that the favorable side-effect profiles of atypical antipsychotics (e.g. clozapine and amisulpride) are related to their ~100-fold faster dissociation from dopamine D₂ receptors (D₂R) compared with typical antipsychotics (e.g. haloperidol and chlorpromazine). Fast dissociation would entail rapidly reversible antagonism; however, this has not been thoroughly studied using functional assays. We compared the reversibilities of D₂R antagonism by 17 compounds using an electrophysiological method to measure dopamine-evoked potassium channel activation via D₂R. Varying rates and amplitudes of D₂R response recovery were observed following antagonist removal. Whereas recovery rates differed 15-fold between atypical drugs, recovery from clozapine and amisulpride antagonism was, unexpectedly, less than twofold faster than from chlorpromazine. The recovery amplitude correlated with calculated water solubility and lipid/water distribution coefficients, suggesting variable drug partitioning into cell membranes. Our data do not support the notion that the rate of reversibility of D₂R antagonism is what distinguishes atypical from typical antipsychotics.

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Introduction
Current antipsychotics display antagonism, or in one case functional selectivity (Urban et al., 2007), at the dopamine D₂ receptor (D₂R) and their use is frequently associated with extrapyramidal side-effects (EPS). EPS are particularly pronounced with typical antipsychotic drugs such as chlorpromazine (the first clinically used antipsychotic) and haloperidol, while being less common with atypical antipsychotics, including clozapine, quetiapine and amisulpride. A number of hypotheses have been put forward to explain these differences, one of the most widely considered being the ‘fast-off hypothesis’ which suggests that the lower EPS risk associated with atypical antipsychotics is related to their faster rates of dissociation from D₂R (Kapur and Seeman, 2001; Vauquelin et al., 2012).

Fast dissociation would produce rapidly reversible antagonism, preserving the physiological dynamics of D₂R signaling. The fast-off hypothesis has recently received much attention from several pharmaceutical companies, which has resulted in the development of rapidly dissociating D₂R ligands, including the ‘dopamine stabilizers’ ACR16 and (−)-OSU6162 (Carlsson et al., 2004; Dyhring et al., 2010; Pettersson et al., 2010), JNJ-37822681 (Langlois et al., 2012) and NS30678 (Dyhring et al., 2010). While the fast-off hypothesis is based mainly on results from radioligand dissociation experiments performed on purified D₂R-containing membranes, the functional reversibility of D₂R antagonism has received little attention. To examine the fast-off hypothesis in the context of signaling, we used an electrophysiology-based assay to compare the reversibility of D₂R antagonism by several clinically used or experimental antipsychotics. The assay is based on D₂R-evoked activation of G-protein-coupled potassium channel (GIRK) currents in live Xenopus oocytes (Sahlholm et al., 2011) and provides a greater temporal resolution than previous studies.
Materials and methods

Molecular biology

Human GIRK1 (Kir3.1) and GIRK4 (Kir3.4) cDNA (provided by Dr Terence Hebert, University of Montreal, Canada) and RGS4 (from the Missouri cDNA Resource Center; www.cdna.org) were in pCDNA3 (Invitrogen, USA), cDNA encoding the human dopamine D2S and D2L receptors were in pXOOM (a gift from Dr Søren-Peter Olesen, University of Copenhagen, Denmark). For in vitro transcription, plasmids were linearized with the appropriate restriction enzymes (GIRK1/4, NotI; RGS4 and D2S/D2L, XhoI) and transcribed in vitro using the T7 mMessage mMachine kit (Ambion, Austin, TX). cRNA concentration and purity were determined using a spectrophotometer.

Oocyte isolation and injection

Oocytes were surgically isolated from female African clawed toads, Xenopus laevis, and injected with cRNA as previously described (Sahlholm et al., 2011): 1 ng of each GIRK subunit cRNA, 40 ng of RGS4 cRNA and 0.2 ng of dopamine D2S or D2L receptor cRNA were injected per oocyte. The surgical procedures had been approved by the Swedish National Board for Laboratory Animals. RGS4 is a GTPase-accelerating protein expressed in striatal neurons (Gold et al., 1997), which speeds up the G-protein cycle such that GIRK channel activity more closely follows receptor occupancy by agonist (Benians et al., 2003).

