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Biochemical evidence that the atypical antipsychotic drugs clozapine and risperidone block 5-HT_{2C} receptors in vivo

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Abstract

Clozapine and risperidone are two atypical antipsychotic drugs which bind, among other receptors, to 5-HT_{2C} receptor subtypes. They inhibit the basal inositol phosphate production in mammalian cells expressing rat or human 5-HT_{2C} receptors. This biochemical effect is indicative of inverse agonist activity at these receptors. There is evidence that 5-HT_{2C} receptors are involved in the control of the activity of central dopaminergic system. Therefore, the effects of clozapine (5 mg/kg ip), risperidone (0.08 mg/kg ip) and of the typical antipsychotic haloperidol (0.1 mg/kg ip) were studied on the extracellular concentration of dopamine (DA) in the nucleus accumbens of chloral hydrate-anesthetized rats, using intracerebral microdialysis. When injected alone, clozapine, risperidone and haloperidol caused only small variations in DA efflux. However, clozapine and risperidone completely prevented the inhibitory action of RO 60-0175 (1 mg/kg ip), a 5-HT_{2C} receptor agonist, on DA release. On the other hand, haloperidol did not affect RO 60-0175-induced decrease in DA release. Taken together, these data indicate that clozapine and risperidone, unlike haloperidol, are capable of blocking 5-HT_{2C} receptors in the nucleus accumbens. It is concluded that the experimental model presented in this study might represent a simple and useful in vivo biochemical method to test the effect of putative atypical antipsychotic drugs on 5-HT_{2C} receptors.

Keywords: Clozapine; Haloperidol; Antipsychotic drugs; 5-HT_{2C} receptors; Dopamine release; Microdialysis

1. Introduction

Currently used antipsychotic drugs are usually divided into two main classes on the basis of their liability to induce neurological side effects after long-term treatment. Drugs defined as typical antipsychotics (e.g. chlorpromazine, haloperidol, trifluopromazine) are known to induce, following repeated administration, various extrapyramidal side effects (EPS) including Parkinson-like syndrome and tardive dyskinesia (Meltzer and Nash, 1991). On the other hand, chronic treatment with atypical antipsychotic drugs (e.g. clozapine, risperidone, sertindole, zotepine) is associated with a low incidence of neurological side effects (Meltzer and Nash, 1991). Moreover, atypical antipsychotic drugs do not increase plasma prolactin levels in humans (Meltzer and Nash, 1991). The hypothesis that typical antipsychotics produce their clinical effects, as well as EPS, by blocking dopamine (DA) D_2 receptors in the mesolimbic and nigrostriatal systems, respectively, (Meltzer and Nash, 1991) is now generally accepted. In contrast, the mechanisms responsible for the clinical effects of atypical antipsychotic drugs are still not clear. The most relevant hypothesis on the mode of action of the atypical antipsychotics is that their action depends on their interaction with central 5-HT_{2A} or 5-HT_{2C} receptor subtypes, more than with D_2 receptors (Meltzer et al., 1989; Meltzer and Nash, 1991; Roth et al., 1992). Clozapine is the prototype atypical antipsychotic drug; however, the pharmacology of this drug has become very complex, because of its affinity for various types of 5-HT, adrenergic, muscarinic, and histaminergic receptors (Roth et al., 1992, 1994, 1998; Meltzer and McGurk, 1999). Nevertheless, whilst the blockade of 5-HT receptors is probably important for the atypical profile of clozapine, the interaction with adrenergic, muscarinic, and histaminer-

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gic receptors does not seem to be relevant for its antipsychotic effect, but may determine the side effect profile of this drug (Meltzer and Nash, 1991). As already mentioned, it is currently argued that atypical antipsychotic drugs including clozapine are characterized by a low affinity for D_2 receptors as compared to 5-HT_{2A} receptors (Meltzer and Nash, 1991; Meltzer et al., 1989; Roth et al., 1992, 1998). However, a direct convincing demonstration of this heuristic model is still lacking. In as much as many atypical antipsychotic drugs bind also to 5-HT_{2C}, 5-HT_6 and 5-HT_7 receptors (Roth et al., 1992, 1998), it is conceivable that these receptors also contribute to the peculiar therapeutic effect of atypical antipsychotics. Thus, Canton et al. (1990) initially showed the high affinity of clozapine and risperidone for 5-HT_{2C} sites in the rat choroid plexus. These findings were subsequently confirmed (Kuoppamäki et al., 1993b; Schotte et al., 1993, 1996) and extended to brain sections (Schotte et al., 1993, 1996), and in transiently expressed 5-HT_{2C} receptors (Roth et al., 1992, 1998). More recently, several atypical antipsychotic drugs including clozapine and risperidone were found to possess inverse agonistic activity at human 5-HT_{2C} receptors (Herrick-Davis et al., 2000). This effect was assayed by using constitutively active mutants of 5-HT_{2C} receptors which are associated with high basal levels of intracellular inositol phosphate (IP) (Herrick-Davis et al., 1999), that are decreased by atypical but not typical antipsychotics (Herrick-Davis et al., 2000).

