Dopamine release induced by atypical antipsychotics in prefrontal cortex requires 5-HT<sub>1A</sub> receptors but not 5-HT<sub>2A</sub> receptors

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Abstract

Atypical antipsychotic drugs (APDs) increase dopamine (DA) release in prefrontal cortex (PFC), an effect probably mediated by the direct or indirect activation of the 5-HT<sub>1A</sub> receptor (5-HT<sub>1A</sub>AR). Given the very low in-vitro affinity of most APDs for 5-HT<sub>1A</sub>Rs and the large co-expression of 5-HT<sub>1A</sub>Rs and 5-HT<sub>2A</sub>Rs (5-HT<sub>2A</sub>Rs) in the PFC, this effect might result from the imbalance of 5-HT<sub>1A</sub>AR and 5-HT<sub>2A</sub>AR activation after blockade of these receptors by APDs, for which they show high affinity. Here we tested this hypothesis by examining the dependence of the APD-induced DA release in medial PFC (mPFC) on each receptor by using in-vivo microdialysis in wild-type (WT) and 5-HT<sub>1A</sub>AR and 5-HT<sub>2A</sub>AR knockout (KO) mice. Local APDs (clozapine, olanzapine, risperidone) administered by reverse dialysis induced a dose-dependent increase in mPFC DA output equally in WT and 5-HT<sub>2A</sub>AR KO mice whereas the DA increase was absent in 5-HT<sub>1A</sub>AR KO mice. To examine the relative contribution of both receptors to the clozapine-induced DA rise in mPFC, we silenced G-protein-coupled receptors (GPCRs) in vivo with N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) while 5-HT<sub>1A</sub>Rs or 5-HT<sub>2A/2C</sub>Rs in the mPFC were selectively protected with the respective antagonists WAY-100635 or ritanserin. The inactivation of GPCRs while preserving ~70% of 5-HT<sub>2A/2C</sub>Rs prevented the clozapine-induced DA rise in mPFC. In contrast, clozapine increased DA in mPFC of EEDQ-treated rats whose 5-HT<sub>1A</sub>Rs were protected (~50% of control rats). These results indicate that (1) 5-HT<sub>1A</sub>Rs are necessary for the APD-induced elevation in cortical DA transmission, and (2) this effect does not require 5-HT<sub>2A</sub>AR blockade by APDs.

Key words: Antipsychotic drugs, dopamine, prefrontal cortex, 5-HT<sub>1A</sub> receptor, 5-HT<sub>2A</sub> receptor.

Introduction

Cognitive impairment, including attention disorders, deficits in working memory and executive functions, is a central feature of schizophrenia lacking adequate treatment (Braff, 1993; Elvevag & Goldberg, 2000; Green et al. 2000). Negative symptoms are also poorly treated with current medications (Weickert et al. 2000), which, by themselves, induce this type of symptomatology (Artaloytia et al. 2006). In particular, atypical antipsychotic drugs (APDs) improve negative symptoms and quality of life more than classical antipsychotics (Sumiyoshi et al. 2006; Woodward et al. 2005). Moreover, some APDs (amisulpride, clozapine, olanzapine, risperidone) proved superior to first-generation drugs when considering their overall efficacy (Leucht et al. 2009).

The ability of some APDs to improve negative/cognitive symptoms (Bubenikova-Valesova et al. 2008; Grayson et al. 2007; Harvey et al. 2008) has been attributed to their capacity to increase dopamine (DA)-mediated transmission in the medial prefrontal cortex (mPFC) and hippocampus of experimental animals.
APDs share a higher in-vitro affinity and in-vivo occupancy of 5-HT$_2$A receptors (5-HT$_2$A Rs) vs. DA D$_2$ receptors (Meltzer et al. 1989; Nyberg et al. 1998; Stockmeier et al. 1993). 5-HT$_2$A Rs are densely expressed in PFC, mainly in projection pyramidal neurons (Amargós-Bosch et al. 2004; López-Giménez et al. 1997; Pazos et al. 1985; Santana et al. 2004), including those projecting to the ventral tegmental area (VTA) (Vázquez-Borsetti et al. 2009). 5-HT$_2$A R stimulation in PFC enhances the activity of pyramidal neurons projecting to the VTA (Puig et al. 2003, 2005) and of VTA dopaminergic neurons (Bortolozzi et al. 2005), leading to an increased mesocortical DA release (Bortolozzi et al. 2005; Gobert & Millan, 1999). Further, the 5-HT$_2$A R antagonist M100907 reduced the firing of DA neurons and DA release in mPFC (Bortolozzi et al. 2005; Minabe et al. 2001; Pehek et al. 2001). Overall, these observations are consistent with the above anatomical finding showing the existence of (a) closed mPFC-VTA loops (Carr & Sesack, 2000) and (b) the expression of 5-HT$_2$A Rs in mPFC pyramidal neurons projecting to the VTA (Vázquez-Borsetti et al. 2009). However, 5-HT$_2$A R blockade has been suggested to be necessary for APDs to enhance DA release in the mPFC (Bonaccorso et al. 2002; Ichikawa et al. 2001; Liégeois et al. 2002). In addition to 5-HT$_2$A R blockade, APDs display variable affinity for other monoamine receptors (see http://kidb.case.edu/pdsp.php; Roth et al. 2003). Agonist activity at 5-HT$_1$A receptors (5-HT$_1$A Rs) by APDs appears to contribute to their superior efficacy in treating non-psychotic symptoms (Bantick et al. 2001; Meltzer & Sumiyoshi, 2008; Millan, 2000; Sumiyoshi et al. 2001a, b; but see Rényi et al. 2001; Yasuno et al. 2003). Hence, although APDs show little or no in-vitro affinity for 5-HT$_1$A Rs ($K_i = 770$ nM for clozapine, >1000 nM for olanzapine and 490 nM for risperidone; Arnt & Skarsfeldt, 1998; Bymaster et al. 1996), these agents increase cortical DA release through 5-HT$_1$A R activation (Díaz-Mataix et al. 2005; Rollema et al. 1997).