Receptor ligands

Clozapine, dopamine, paliperidone, risperidone and (−)-sulpiride were purchased from Sigma-Aldrich (USA), asenapine, chlorpromazine, haloperidol, N-desmethylclozapine and olanzapine were from Abcam chemicals (UK), (−)-OSU6162 was from Tocris Bioscience (UK), amisulpride andquetiapine were from Axon MedChem BV (The Netherlands), (−)-3-PPP and remoxipride were gifts from Astra (Sweden), and ACR16, NS30678, and JNJ-37822681 were custom synthesized by Axon MedChem BV. (−)-3-PPP, (−)-OSU6162, ACR16, amisulpride, asenapine, chlorpromazine, dopamine, JNJ-37822681, NS30678 and remoxipride were dissolved in distilled water, whereas the other ligands were dissolved in dimethylsulfoxide (DMSO). Ligands were diluted in the recording solution to obtain the desired concentrations. The maximum final concentration of DMSO used in any experiment did not exceed 0.3% v/v.

Electrophysiology

The electrophysiological experiments were performed at room temperature (20–22 °C), 5–7 d after cRNA injection using a two-electrode voltage-clamp setup (CA-1 amplifier, Dagan, USA) as previously described (Sahlholm et al., 2011). Data were acquired at 134 Hz using pCLAMP 8 (Molecular Devices, USA) software. A high-potassium solution (64 mM NaCl, 25 mM KCl, 0.8 mM MgCl2, 0.4 mM CaCl2, 15 mM Hepes, 1 mM ascorbic acid, adjusted to pH 7.4), giving a K+ reversal potential of about −40 mV, was used for GIRK current recording. Ascorbic acid was present in order to prevent the oxidation of dopamine. Single −80 mV pulses were applied to study GIRK current responses to D2R agonist. Ligands were added to the 20 μl recording chamber by superfusion at 1.5 ml/min using a pressure-driven, computer-controlled perfusion system (SmartSquir; AutoMate Scientific, USA). At the beginning of every protocol, 100 nM dopamine was applied to provide a baseline response, after which the antipsychotic of interest was applied in the continued presence of dopamine. To obtain response recovery data, the antipsychotic ligand was applied during 125 s, after which the antipsychotic was washed out while dopamine was maintained at 100 nM. For antagonist concentration–response data, following an initial application of 100 nM, three to five increasing concentrations of the antipsychotic ligand were applied consecutively, in the continued presence of 100 nM dopamine, at 50 s intervals. For asenapine, haloperidol, paliperidone and risperidone, which displayed slow inhibition kinetics, 100 s intervals were used instead. For each oocyte, the current amplitude at the end of each antagonist application interval was normalized to the control response to 100 nM dopamine obtained in the same oocyte. The dopamine-evoked current response was determined by subtracting the basal (agonist-independent) current from the experimental record. For dopamine concentration–response data, four or five increasing concentrations of dopamine were applied at 25 s intervals, ending with a response-saturating concentration (100 μM) of dopamine (Supplementary Fig. S1). For each oocyte, the dopamine-evoked current response to each concentration was normalized to the response evoked by 100 μM dopamine obtained in the same oocyte.

Data analysis

Variable-slope sigmoidal concentration–response curves were fitted to the concentration–response data
using GraphPad Prism (Graphpad Software, USA). The following equation was used for fitting:

\[ Y = \frac{1}{1 + 10^{\log EC_{50} - X/M}}, \]

where \( Y \) is the response as a fraction of 1, \( X \) is the logarithm of the ligand concentration and \( n \) is the Hill slope. When fitting the data for quetiapine and \( N \)-desmethylclozapine, \( n \) was constrained to \(-1\).

Analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparisons post hoc test was used to compare rates (\( T_{1/2} \)) and extents of recovery between different antipsychotics, and between antipsychotics and control conditions (dopamine washout and reapplication). Water solubility (cLogS) and lipid/water distribution (cLogD) coefficients at pH 7.4 were calculated from antagonist structures using online prediction tools; ACD/I-Lab (Advanced Chemistry Development Inc., Canada; https://ilab.acdlabs.com). The correlation of the extent of recovery with cLogS and cLogD was evaluated using Spearman correlation analysis in GraphPad Prism.

Results

The reversibility of antipsychotic antagonism was quantified using two parameters: the rate of recovery of the response to 100 nM dopamine (i.e. the time to reach half-maximal recovery) and the extent of response recovery relative to a control response (Fig. 1a). The antagonist concentrations were chosen based on concentration–response relationships (Table 1; see ‘Materials and Methods’), to achieve near-maximal inhibition. Unless otherwise stated, experiments were carried out using the short isoform of the human D2R, hD2S.

Clozapine, \( N \)-desmethylclozapine and quetiapine were found to inhibit agonist-independent GIRK currents (measured in oocytes expressing GIRK1/4 subunits without D2R) by about 10% at 10 \( \mu \text{M} \) (as previously described for clozapine by Heusler et al., 2007). This inhibition was readily reversible upon washout (\( T_{1/2} = 10.1 \pm 0.8 \text{ s for clozapine} \)); however, to minimize any influence of direct GIRK inhibition on the readout of reversibility of D2R antagonism, we chose not to use these compounds at higher concentrations than 10 \( \mu \text{M} \). The other compounds had no discernible effect on agonist-independent GIRK currents at the concentrations used.

For several antagonists, the response to dopamine following antagonist washout recovered to significantly smaller amplitudes than the control response. However, in all instances where recovery was observed, the response reached near steady-state conditions before the end of the recording period.

Recovery of D2R response from antipsychotic antagonism

There was no significant recovery from antagonism by haloperidol, JNJ-37822681 and risperidone, whereas there was complete recovery (not significantly different from the control response) following (−)-OSU6162, ACR16, remoxipride, (−)-3-PPP, (−)-sulpiride and \( N \)-desmethylclozapine. Antagonism by amisulpride, chlorpromazine, clozapine, NS30678, olanzapine and quetiapine was not fully reversible (ranging from 38% to 58%) and recovery from asenapine and paliperidone was 18% and 16%, respectively (Fig. 1a, Table 1). The rates of recovery from antagonism by (−)-OSU6162, (−)-3-PPP, ACR16 and remoxipride were significantly faster than from clozapine antagonism by four- to ten-fold. The rates of recovery from amisulpride, asenapine, \( N \)-desmethylclozapine, NS30678, olanzapine and quetiapine did not differ significantly from clozapine, while recovery from chlorpromazine, paliperidone and (−)-sulpiride was significantly slower (Fig. 1a, Table 1). Surprisingly, however, the rate of recovery from chlorpromazine antagonism was less than twofold slower than from clozapine antagonism and was not significantly different from amisulpride. Similar results were obtained when the long isoform of the D2R, hD2L, was used instead of hD2S (chlorpromazine, amisulpride, clozapine, remoxipride and (−)-OSU6162 were tested; Supplementary Table S1).

Competitive and non-competitive effects of clozapine antagonism

To investigate the mechanisms behind the slower and incomplete response recovery from clozapine as compared with remoxipride, excess remoxipride was applied before and during clozapine to compete for D2R binding. Upon washout of both antagonists, the rate of recovery was similar to that with remoxipride alone; however, the extent of recovery was similar to that with clozapine alone (Fig. 1b, c). This indicates the presence of two components of clozapine antagonism: one is competitive, readily reversible, and measurable as the rate of recovery, whereas the other is non-competitive and long-lasting, resulting in partial recovery.