Substantial evidence indicates that 5-HT_{2C} receptors exert both tonic and phasic control of mesocorticolimbic dopaminergic function (Di Matteo et al., 2001). In particular, the 5-HT_{2C} receptor agonist RO 60-0175 [(*S*)-2-(chloro-5-fluoro-indol-1-yl)-1-methylethylamine 1:1 $C_4H_4O_4$] decreases in vivo DA release in the nucleus accumbens and the frontal cortex (Di Matteo et al., 1999, 2000a,b, Millan et al., 1998). Therefore, atypical antipsychotic drugs such as clozapine and risperidone might antagonize this effect, which represents a suitable parameter of central 5-HT_{2C} receptor antagonism (Di Matteo et al., 1999, 2000a,b). To test this hypothesis, extracellular levels of DA were monitored in the rat nucleus accumbens, by using intracerebral microdialysis coupled to HPLC with electrochemical detection. The ability of clozapine and risperidone to prevent RO 60-0175-induced decrease in DA outflow was assayed in anesthetized rats, and compared to the effect of the typical antipsychotic haloperidol.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (Consorzio Mario Negri Sud, Italy) weighing 340–380 g were used. The animals were kept at constant room temperature (21 ± 1 °C) and relative humidity (60 ± 5%) under a regular light/dark schedule (light 08:00–20:00 h). Food and water were freely available. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., suppl. 40, 18 Febbraio 1992) and international laws and policies (*EEC Council Directive* 86/609, OJ L 358,1, Dec. 12, 1987; *NIH Guide for the Care and Use of Laboratory Animals*, NIH Publication N. 85-23, 1985 and Guidelines for the Use of Animals in Biomedical Research, Thromb. Haemost. 58, 1078–1084, 1987).

2.2. Microdialysis

Rats were anesthetized with chloral hydrate (400 mg/kg ip) and then placed on a stereotaxic instrument (David Kopf Instruments, Tujunga, USA). Supplemental doses of anesthetic were administered intraperitoneally during the experiment. A microdialysis probe (CMA/12, 2 mm length, 500 μm outer diameter, Carnegie Medicin, Stockholm, Sweden) was implanted into the left nucleus accumbens (AP = 2.5; L = 1.4; V = − 8 from the dura surface and respect to the bregma), according to the atlas of Paxinos and Watson (1986). The probe was perfused at a constant rate of 1 μl/min by means of a microperfusion pump (Harvard Apparatus syringe infusion pump 22, USA) with an artificial cerebrospinal fluid (aCSF) composed of 147 mM Na^+, 2.7 mM K^+, 1 mM Mg^{2+}, 1.2 mM Ca^{2+}, 154.1 mM Cl^-, adjusted to pH 7.4 with 2 mM sodium-phosphate buffer. The aCSF was filtered through type GS (0.22 μm) Millipore glass filters before use. Every 20 min samples of perfusate were collected and immediately assayed by HPLC with electrochemical detection.

2.3. HPLC analysis

Dialysate samples were analyzed by reversed-phase HPLC coupled with electrochemical detection. The mobile phase was composed of 70 mM NaH_2PO_4, 0.1 mM Na_2EDTA, 0.7 mM triethylamine, 0.1 mM octylsulfonic acid, and 10% methanol, adjusted to pH 4.8 with orthophosphoric acid. This mobile phase was delivered at 1 ml/min flow rate (Pump 420, Kontron Instruments, Milano, Italy) through a Hypersil column (C18, 4.6 × 150 mm, 5 μm, Sigma Aldrich, USA). Samples were injected manually into the HPLC and detection of DA was carried out with a coulometric detector (Coulochem II, ESA, Bedford, MA, USA) coupled to a dual electrode analytic cell (model 5014). The potential of the first electrode was set at − 175 mV and the second at + 175 mV. Under these conditions, the sensitivity for DA was 0.95 pg/20 μl with a signal to noise ratio of 3:1.