Given the large co-expression of 5-HT$_1$A and 5-HT$_2$A receptors in PFC (Amargós-Bosch et al. 2004) and their opposite role in modulating pyramidal neuron activity (Aghajanian & Marek, 1997; Amargós-Bosch et al. 2004; Araneda & Andrade, 1991; Ashby et al. 1994; Puig et al. 2005), the apparent in-vivo action of APDs at 5-HT$_1$A Rs might be due to blockade of 5-HT$_2$A Rs in cells co-expressing both receptors, thus enhancing 5-HT$_1$A R-mediated neurotransmission. Here we examined this possibility using control mice and mice lacking 5-HT$_1$A or 5-HT$_2$A receptors. We also used an in-vivo rat model consisting in the inactivation of G-protein-coupled receptors (GPCRs) with the alkylation agent N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), while selectively protecting 5-HT$_1$A or 5-HT$_2$A/SC receptors in the mPFC (Amargós-Bosch et al. 2004).

Materials and methods

Animals

Male albino Wistar rats (250–320 g) were from Iffa Credo (France). Male homozygous 5-HT$_2$A R knockout (KO) mice were generated at Princeton University (Parks et al. 1998) and 5-HT$_2$A KO mice were generated at Columbia University (Fiorica-Howells et al. 2002). Both genotypes were gradually backcrossed to C57BL/6 background. From these initial sources, some 5-HT$_1$A R KO and 5-HT$_2$A R KO mice were transferred to develop a stable colony in our animal facilities. Wild-type (WT) mice of the same genetic background (C57BL/6) were also used. Mice were aged 10–15 wk at the time of experiments. Animals were maintained in a temperature-controlled room with a 12-h light/dark cycle (lights on 08:00 hours). Food and water were available ad libitum. Animal care followed the European Union regulations (O.J. of E.C. L358/1 18/12/1986) and was approved by the Institutional Animal Care and Use Committee of the School of Medicine, University of Barcelona.

Drugs and reagents

All reagents used were of analytical grade and were obtained from Merck (Germany). 5-HT oxalate, clozapine, 1-[2,5-dimethoxy-4-iodophenyl-2-amino-propanol] (DOI), dopamine hydrochloride, EEDQ, risperidone, ritanserin, 6-chloro-5-methyl-1-[6-(2-methylpyridin-3-yloxy) pyridin-3-yl carbamoyl] indoline (SB-242084), spiperone and N-[2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl]-N-(2-pyridyl) cyclohexane carboxamide 3 HCl (WAY-100635) were from Sigma/RBI (Spain). BAY x 3792, citalopram HBr.
and olanzapine were from Bayer, Lundbeck A/S (Denmark) and Eli Lilly (USA), respectively. To assess local effects in microdialysis experiments, drugs were dissolved in perfusion fluid [aCSF (artificial cerebrospinal fluid, mS): NaCl, 125; KCl, 2.5; CaCl₂, 1.26 and MgCl₂ 1.18] and administered by reverse dialysis at the stated concentrations (uncorrected for membrane recovery). Clozapine, olanzapine and risperidone were initially dissolved in a drop of acetic acid and diluted to appropriate concentrations in aCSF. All other drugs were dissolved in distilled water, saline or aCSF, as required. Concentrated solutions (1 mM; pH adjusted to 6.5–7 with NaHCO₃ when necessary) were stored at −80 °C and working solutions were prepared daily by dilution in aCSF. Control mice and rats were perfused with aCSF. Bars in the figures show the period of drug administration, corrected for the void volume of the system.

Microdialysis procedures

Microdialysis experiments in rats and mice were conducted as described previously (Amargós-Bosch et al. 2004; Bortolozzi et al. 2003). Rats were anaesthetized with sodium pentobarbital (60 mg/kg i.p.) and implanted with 4-mm concentric dialysis probes (Cuprophan) in the mPFC [coordinates in mm: AP +3.2, L −0.8, DV −6.0 (Paxinos & Watson, 2005)]. Experiments were performed in awake animals ~20 h after surgery. Probes were perfused with aCSF at 1.5 μl/min. After an initial 100 min stabilization period, four baseline samples were collected (20 min each) before local drug administration, and successive dialysate samples were collected. For mice, the surgical and microdialysis procedures were identical to those described for rats, except for the dose of anaesthesia (40 mg/kg i.p.), length of dialysis membrane (2 mm), and the brain coordinates (in mm) of the mPFC: AP +2.2, L −0.2, DV −3.4 (Franklin & Paxinos, 1997).

Monoamine concentration in dialysate samples was determined by HPLC with electrochemical detection (Hewlett Packard 1049; +0.75 for DA, +0.6 V for 5-HT) as described previously (Bortolozzi et al. 2003; Díaz-Mataix et al. 2005). Detection limits were 2–3 fmol for DA and 5-HT.

