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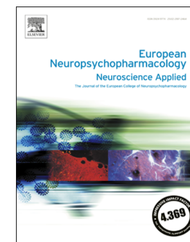

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The fast-off hypothesis revisited: A functional kinetic study of antipsychotic antagonism of the dopamine D_2 receptor

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Abstract
Newer, "atypical" antipsychotics carry a lower risk of motor side-effects than older, "typical" compounds. It has been proposed that a ~100-fold faster dissociation from the dopamine D_2 receptor (D_2R) distinguishes atypical from typical antipsychotics. Furthermore, differing antipsychotic D_2R affinities have been suggested to reflect differences in dissociation rate constants (k_{off}), while association rate constants (k_{on}) were assumed to be similar. However, it was recently demonstrated that lipophilic accumulation of ligand in the cell interior and/or membrane can cause underestimation of k_{off}, and as high-affinity D_2R antagonists are frequently lipophilic, this may have been a confounding factor in previous studies. In the present work, a functional electrophysiology assay was used to measure the recovery of dopamine-mediated D_2R responsivity from antipsychotic antagonism, using elevated concentrations of dopamine to prevent the potential bias of re-binding of lipophilic ligands. The variability of antipsychotic k_{on} was also reexamined, capitalizing on the temporal resolution of the assay. k_{on} was estimated from the experimental recordings using a simple mathematical model assumed to describe the binding process. The time course of recovery from haloperidol (typical antipsychotic) was only 6.4- to 2.5-fold slower than that of the atypical antipsychotics, amisulpride, clozapine, and quetiapine, while antipsychotic k_{on}s were found to vary more widely than previously suggested. Finally, affinities calculated using our k_{on} and k_{off} estimates correlated well with functional potency and with affinities reported from radioligand binding studies. In light of these findings, it appears unlikely that typical and atypical antipsychotics are primarily distinguished by their D2R binding kinetics.
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1. Introduction

The dopamine D_2 receptor (D_2R), a G protein-coupled receptor which signals via inhibitory $G_{i/o}$ proteins, is crucially involved in psychotic disorders such as schizophrenia. Traditionally, antipsychotics have been divided into typical (e.g., haloperidol and chlorpromazine) and atypical (e.g., clozapine, quetiapine, and amisulpride) compounds based on their propensity to generate motor side-effects (extrapyramidal symptoms; EPS). These often severe adverse effects diminish the willingness of patients to comply with medication, and thus constitute a major limitation to the clinical utility of antipsychotics.

The lower liability of the atypical antipsychotics to give rise to these side-effects has been suggested to be a consequence of their faster dissociation rates (k_{off}s) from D_2R, which would produce rapidly reversible antagonism, preserving the physiological dynamics of D_2R signaling (Kapur and Seeman, 2001). The idea that a fast k_{off} is associated with a favorable side-effect profile has prompted several pharmaceutical companies to develop new fast-dissociating D_2R antagonists (Dyhring et al., 2010; Koprich et al., 2013; Langlois et al., 2012; Pompeu et al., 2015). Moreover, radioligand dissociation experiments indicated that the wide variability (>100-fold) in D_2R affinities of clinically used antipsychotics could largely be accounted for by differences in k_{off} and since affinity, expressed as the dissociation constant K_d, is determined by the ratio of dissociation and association rate constants ($K_d = k_{off}/k_{on}$), differences in k_{on} should be negligible (Kapur and Seeman, 2000). Hence, by extension of the "fast-off" hypothesis cited above, low-affinity D_2R antagonists were postulated to be atypical antipsychotics. However, k_{on} was not measured directly, but only estimated from the ratio k_{off}/K_d (Kapur and Seeman, 2000).