Extent of recovery correlates with calculated lipid/water distribution and water solubility coefficients

A plausible mechanism for such non-competitive, long-lasting antagonism is partitioning of the
antipsychotic into the cell membrane, supported by observations from recent live-cell radioligand-binding experiments (Packeu et al., 2010; Vauquelin et al., 2012). In these studies, haloperidol exhibited non-competitive binding characteristics and partitioned into membranes, whereas sulpiride did not. Clozapine however, displayed intermediate properties, similar to our own findings. Differential antagonist partitioning into membrane lipids may be due to differences in ligand lipid/water solubility (Barton et al., 1991; Van Craenenbroeck et al., 2005; Vauquelin et al., 2012). Indeed, the extent of recovery correlated negatively with the calculated lipid/water distribution coefficient (cLogD) and positively with the calculated water solubility coefficient (cLogS), indicating that highly lipophilic and less water-soluble ligands tend to allow less response recovery in our assay (Supplementary Fig. S2).

Discussion

The most surprising finding of the present study was the relatively rapid reversibility of chlorpromazine antagonism, which contrasts sharply with the slow dissociation rates reported from radioligand studies with this compound (Leysen and Gommeren, 1984; Seeman and Tallerico, 1999). Whereas earlier studies have reported differences in dissociation rates of the

**Fig. 1.** Differential rates and extents of D2R response recovery following antipsychotic washout. (a) Averaged GIRK current traces recorded in *Xenopus* oocytes expressing the dopamine D2S receptor, GIRK1/4 channels, and Regulator of G protein Signaling 4 (RGS4; see Materials and Methods), showing recovery from inhibition by ACR16, clozapine, chlorpromazine and haloperidol, as indicated. Antipsychotic recovery into the cell membrane, supported by observations from recent live-cell radioligand-binding experiments (Packeu et al., 2010; Vauquelin et al., 2012). In these studies, haloperidol exhibited non-competitive binding characteristics and partitioned into membranes, whereas sulpiride did not. Clozapine however, displayed intermediate properties, similar to our own findings. Differential antagonist partitioning into membrane lipids may be due to differences in ligand lipid/water solubility (Barton et al., 1991; Van Craenenbroeck et al., 2005; Vauquelin et al., 2012). Indeed, the extent of recovery correlated negatively with the calculated lipid/water distribution coefficient (cLogD) and positively with the calculated water solubility coefficient (cLogS), indicating that highly lipophilic and less water-soluble ligands tend to allow less response recovery in our assay (Supplementary Fig. S2).

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Means±S.E.M. of current responses measured in three to eight oocytes are shown. Whereas the current signal was sampled at 134 Hz, symbols are plotted with a 5 s interval for clarity. Labeled bars indicate application of dopamine and D2R antagonists: DA, dopamine; ant, antagonist, hal, haloperidol; cpz, chlorpromazine; clz, clozapine. (b) Averaged GIRK current traces, as in (a), showing response recovery from inhibition by 300 μM remoxipride applied alone, and when 10 μM clozapine was co-applied. Labeled bars indicate application of dopamine and D2R antagonists during the remoxipride–clozapine co-application protocol (filled squares): DA, dopamine; clz, clozapine; rem, remoxipride. (c) Bar graphs showing the increase in rate of recovery (left panel) and no change in extent of recovery (right panel) when 10 μM clozapine was co-applied with 300 μM of remoxipride, compared with 10 μM clozapine alone. Data were obtained from six to eight oocytes. Statistical significance was assessed by analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparisons post hoc test. Asterisks denote statistically significant differences in rate of recovery (T1/2) compared with clozapine alone (left panel) and extent of recovery compared with remoxipride alone (right panel): **, p<0.01; ***, p<0.001; n.s., not significant.
order of 100-fold between the typical antipsychotic chlorpromazine and the atypical drugs amisulpride, clozapine and quetiapine (Seeman and Tallerico, 1999; Seeman 2005), the present study found rates of recovery from all four drugs to differ by less than two-fold. The fact that we observed significantly faster recovery rates with some other compounds, such as remoxipride, suggests that the small difference between chlorpromazine and clozapine was not due to a limiting effect of the solution exchange rate at the D2R. There is recent evidence that dissociation rates measured in radioligand experiments might be confounded by receptor rebinding following dissociation, by drug either present in an unstirred buffer layer close to the plasma membrane or dissolved in the membrane itself (Vauquelin et al., 2012). In the present assay, the combination of continuous buffer flow and the presence of competing dopamine may to some extent prevent antagonist rebinding, and thus reveal more accurate estimates of the relative rates of antagonist reversibility. Nevertheless, it appears that drug partitioning into the cell occurred also in our study, giving rise to non-competitive effects (see below). The high temporal resolution of the present assay further allowed us to distinguish the markedly faster rates of recovery from ACR16 and (−)-OSU6162, as compared with quetiapine, differences that were not resolved by a recent functional study based on calcium release, reporting data with 5 min intervals (Dyhring et al., 2010).