At the end of the experiments, animals were killed by an overdose of anaesthetic. Brains were quickly removed and frozen in dry ice before being sectioned (40 μm) with a cryostat (HM500-Om Microm, Germany) in coronal planes. Brain sections were stained with Neutral Red to verify the correct placement of probes.

Silencing of GPCRs in vivo with selective protection of 5-HT₁ARs or 5-HT₂A/₃C-Rs in mPFC

We used a previously described strategy to selectively protect 5-HT₁ARs or 5-HT₂A/₃C-Rs in mPFC from the overall inactivating effect of EEDQ on GPCRs (Amargós-Bosch et al. 2004). EEDQ was systemically administered to inactivate GPCRs while selectively protecting one or other receptor by the local perfusion of selective antagonists (WAY-100635 for 5-HT₁ARs, ritanserin for 5-HT₂A/₃C-Rs) through the microdialysis probes. EEDQ alkylates several GPCRs and inactivates their function (Gozlán et al. 1994), except those whose binding pockets are occupied. Thus, the perfusion of WAY-100635 or ritanserin in mPFC confers a selective protection of 5-HT₁ARs or 5-HT₂A/₃C-Rs in mPFC, respectively, during EEDQ treatment. These two experimental groups are designated as GPCR-silenced + 5-HT₁AR-protected and GPCR-silenced + 5-HT₂A/₃C-R-protected, respectively.

Three to four hours after implantation, microdialysis probes were perfused with WAY-100635 (300 μM) for 3 h at 1.5 μl/min (5-HT₁AR protection). One hour after starting the perfusion, EEDQ (dissolved in ethanol/water 1:1) was administered at 6 mg/kg i.p. The same procedure was applied to protect 5-HT₂A/₃C-Rs using the 5-HT₂A/₃C-R antagonist ritanserin (300 μM). Control rats received vehicle intraperitoneally and aCSF through the dialysis probes. On the following day, histological or microdialysis experiments were performed. For autoradiographic studies, 14-μm-thick coronal sections were cut, thaw-mounted onto 3-aminopropyltriethoxysilane (APTS; Sigma/RBI, Spain) coated slides, and kept at −20 °C until required. All experiments with control and EEDQ-treated rats were run in parallel.

Receptor autoradiography

To determine the extent of regional 5-HT₁AR or 5-HT₂A/₃C-R protection in EEDQ-treated rats we performed receptor autoradiography for 5-HT₁A and 5-HT₂A/₃C receptors using the ligands [³H]8-OH-DPAT (227.0 Ci/mmol) and [³H]mesulergine (83.0 Ci/mmol), respectively, from Amersham (GE Healthcare, Spain). Fresh frozen coronal sections of PFC from control and EEDQ-treated rats were used. Incubation conditions for [³H]8-OH-DPAT were as previously described (Mengod et al. 1996). Non-specific binding was defined as that remaining in presence of 10⁻⁸ M 5-HT. Incubation conditions for [³H]mesulergine were as previously described (López-Giménez et al. 2002; Pazos et al. 1985). Non-specific binding was defined as that remaining in the presence of 10⁻⁸ M.
mianserin. After incubation and washing, tissue sections were dipped in distilled, ice-cold water and dried rapidly under a cold air stream. Tissues were exposed to tritium-sensitive film (Kodak Biomax MR; Kodak, USA) together with plastic \(^{3}H\) standards for 60 d at 4 °C. All tissue sections used for quantification of receptor sites were processed simultaneously under the same conditions.

5-HT\(_{1A}\), 5-HT\(_{2A}\) and 5-HT\(_{2C}\) receptors were examined in mice brain by receptor autoradiography as previously described (López-Giménez et al. 2002; Mengod et al. 1996; Pazos et al. 1985) using (a) \(^{3}H\)8-OH-DPAT for 5-HT\(_{1A}\)Rs, (b) \(^{3}H\)mesulergine (plus \(10^{-7}\) M of the selective 5-HT\(_{2C}\)R antagonist SB242084) for 5-HT\(_{2A}\)Rs, and (c) \(^{3}H\)mesulergine (plus \(10^{-7}\) M of the 5-HT\(_{2A}\)R antagonist spiperone) for 5-HT\(_{2C}\)Rs. Quantitative analysis of the autoradiograms was done with AIS computerized image analysis system (Imaging Research Inc., Canada).

**Data and statistical analysis**

Microdialysis results are expressed as fmol/30-μl fraction for DA and 5-HT and shown in the Figures as percentages of baseline (individual means of four pre-drug fractions). Area under the curve (AUC) of selected time periods (fractions 6–16) was also calculated. Statistical analysis was performed using one- or two-way ANOVAs for repeated measures or AUC of DA or 5-HT values followed by Newman–Keuls post-hoc test.

Quantitative autoradiographic measurements obtained from the different radioligands were analysed using one-way ANOVA followed by Newman–Keuls post-hoc test or Student’s \(t\) test, as appropriate. Data are expressed as means ± S.E.M. Statistical significance has been set at the 95% confidence level (two tailed).

**Results**

**Basal values of DA and 5-HT in mPFC dialysates**

Basal extracellular levels of DA and 5-HT in dialysates from mPFC of mice and rats are shown in Table 1. Non-significant differences were found between mice genotype or between control and EEDQ-pretreated rats.