We recently investigated the relative rates of D_2R response recovery from antagonism by a range of antipsychotics using a *Xenopus* oocyte electrophysiology assay (Sahlholm et al., 2014). This assay is based on the activation of G protein-coupled inwardly rectifying potassium channels (GIRK) by $G_{\beta\gamma}$ subunits liberated from $G_{\alpha i/o}$ proteins activated by the D_2R. The fraction of open channels serves as a functional readout of receptor occupancy by dopamine (DA) with second-scale temporal resolution. We reported that the differences in rates of recovery from inhibition by the typical antipsychotic, chlorpromazine, and the atypical compounds, amisulpride, clozapine, and quetiapine, were on the order of 2-fold rather than 100-fold as suggested by the proponents of the fast-off hypothesis (Kapur and Seeman, 2001). We also observed that ligands with high calculated lipophilicity and low water solubility exhibited a component of long-lasting antagonism which could not readily be overcome by replacing the extracellular buffer. This is in agreement with recent reports that lipophilic membrane accumulation and subsequent re-binding of ligand to its receptor can lead to falsely low estimates of ligand k_{off} (Packeu et al., 2010). Lipophilic ligands such as haloperidol have been shown to cross- or accumulate in-cell membranes, whereas hydrophilic ligands such as sulpiride do not (Packeu et al., 2010; Rayport and Sulzer, 1995). Thus, by diffusing from within the cell interior or laterally in the membrane, the effective concentration of antagonist might remain high at D_2R receptors on the cell surface even after it has been washed out from the extracellular buffer.

Given the impact of the "fast-off" hypothesis on ongoing efforts to create improved antipsychotics, we explored the long-lasting D_2R antagonism afforded by some highly lipophilic compounds such as haloperidol. Moreover, we reexamined the variability of k_{on} among different antipsychotics, capitalizing on the high temporal resolution of the GIRK assay. To estimate k_{on}, a mathematical expression was derived based on a simple three-state kinetic scheme, assuming binding of DA and antagonist to the same receptor site. Finally, the usefulness of our kinetic measurements was tested by calculating kinetic K_d values for the antipsychotics in question, and comparing these K_d values with inhibition constants (K_is) obtained both from equilibrium radioligand binding studies reported in the literature and from cumulative inhibition experiments using the GIRK assay.

2. Experimental procedures

2.1. 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). cDNA encoding the human dopamine D_{2S} and D_{2L} 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 (GIRK 1/4, NotI; RGS4 and D_{2S}/D_{2L}, XhoI) and transcribed in vitro using the T7 mMessage mMachine kit (Ambion, Austin, TX). cRNA concentration and purity were determined using a spectrophotometer.

2.2. Oocyte isolation and injection

Oocytes were surgically isolated from female African clawed toads, *Xenopus laevis*, or purchased from EcoCyte Bioscience (Castrop-Rauxel, Germany), and injected with cRNA as previously described (Sahlholm et al., 2011). The surgical procedures have been approved by the Swedish National Board for Laboratory Animals. 1 ng of each GIRK1/4 subunit cRNA, 40 ng of RGS4 cRNA, and 0.2 ng of dopamine D_{2S} or D_{2L} receptor cRNA were injected per oocyte.

2.3. Receptor ligands

Clozapine, DA, and paliperidone were purchased from Sigma-Aldrich (St. Louis, MO), asenapine, chlorpromazine, haloperidol, N-desmethylclozapine, and olanzapine were from Abcam chemicals (Cambridge, UK), amisulpride and quetiapine were from Axon MedChem BV (Groeningen, The Netherlands), and remoxipride was a gift from Astra (Södertälje, Sweden). JNJ-37822681 was custom synthesized by Axon MedChem BV. Amisulpride, asenapine, chlorpromazine, DA, JNJ-37822681, and remoxipride were dissolved in distilled water, whereas the other ligands were dissolved in 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.

2.4. Electrophysiology

The electrophysiological experiments were performed at room temperature (20-22 °C), 5-7 days after cRNA injection using a two-electrode voltage-clamp setup (CA-1 amplifier, Dagan, Minneapolis, MN) as previously described (Sahlholm et al., 2011). Data were acquired at 134 Hz using Molecular Devices software (pClamp 8.2). A high-potassium solution (in mM; 64 NaCl, 25 KCl, 0.8 $MgCl_2$, 0.4 $CaCl_2$, 15 HEPES, 1 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

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