2.4. Drug treatments

All pharmacological treatments were performed following the stabilization of DA levels in the perfusate. A stable baseline, defined as three consecutive samples in which

DA content varied by <10%, was generally obtained 150–180 min after the beginning of the perfusion (stabilization period). Clozapine and RO 60-0175 were dissolved in 200 μl 10% acetic acid, made up to almost required volume with 0.9% saline and brought to pH 6. Haloperidol was freshly diluted in physiological saline (NaCl 0.9%). Risperidone was dissolved in 0.9% NaCl containing 8% hydroxypropyl-β-cyclodextrin by weight and 25 mM citric acid. Control rats were injected with an equal volume of vehicle only. All drugs and vehicles were given intraperitoneally in a volume of 2 ml/kg body weight. The dose of RO 60-0175 (1 mg/kg ip) was chosen on the basis of previous studies carried out in our laboratory (Di Matteo et al., 1999, 2000a,b). The doses of clozapine (5 mg/kg ip), risperidone (0.08 mg/kg ip), and haloperidol (0.1 mg/kg ip) were chosen on the basis of a preliminary experiment showing that they caused minimal changes of basal DA release.

2.5. Histology

At the end of the experiments, the rats were perfused transcardially with 0.9% saline solution (60 ml), followed by 4% paraformaldehyde–saline solution (60 ml). The brains were removed and stored in 4% paraformaldehyde for some days. Coronal sections (40 μm) were cut using a cryostat microtome and stained with formal-thionin. The placement of the probe was verified under a microscope.

2.6. Data analysis

In dialysis experiments, DA content in each sample was expressed as percentage of the average baseline level calculated from three fractions collected before drug administration. Data correspond to mean ± S.E.M. values of the percentage obtained in each experimental group. The statistical analysis of the time course was performed by two-factor mixed analysis of variance (ANOVA; split-plot design) Treatment × Time or Pretreatment × Treatment, followed by Tukey's test to permit adequate multiple comparisons.

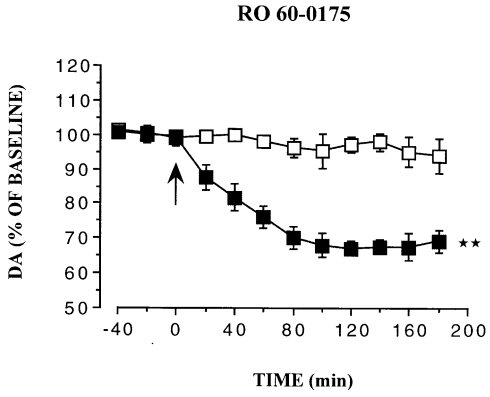

Fig. 1. Time course of the effect of intraperitoneal administration of 1 mg/kg of RO 60-0175 (■) on extracellular DA levels in the nucleus accumbens. (□) Control group treated with vehicle. RO 60-0175 was administered at the time indicated by vertical arrow. Each data point represents mean percentage ± S.E.M. of the baseline value calculated from three samples before drug injection. Each experiment was carried out on five to six animals per group. ** $P<.01$ versus control group; two-factor mixed ANOVA, split-plot design, followed by Tukey's test.