**5-HT\(_{1A}\)R and 5-HT\(_{2A}\)R KO mice: receptor autoradiography and neurochemical analysis**

The lack of 5-HT\(_{1A}\)Rs in these KO mice was previously assessed by autoradiography, electrophysiology and microdialysis procedures (Amargós-Bosch et al. 2004). Here, we extended previous autoradiographic observations. \(^{3}H\)8-OH-DPAT binding to 5-HT\(_{1A}\)Rs showed a high density in PFC, hippocampus and raphe nuclei of WT mice (Fig. 1, panels A1–A3). Homozygous 5-HT\(_{1A}\)AR KO mice showed no specific binding in either region (Fig. 1, panels A4–A6).

The absence of 5-HT\(_{2A}\)Rs in these KO mice was evaluated by receptor autoradiography and by the neurochemical response to the preferential 5-HT\(_{2A}\)R agonist DOI. Autoradiographic analysis of 5-HT\(_{2A}\)Rs revealed the presence of a strong signal in the frontal cortex and claustrum of WT mice (Fig. 2, panels A1–A2). Homozygous 5-HT\(_{2A}\)AR KO mice showed no specific binding in either region (Fig. 2, panels A3–A4). Quantitative assessments of 5-HT\(_{1A}\)R and 5-HT\(_{2C}\)R density are shown in Table 2. No genotype differences between the densities of 5-HT\(_{2C}\)Rs and 5-HT\(_{1A}\)Rs were found in receptor-rich areas such as the choroid plexus and PFC, respectively (Fig. 2b, c).

Perfusion of aCSF did not significantly alter DA and 5-HT output in the mPFC of WT and 5-HT\(_{2A}\)R KO mice (DA, \(n = 6\) and 5, respectively; 5-HT, \(n = 5\) for each genotype) (Fig. 3a, b). Local administration of DOI (100 μM for 5-HT and 300 μM for DA; see Bortolozzi et al. 2003, 2005) enhanced 5-HT and DA output in the mPFC of WT mice (Fig. 3c, d). DOI induced a maximal elevation of the 5-HT output to 205 ± 19% of baseline (\(n = 10\)) \([F(15, 135) = 7.71, p < 0.001]\). Similarly, DOI elevated DA output to 192 ± 23% of baseline (\(n = 10\)) \([F(15, 135) = 6.46, p < 0.001]\). Neither of these effects was observed when DOI was perfused in the mPFC of 5-HT\(_{1A}\)R KO mice (Fig. 3c, d).

**Effect of atypical antipsychotics on mPFC DA output in 5-HT\(_{1A}\)R and 5-HT\(_{2A}\)R KO mice**

These experiments were conducted to examine whether the increase in DA output induced by APDs...
in mPFC is primarily associated with activation of 5-HT₁ARs or with blockade of 5-HT₂ARs. Local administration of clozapine (300 μM), olanzapine (100 μM) (Díaz-Mataix et al. 2005) and risperidone (100 μM) by reverse dialysis increased the DA concentration similarly in the mPFC of WT and 5-HT₂AR KO mice. Two-way ANOVAs revealed a significant effect of time and non-significant effects of genotype and time x genotype interaction (Fig. 4a–c). The maximal effect induced by clozapine on mPFC DA output was 428 ± 52% of baseline in WT mice (n = 9) and 442 ± 54% of baseline in 5-HT₂AR KO mice (n = 9) [time effect: F(15, 240) = 37.22, p < 0.0001]. Olanzapine perfusion increased DA output to 363 ± 61% of basal values in mPFC of WT mice (n = 6) and to 348 ± 75% in 5-HT₂AR KO mice (n = 7) [time effect: F(15, 165) = 10.84, p < 0.0001], and the maximal elevation of mPFC DA release produced by risperidone was 283 ± 71% of

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**Fig. 1.** Representative autoradiograms of [³H]8-OH-DPAT binding in the brains of wild-type (WT) (A1–A3, upper panels) and 5-HT₁AR knockout (KO) mice (A4–A6, lower panels). In WT mice, note the high density for 5-HT₁ARs in (A1) prefrontal cortex [cingulated (Cg1), prelimbic (PrL), infralimbic (IL); AP: 1.78 mm], (A2) hippocampus (AP: −2.06 mm) and (A3) dorsal raphe nuclei (DR) and entorhinal cortex (Ent) (AP: −4.36 mm). Parallel sections of the corresponding null mutant mice (KO) show a lack of [³H]8-OH-DPAT binding (A4–A6). Scale bar, 1 mm.

**Fig. 2.** Representative autoradiograms of [³H]mesulergine (a, b) and [³H]8-OH-DPAT (c) binding in the brains of wild-type (WT) (upper panels) and 5-HT₂AR knockout (KO) mice (lower panels). In WT mice, note the expected high density for 5-HT₂ARs as visualized with [³H]mesulergine plus 10⁻⁷ M SB242084 in (A1) prefrontal cortex [cingulated (Cg1). Motor (M1, M2), AP: 2.10 mm] and, (A2) frontal cortex (FCx) and claustrum (CL) (AP: 0.74 mm). Parallel sections of the corresponding null mutant mice (KO) show a conspicuous lack of binding (A3-A4). No differences between genotypes were noted for 5-HT₆CRs as visualized with [³H]mesulergine plus 10⁻⁷ M spiperone in the choroid plexus (ChP) (B1-B2) nor for 5-HT₁ARs, as visualized with [³H]8-OH-DPAT in prefrontal cortex (C1–C2) in WT and 5-HT₂AR KO mice, respectively. Scale bar, 1 mm.
Table 2. 5-HT₁AR and 5-HT₂C labelling in different brain regions from WT and 5-HT₁AR KO mice