Table 1. Differential rates and extents of dopamine D2S receptor response recovery following antipsychotic washout

<table>
<thead>
<tr>
<th>Compound</th>
<th>$T_{1/2}$ (s)</th>
<th>Extent of recovery (fraction of baseline response)</th>
<th>pIC50 ±S.E.M. (IC50 nM)</th>
<th>Antagonist test concentration</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (dopamine washout and re-application)</td>
<td>2.0±0.2***</td>
<td>1.09±0.04§§ §§ §§§</td>
<td>n.a.</td>
<td>n.a.</td>
<td>5</td>
</tr>
<tr>
<td>Typical antipsychotics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>89.5±8.0**</td>
<td>0.40±0.06†††</td>
<td>7.03±0.17 (93.1)</td>
<td>1 μM</td>
<td>6</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>n.d.</td>
<td>−0.08±0.18 ††† ††† ††† §§ §§ §§</td>
<td>7.80±0.06 (15.7)</td>
<td>300 nM</td>
<td>4</td>
</tr>
<tr>
<td>Atypical antipsychotics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amisulpride</td>
<td>79.5±5.5</td>
<td>0.50±0.05†††</td>
<td>7.48±0.09 (33.1)</td>
<td>1 μM</td>
<td>6</td>
</tr>
<tr>
<td>Asenapine</td>
<td>78.8±6.0</td>
<td>0.18±0.02†††</td>
<td>7.79±0.15 (16.3)</td>
<td>300 nM</td>
<td>4</td>
</tr>
<tr>
<td>Clozapine</td>
<td>53.0±7.6</td>
<td>0.45±0.05†††</td>
<td>5.86±0.13 (1387)</td>
<td>10 μM</td>
<td>8</td>
</tr>
<tr>
<td>N-desmethylclozapine</td>
<td>45.8±3.7</td>
<td>0.80±0.05§§§</td>
<td>5.84±0.09 (1457)</td>
<td>10 μM</td>
<td>4</td>
</tr>
<tr>
<td>Olanzapine</td>
<td>73.1±3.5</td>
<td>0.38±0.02†††</td>
<td>6.68±0.07 (209.2)</td>
<td>3 μM</td>
<td>6</td>
</tr>
<tr>
<td>Paliperidone</td>
<td>188.5±17.9***</td>
<td>0.16±0.03†††</td>
<td>7.90±0.08 (12.6)</td>
<td>1 μM</td>
<td>3</td>
</tr>
<tr>
<td>Remoxipride</td>
<td>12.2±0.4**</td>
<td>1.01±0.04§§§</td>
<td>6.02±0.09 (963.5)</td>
<td>10 μM</td>
<td>3</td>
</tr>
<tr>
<td>Risperidone</td>
<td>n.d.</td>
<td>−0.01±0.03††† ††† §§ §§</td>
<td>7.48±0.07 (32.9)</td>
<td>1 μM</td>
<td>5</td>
</tr>
<tr>
<td>(−)-Sulpiride</td>
<td>84.0±6.0**</td>
<td>0.80±0.07§§§</td>
<td>7.25±0.13 (56.3)</td>
<td>1 μM</td>
<td>8</td>
</tr>
<tr>
<td>Quetiapine</td>
<td>59.7±2.2</td>
<td>0.42±0.07†††</td>
<td>5.69±0.08 (2049)</td>
<td>10 μM</td>
<td>3</td>
</tr>
<tr>
<td>Experimental compounds</td>
<td></td>
<td></td>
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<tr>
<td>(−)-3PPP</td>
<td>7.8±0.9***</td>
<td>0.88±0.06§§§</td>
<td>6.48±0.08 (332.7)</td>
<td>10 μM</td>
<td>5</td>
</tr>
<tr>
<td>ACR16</td>
<td>8.2±1.8***</td>
<td>1.04±0.05§§§</td>
<td>4.60±0.05 (25270)</td>
<td>300 μM</td>
<td>4</td>
</tr>
<tr>
<td>(−)-OSU6162</td>
<td>5.4±0.5**</td>
<td>0.94±0.05§§§</td>
<td>5.26±0.10 (5491)</td>
<td>100 μM</td>
<td>4</td>
</tr>
<tr>
<td>NS30678</td>
<td>65.7±8.1</td>
<td>0.58±0.12†††</td>
<td>7.38±0.07 (41.6)</td>
<td>1 μM</td>
<td>5</td>
</tr>
<tr>
<td>JNJ-37822681</td>
<td>n.d.</td>
<td>−0.12±0.14††† ††† §§</td>
<td>6.69±0.03 (204.4)</td>
<td>3 μM</td>
<td>5</td>
</tr>
</tbody>
</table>