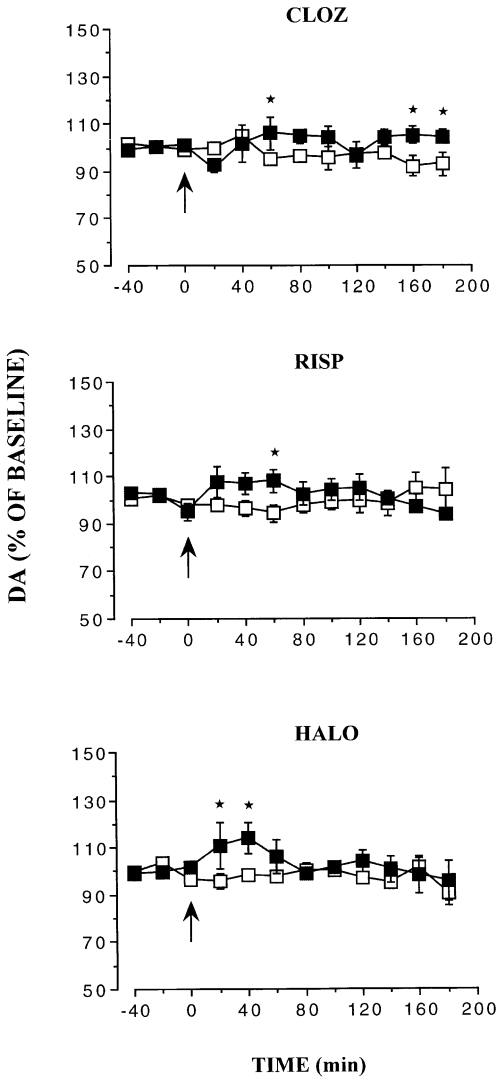

Fig. 2. Time course of the effect of intraperitoneal administration of 5 mg/kg of clozapine (CLOZ), 0.08 mg/kg risperidone (RISP) and 0.1 mg/kg haloperidol (HALO) on extracellular DA levels in the nucleus accumbens. The symbol (■) indicates treatment with antipsychotic drugs. (□) Control group treated with vehicle. Clozapine, risperidone and haloperidol were administered at the time indicated by vertical arrows. Each data point represents mean percentage ± S.E.M. of the baseline value calculated from three samples before drug injection. Each experiment was carried out on five animals per group. $P<.05$, versus control group; two-factor mixed ANOVA, split-plot design, followed by Tukey's test.

2.7. Drugs

RO 60-0175 was kindly donated by Dr. Eva-Maria Gutknecht, F. Hoffmann-La Roche, Basel, Switzerland. Clozapine was from Sandoz Pharma Ag Basel/Schweiz,

Switzerland. Haloperidol as the commercially available solution (Haldol 5 mg/ml, Janssen-Cilag S.p.A, Cologno Monzese, Milano, Italy). Risperidone was from Sigma RBI, St. Louis, MO, USA.

3. Results

Absolute basal level of dialysate DA content, taken as the mean ± S.E.M. of the three values preceding any pharmacological treatment, without considering the probe recovery, was 4.26 ± 0.05 pg/20 μl ($n = 21$) in the nucleus accumbens.

Administration of the 5-HT_{2C} receptor agonist RO 60-0175 (1 mg/kg ip) ($n = 6$) significantly decreased accumbal DA release, progressively reaching a plateau up to 120 min after its administration (− 32.9%, below baseline). As illustrated in Fig. 1, extraneuronal DA concentrations remained stably reduced for up to 3 h after treatment (two-factor mixed ANOVA, split-plot design, $F_{1,10} = 149.9$, $P < .001$). Administration of the vehicle used to dissolve RO 60-0175 did not cause any significant change in DA outflow ($n = 5$) (Fig. 1).

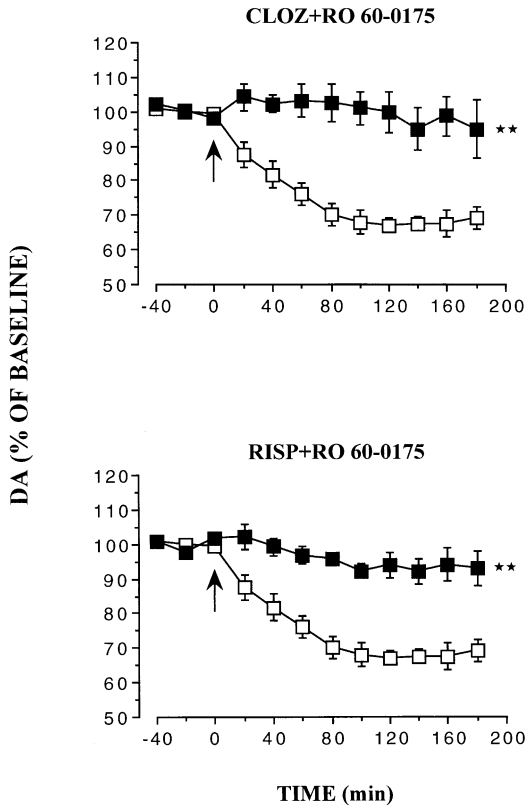

Fig. 3. Time course of the effect of RO 60-0175 (1 mg/kg IP) (□) and pretreatment (■) with clozapine (CLOZ; 5 mg/kg ip) or risperidone (RISP; 0.08 mg/kg ip) on extracellular DA levels in the nucleus accumbens. RO 60-0175 was administered at the time indicated by vertical arrows. Clozapine and risperidone were given 20 and 10 min before RO 60-0175, respectively. Each data point represents mean percentage ± S.E.M. of the baseline value calculated from three samples before RO 60-0175 injection. Each experiment was carried out on five to six animals per group. ** $P < .01$ versus RO 60-175 group alone; two-factor mixed ANOVA, split-plot design, followed by Tukey's test.