<table>
<thead>
<tr>
<th></th>
<th>5-HT₁AR [³H]8-OHPAT (fmol/mg tissue)</th>
<th>5-HT₂C [³H]Mesulergine +10⁻⁷ M spiperone (fmol/mg tissue)</th>
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<tr>
<td></td>
<td>Prefrontal cortex</td>
<td>Frontal cortex</td>
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<tr>
<td>WT</td>
<td>34.1±0.5</td>
<td>23.1±1.1</td>
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<tr>
<td>5-HT₁AR KO</td>
<td>32.5±1.0</td>
<td>23.2±1.7</td>
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5-HT₁AR labelling by [³H]8-OHPAT and 5-HT₂C labelling by [³H]mesulergine +10⁻⁷ M spiperone were measured in different brain regions including prefrontal cortex (AP: 2.1 mm), frontal cortex and claustrum (AP: ~0.74 mm) and lateral and medial choroid plexuses (AP: about −1.58 mm). Results, expressed as fmol/mg tissue, are the means±S.E.M. of 4–8 observations per mouse (n = 4) (one or two observations for each hemisphere of two consecutive sections per animal and four animals per group). Non-significant differences were observed between both genotypes (Student’s t test).

Fig. 3. Local effect of the 5-HT₁A/₂C agonist DOI (100–300 µM) on the output of 5-HT (c) and DA (d) in the mPFC of wild-type (WT) and 5-HT₁AR knockout (KO) mice. The perfusion of DOI increased 5-HT and DA levels in mPFC of WT mice (n = 10). Both effects were absent in 5-HT₁AR KO mice (n = 7–10). The administration of aCSF did not alter prefrontal 5-HT (a) and DA (b) in either genotype (n = 5–6). Data are expressed as mean ± S.E.M. See Results section for statistical analysis.

Baseline in WT mice (n = 5) and 310 ± 68 % of baseline in 5-HT₁AR KO mice (n = 5) [time effect: F(15, 120) = 10.85, p < 0.0001].

However, clozapine (300 µM), olanzapine (100 µM) and risperidone (100 µM) were unable to increase DA output in the mPFC of 5-HT₁AR KO mice (n = 4–6) (Fig. 4a–c). Two-way ANOVAs revealed significant differences in the effects of APDs between the strains of mice: (a) clozapine [genotype effect: F(2, 19) = 7.83, p < 0.01; time effect: F(15, 285) = 21.88, p < 0.0001; time × genotype interaction: F(30, 285) = 3.79, p < 0.0001], (b) olanzapine [genotype effect: F(2, 14) = 5.65, p < 0.05; time effect: F(15, 210) = 7.42, p < 0.0001; time × genotype interaction: F(30, 210) = 2.05, p < 0.001] and (c) risperidone [genotype effect: F(2, 13) = 8.66, p < 0.01; time effect: F(15, 195) = 10.41, p < 0.0001; time × genotype interaction: F(30, 195) = 3.73, p < 0.0001].

In addition, the local perfusion of clozapine and olanzapine at increasing concentrations (30–100–300 µM) significantly raised DA concentration in mPFC of both WT and 5-HT₁AR KO, but not in 5-HT₁AR KO mice in a concentration-dependent...
manner (Fig. 5a, b). Two-way ANOVAs of AUC revealed significant differences in the effects of APDs on the different genotypes: (a) clozapine [concentration effect: $F(2, 40) = 8.42, p < 0.001$; genotype effect: $F(2, 40) = 16.02, p < 0.0001$] and (b) olanzapine [concentration effect: $F(2, 31) = 5.40, p < 0.01$; genotype effect: $F(2, 31) = 11.60, p < 0.001$]. Unlike DA, the concentration of 5-HT in mPFC dialysates was similarly affected by clozapine (300 $\mu M$) and olanzapine (100 $\mu M$) in the three genotypes. A marginal reduction was noted in WT and 5-HT$_{2A}$R KO, but not in 5-HT$_{1A}$R KO mice ($p=0.09$ for clozapine; $p=0.17$ for olanzapine; one-way ANOVA of AUCs) (Fig. 5c).

**Effect of clozapine on mPFC DA output after GPCR silencing with selective protection of 5-HT$_{1A}$ or 5-HT$_{2A/C}$ receptors in rats**

To assess the involvement of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors in the clozapine-induced increase of DA output in rat PFC, we examined its effect in control
rats and in rats whose GPCRs were previously inactivated by EEDQ together with a selective protection of 5-HT1A or 5-HT2A/2C receptors in mPFC (Amargós-Bosch et al. 2004, see Methods section).

Clozapine perfusion (300 μM) increased the DA output in control rats (n = 10) to 170 ± 14% of baseline but to a much lower extent (a transient increase to 121 ± 24% of baseline) in rats with preserved 5-HT2A/2C CRs (n = 5) (Fig. 6a). Two-way ANOVAs revealed a significant group effect [F(1, 13) = 9.41, p < 0.009], time effect [F(15, 195) = 3.98, p < 0.0001] and time × group interaction [F(15, 195) = 3.23, p < 0.0001].