Data are presented as mean±S.E.M. Data were obtained from three to eight oocytes using a near-maximally effective concentration of the antagonist. The control response was determined as the response recovery following washout and re-application of dopamine. Data were analyzed by ANOVA followed by the Tukey–Kramer multiple comparisons post hoc test. n.d., not determinable; n.a., not applicable. Symbols denote statistical significance; $T_{1/2}$ statistically different from clozapine: **, $p<0.01$; ***, $p<0.001$. Extent of recovery statistically different from control: †††, $p<0.01$. Extent of recovery statistically different from clozapine: §, $p<0.05$; §§, $p<0.01$; §§§, $p<0.001$. 

Reversibility of dopamine D2 receptor antagonism
the notion that the rate of recovery from clozapine alone was not limited by the rate of solution exchange. However, the extent of recovery from clozapine antagonism was not increased under these conditions, suggesting the presence of a non-competitive mechanism. Based on this observation, and on the findings of Vauquelin and colleagues (Packeu et al., 2010; Vauquelin et al., 2012), the variable extents of recovery observed with different compounds suggest differential partitioning into cell membranes. In support of this assumption, calculated cLogS and cLogD values for the different antagonists were found to correlate with the extents of response recovery. It is also noteworthy that a greater response recovery was seen with the two antipsychotic metabolites, N-desmethylclozapine and paliperidone (9-hydroxyrisperidone), compared with the two antipsychotic antagonists have all been found to antagonize D2R arrestin recruitment and internalization (Heusler et al., 2008; Masri et al., 2008) and the weak partial agonists used in the present study, (−)-3-PPP and N-desmethylclozapine, allowed complete response recovery.

It should be noted that the radioligand study by Langlois et al. (2012) found JNJ-37822681 to dissociate faster than clozapine, and haloperidol to dissociate faster than paliperidone. Whereas the present study examined whole, live cells, Langlois et al. performed their experiments on purified membranes. It is possible that JNJ-37822681 and haloperidol undergo extensive partitioning into the cell cytoplasm and that for this reason their antagonistic effects are not readily washed out in our assay, precluding us from recording any response recovery. Partitioning of antipsychotics into cells may have important consequences in vivo (Lester et al., 2012), where further investigation is needed.

In conclusion, the present study has demonstrated a wide variability in the rates and extents of response recovery following antagonism by different antipsychotic D2R ligands. However, the small differences observed between chlorpromazine on the one hand and amisulpride, clozapine and quetiapine on the other do not support the notion that the rate of reversibility of D2R antagonism is the distinguishing feature of atypical vs. typical antipsychotics. Instead, other factors, such as engagement of serotonin receptors, functional selectivity for D2R signaling pathways, or subpopulation- or brain region-selective D2R occupancy, may be the critical determinants of antipsychotic atypicality (Ogren et al., 1994; Urban et al., 2007; Meltzer et al., 2012; Miyamoto et al., 2012).

**Supplementary material**

For supplementary material accompanying this paper, visit http://dx.doi.org/10.1017/S1461145713000801.

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**Statement of Interest**

None.

**References**