We also tested the role of mPFC 5-HT1A CRs in the clozapine-induced DA release in rats using the EEDQ model. 5-HT1A CRs were unilaterally protected in rat mPFC by local administration of WAY-100635 (300 μM) during EEDQ treatment (see Methods section). In this group of rats, clozapine (300 μM) elicited a significant DA elevation (299 ± 57% of baseline, n = 6) which was greater than in control rats (170 ± 14% of baseline, n = 10; see above) (Fig. 6b). Two-way ANOVAs revealed a significant effect of group [F(1, 14) = 8.17, p < 0.01], time [F(15, 210) = 14.08, p < 0.0001] and time × group interaction [F(15, 210) = 3.51, p < 0.0001].

Previous studies have shown that clozapine reversed the increase of cortical 5-HT release induced by DOI (Bortolozzi et al. 2003). Since DOI also elevates DA release in mPFC by a 5-HT2A CR-dependent mechanism (Bortolozzi et al. 2005), we examined whether clozapine was able to counteract the increase in DA output induced by DOI, despite its ability to increase DA output by itself. DOI (300 μM) was locally administered in the mPFC of control rats and of EEDQ-treated rats with protected 5-HT1A/2A/2C CRs (n = 6 and 8, respectively). DOI increased DA output in control rats and in those treated with EEDQ (with protected 5-HT1A/2A/2C CRs). Two-way ANOVAs revealed a significant effect of time [time effect: F(9, 108) = 18.88, p < 0.0001; non-significant effects of the group or time × group interactions; fractions 1–10] (Fig. 7a). The co-perfusion of clozapine (300 μM) reversed the effect of DOI on DA output in the mPFC of control rats but not of those treated with EEDQ with protected 5-HT2A/2C CRs [group effect: F(1, 12) = 11.58, p < 0.005; time effect: F(15, 180) = 8.87, p < 0.0001; time × group interaction: F(15, 180) = 3.69, p < 0.0001; fractions 1–16] suggesting that this effect does not involve blockade of 5-HT2A CRs.

To examine the involvement of 5-HT1A CRs in the clozapine-mediated reversal of DOI action on PFC DA output, we conducted additional experiments in which we evaluated the ability of the 5-HT1A agonist BAY x 3702 to antagonize the DOI-mediated DA increase. The local perfusion of 30 μM BAY x 3702 reversed the DA elevation in PFC induced by local DOI administration [group effect: F(1, 7) = 31.60, p < 0.0008; time effect: F(15, 105) = 7.26, p < 0.0001; time × group interactions: F(15, 105) = 3.04, p < 0.0001] (Fig. 7b).

**Autoradiographic examination of 5-HT1A and 5-HT2A/2C receptors in the GPCR-silencing model**

We performed additional autoradiographic experiments in rats not subjected to drug infusion (except for WAY-100635 or ritanserin administration during EEDQ treatment) to determine the site and extent of 5-HT1AR and 5-HT2A/2C-R protection in the
ANOVA indicated that the same hemisphere of control rats (n of control rats in the same hemisphere (2 contralateral (unprotected) side, 5-HT mBAY x 3702 (30 

Dialysate DA (µM) antagonized the increase of DA output in (Fig. 8). (a) The perfusion of 300 µM DOI (5-HT2A/2C agonist) increased DA output in the mPFC of control rats and of rats treated with EEDQ and whose mPFC 5-HT2A/2C R were unilaterally preserved by prior ritanserin administration. The perfusion of clozapine (300 µM) reversed the effect of DOI only in control rats (n = 6-8) but not in those with protected 5-HT2A/2C R. (b) In control rats, the local perfusion of BAY x 3702 (30 µM) antagonized the increase of DA output in mPFC induced by the local administration of DOI (300 µM) (n = 5). Data are expressed as mean ± S.E.M. See Results section for statistical analysis.

EEDQ-treated rats. EEDQ evoked a massive reduction of 5-HT1A R and 5-HT2A/2C R density (Figs. 8 and 9).

Local, unilateral ritanserin perfusion by reverse dialysis in the mPFC partially avoided 5-HT2A/2C R inactivation by EEDQ. Densities of 5-HT2A/2C Rbinding sites in the ipsilateral (protected) mPFC at AP coordinates (in mm) 3.20–3.70 ranged from 61% to 83% (mean 71 ± 4%, n = 4 rats) relative to mPFC of control rats in the same hemisphere (n = 2). In the contralateral (unprotected) side, 5-HT2A/2C R-binding sites were 31 ± 2% (n = 4 rats) relative to the mPFC in the same hemisphere of control rats (n = 2). One-way ANOVA indicated that [3H]mesulergine binding in ipsilateral mPFC of the GPCR-silenced + 5-HT2A/2C R-protected group was significantly different from contralateral mPFC (p < 0.001) (Fig. 8a, b). Coronal sections from an AP coordinate distant from local ritanserin administration (e.g. 4.20 mm), revealed a marginally significant difference between ipsilateral and contralateral sides (68 ± 3 vs. 43 ± 3% relative to PFC in the same hemispheres of control rats, respectively, p = 0.058).

Figure 9a shows autoradiograms of 5-HT1A R in PFC at AP 3.20–3.70 mm from different groups of rats: control (n = 2), GPCR-silenced (n = 2), GPCR-silenced + 5-HT2A/2C R-protected (n = 4) and GPCR-silenced + 5-HT1A R-protected (n = 4). WAY-100635 perfusion partially protected 5-HT1A R from inactivation by EEDQ. One-way ANOVA indicated that [3H]8-OH-DPAT binding in ipsilateral PFC of the GPCR-silenced + 5-HT1A R-protected group was significantly different from contralateral PFC (51 ± 7 vs. 17 ± 4% relative to ipsilateral and contralateral cortices of control rats, respectively, p < 0.001) (Fig. 9b). In this case, 5-HT1A R density was also significantly different between both ipsilateral and contralateral mPFC at AP ~4.20 mm of GPCR-silenced + 5-HT1A R-protected rats (data not shown).

Discussion

The main finding of the present study is that APDs such as clozapine, olanzapine and risperidone do not require interaction with 5-HT1A R to elevate DA release in rodent mPFC. This observation is relevant to understanding the neurobiological basis of the superior therapeutic action of these APDs in schizophrenia (Leucht et al. 2009) and may help to develop new drugs overcoming the limitations of existing treatments.

Methodological considerations

Two experimental models have been used in the present study: (a) mice lacking 5-HT1A or 5-HT2A receptors, and (b) rats, whose GPCRs were inactivated by EEDQ using selective protection of 5-HT1A or 5-HT2A/2C receptors.

The lack of 5-HT1A R in KO mice (Parks et al. 1998) was assessed by receptor autoradiography, electrophysiology and microdialysis (Amargos-Bosch et al. 2004; present study). Here we extend these observations to 5-HT1A R KO mice (Fiorica-Howells et al. 2002). A preliminary account of these data has been presented previously (Bortolozzi et al. 2007a). We show the absence of compensatory changes of 5-HT1A R and 5-HT2C R proteins in mice lacking 5-HT1A R, similarly to Popa et al. (2005) who reported an unaltered 5-HT1A R mRNA expression in 5-HT1A R KO mice. Consistent with the autoradiographic data, the preferential
5-HT2A agonist DOI did not increase 5-HT and DA release in the mPFC of 5-HT2A KO mice, an effect requiring the activation of post-synaptic 5-HT2A Rs on pyramidal cells projecting to the midbrain monoaminergic nuclei (Bortolozzi et al. 2005; Martín-Ruiz et al. 2001; Vázquez-Borsetti et al. 2009).

To examine the role of 5-HT1A and 5-HT2A/2C receptors in rat PFC, we used a previously described model (Amargós-Bosch et al. 2004), consisting in the selective protection of one or other receptor from the inactivating action of EEDQ (Battaglia et al. 1987; Gozlan et al. 1994; Keck & Lakoski, 2000) through the local administration of antagonists (WAY-100635 or ritanserin, respectively) to occupy 5-HT1A or 5-HT2A/2C receptors in mPFC during EEDQ treatment. This model is far from the specificity of KO mice yet it allows for a preliminary examination of the involvement of 5-HT1A and 5-HT2A receptors on the effects of APDs in the rat brain.

The present autoradiographic data indicate that (1) EEDQ produces a massive loss of 5-HT1A and 5-HT2A/2C receptors in vivo, and (2) the local protection of 5-HT1A or 5-HT2A/2C receptors by the respective antagonists was relatively successful, as shown by differences in receptor density between (a) ipsilateral (protected) and contralateral (unprotected) mPFCs,
and (b) the ipsilateral side in EEDQ-treated and control rats. The fact that receptor densities in protected sides were lower than in control rats may be partly due to the damage caused by the dialysis probe, which forced us to use coronal sections relatively distant from the administration site, and thus, with lower antagonist occupancy than sites close to the microdialysis probes receiving a higher antagonist concentration.

5-HT_{1A}Rs have a great sensitivity to EEDQ in vitro (Gozlan et al. 1994). The present in-vivo data are consistent with this view, since EEDQ reduced 5-HT_{1A} and 5-HT_{2A/2C} receptor densities to 15% and 30% of controls, respectively. Similar differences have been noted for DA D_{1} and D_{2} receptors (see Cox & Waszczak, 1993; Hemsley & Crocker, 2001 and references therein). Interestingly, despite measured receptor densities in protected sides being lower than 100% of controls, DOI increased DA release to the same extent in the mPFC of control rats and of those receiving EEDQ + ritanserin, indicating that local 5-HT_{2A/2C}Rs remained entirely functional using this experimental paradigm. Similar results have been reported for 5-HT_{1A}R agonists (Amargós-Bosch et al. 2004).

Role of 5-HT_{1A} and 5-HT_{2A} receptors in the APD-induced DA release

Despite the diverse pharmacological profiles of APDs (Arnt & Skarsfeldt, 1998), they share the ability...
to increase DA release in rodent mPFC through 5-HT1AR activation (Bortolozzi et al. 2007b; Dı´az-Mataix et al. 2005; Ichikawa et al. 2001; Li et al. 2009; Rollema et al. 1997, 2000). This effect was attributed to simultaneous blockade of 5-HT1A and D2 receptors (Ichikawa et al. 2001) yet it seems to depend exclusively on the activation of 5-HT1ARs in mPFC (Bortolozzi et al. 2007b; Dıazor-Mataix et al. 2005). In the present study, we further confirm these previous observations in 5-HT1AR KO mice and show that 5-HT2AAR blockade is not a requirement. Further, we extend these observations to rat mPFC, where clozapine increased local DA release in presence of ~50% of mPFC 5-HT1ARs but not in rats whose 5-HT1ARs were inactivated to 15% of controls by EEDQ treatment. The greater DA increase induced by clozapine in rats whose GPCRs were silenced by EEDQ – yet with preserved 5-HT1ARs – suggests an additional regulatory role of other receptors in the clozapine-evoked DA release (e.g. DA D2, α2-adrenoceptors) once the DA increase has been induced by 5-HT1AR stimulation. It has been suggested that WAY-100635 may also bind to DA D2 receptors in addition to 5-HT1ARs (Chemel et al. 2006; Martel et al. 2007) and therefore, some protection for D2 receptors may exist in the EEDQ + WAY-100635 model. Thus, it cannot be excluded that D2 receptors play a role in the clozapine-induced cortical DA release using this model in rat mPFC despite its effects being totally absent in 5-HT1AR KO mice (Dıazor-Mataix et al. 2005; present study).

The apparent bell-shaped dose–effect relationship of olanzapine on DA release (Fig. 5b) suggests the involvement of other prefrontal monoaminergic receptors (e.g. α2-adrenoceptors; Amarga-Bosch et al. 2003) for which olanzapine shows ns affinity (Arnt & Skarsfeldt, 1998).

The similar in-vivo DA increases in PFC produced by several APDs (Dıazor-Mataix et al. 2005; Ichikawa et al. 2001), does not bear a relationship with their in-vitro affinities for 5-HT1ARs, e.g. high for ziprasidone, low for clozapine and risperidone (yet clozapine occupies 5-HT1ARs in vivo; Chou et al. 2003) or negligible for olanzapine (Arnt & Skarsfeldt, 1998; Bymaster et al. 1996; Newman-Tancredi et al. 1998). The exact way by which APDs lacking in-vitro affinity interact in vivo with 5-HT1AR-mediated neurotransmission is unclear. The DA output induced by APDs and 5-HT1A agonists was cancelled by co-perfusion with the GABA_A antagonist bicuculline, suggesting the involvement of 5-HT1ARs in GABA interneurons (Dıazor-Mataix et al. 2005). Given the inhibitory nature of 5-HT1ARs, a preferential action of APDs on 5-HT1ARs located on GABA interneurons would eventually result in an increased excitatory cortical output to the VTA to enhance DA neuron activity (Gessa et al. 2000), an effect qualitatively similar to that of selective 5-HT1A agonists (Dıazor-Mataix et al. 2005, 2006).

The increase in mPFC DA release produced by APDs might theoretically result from an interaction between 5-HT1ARs and 5-HT1A receptors in neurons co-expressing these receptors (Amargós-Bosch et al. 2004). Thus, 5-HT2AR blockade by APDs might alter the physiological balance between 5-HT1AR and 5-HT2A receptors, resulting in an increase of 5-HT1AR-mediated neurotransmission. However, the present data do not support this possibility, since the APDs clozapine, olanzapine and risperidone increased a similar DA release in the mPFC of mice lacking 5-HT1ARs (with no alteration of 5-HT1AR density) and in WT controls. These results in mice were confirmed by rat data showing that clozapine was ineffective in enhancing DA output in the mPFC of rats whose 5-HT1ARs were inactivated (~15% of controls), yet whose 5-HT2AARs were protected (~70% of controls).

Interestingly, and despite its ability to stimulate DA release when given alone, clozapine counteracted the increase in PFC DA output induced by DOI, suggesting a different effect in PFC in basal or stimulated conditions. This pattern is similar to that observed in some electrophysiological studies, where clozapine displays a state-dependent action, reducing neuronal hyperactivity (e.g. Homayoun & Moghaddam, 2007; Kargieman et al. 2007; Schwieler & Erhardt, 2003). Thus, clozapine would activate the mesocortical DA system from basal conditions but would dampen cortical hyperactivity.

Clozapine could not reverse the effect of DOI in GPCR-silenced + 5-HT2A/2C-R-protected rats. In this group, 5-HT2ARs were entirely functional, as indicated by the local effect of DOI on DA output, comparable to that in controls. The inability of clozapine to counteract DOI’s effect in these rats suggests the involvement of other receptors, different from 5-HT1ARs, to reverse the action of DOI in control rats. Although we could not perform a systematic study, the comparable effect of clozapine and the selective 5-HT1AR agonist BAY x 3702 suggests the involvement of 5-HT1ARs.

Overall, these findings indicate that the stimulation of mesocortical DA release by APDs does not require the presence of 5-HT2ARs and suggest that these drugs activate 5-HT1ARs to enhance cortical DA neurotransmission. Alternatively, it is conceivable that other receptor–receptor interactions might explain this marked discrepancy between in-vitro and in-vivo actions of APDs at 5-HT1ARs.
**Functional consequences**

The present data, obtained in mice and rats using two different experimental models (permanent 5-HT receptor KO mice and GPCR inactivation with selective protection of 5-HT receptors) indicate that blockade of 5-HT2ARs by APDs is not a necessary step to elevate DA release and that this effect is mediated by indirect activation of 5-HT1A Rs in PFC. The molecular/cellular basis of the present in-vivo results is not known.

These observations do not preclude at all that 5-HT2AR blockade by APDs participates in their therapeutic action. Our conclusions are restricted to the role of 5-HT receptors required to enhance mesocortical DA, an effect potentially important for the actions of APDs on negative symptoms and cognitive deficits of schizophrenia patients. Given the lack of adequate treatment of these problems, further detailed studies are required to examine the ability of APDs – and in particular, of clozapine – to stimulate cortical 5-HT1A-R-mediated neurotransmission despite their low or negligible in-vitro affinity.

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**Statement of Interest**

None.

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