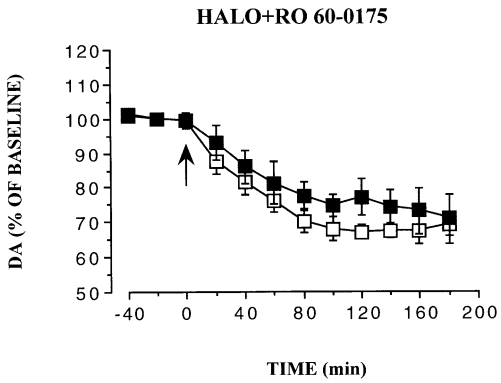

Fig. 4. Time course of the effect of RO 60-0175 (1 mg/kg IP) (□) and pretreatment (■) with haloperidol (HALO; 0.1 mg/kg ip) on extracellular DA levels in the nucleus accumbens. RO 60-0175 was administered at the time indicated by vertical arrow. Haloperidol was given 20 min before RO 60-0175. Each data point represents mean percentage ± S.E.M. of the baseline value calculated from three samples before RO 60-0175 injection. Each experiment was carried out on five animals per group. Haloperidol did not cause any significant change of RO 60-0175 effect.

As reported in Fig. 2, the intraperitoneal administration of all antipsychotic drugs used in this study, caused only modest modifications in basal DA efflux during the whole time of the experiment (3 h). In detail: clozapine (5 mg/kg ip) produced a slight increase in extracellular DA release ($F_{1,9} = 2$, two-factor mixed ANOVA, split-plot design, $n = 5$) with a significant rise (+6%, respect to baseline, $P < .05$) only at 60, 160 and 180 min after its administration. Risperidone (0.08 mg/kg ip) produced a significant increase (+8% relative to baseline) in DA release ($P < .05$) only 60 min after its injection ($F_{1,9} = 0.5$, two-factor mixed ANOVA, split-plot design, $n = 5$), while administration of haloperidol (0.1 mg/kg ip) produced a significant modification ($P < .05$) of DA release (+10% and +14%, relative to baseline, respectively), at 20 and 40 min after its injection ($F_{1,9} = 2$, two-factor mixed ANOVA, split-plot design, $n = 5$). All the vehicles used to dissolve these drugs did not cause any significant change in extraneuronal DA levels (Fig. 2).

Intraperitoneal administration of 5 mg/kg clozapine, 20 min before the 5-HT_{2C} receptor agonist RO 60-0175 (1 mg/kg ip), completely blocked its inhibitory effect on accumbal DA release ($F_{1,10} = 42$, $P < .001$; two-factor mixed ANOVA, split-plot design, treatment × pretreatment, $n = 5$) (Fig. 3). An overall significant interaction of risperidone (0.08 mg/kg ip; 10 min pretreatment) on the RO 60-0175 (1 mg/kg ip) induced extracellular DA decrease was also found ($F_{1,10} = 101.7$, $P < .001$; two-factor mixed ANOVA, split-plot design, Treatment × Pretreatment, $n = 5$) (Fig. 3).

Haloperidol (0.1 mg/kg ip; 20 min pretreatment) failed to significantly modify the decrease of DA release induced by RO 60-0175 (1 mg/kg ip) in the nucleus accumbens

($F_{1,10}=2$, not significant; two-factor mixed ANOVA, split-plot design, treatment × pretreatment, $n=5$) (Fig. 4).

4. Discussion

It is now well established that all effective antipsychotic drugs block, to some degree, D_2 receptors in the brain (Meltzer and Nash, 1991). Although the inhibitory effect of typical antipsychotics on D_2 receptors seems to correlate with their clinical action (Creese et al., 1976; Seeman et al., 1976), this effect alone cannot explain the therapeutic efficacy of atypical antipsychotic drugs. Thus, clozapine, the prototypic atypical antipsychotic drug, has a weak affinity for D_2 receptors yet it appears to be more effective than typical antipsychotics in reducing schizophrenic symptomatology (Meltzer and McGurk, 1999). Clozapine binds also to D_1, D_3, D_4 and 5-HT_{2A}, 5-HT_{2C}, 5-HT_6, 5-HT_7 receptors (Roth et al., 1992, 1994, 1998; Meltzer and McGurk, 1999). However, the relative contribution of these receptors subtype to the atypical profile of clozapine is as yet unclear. Moreover, risperidone is an effective atypical antipsychotic drug (Chouinard et al., 1993) which displays higher affinity for 5-HT_{2A} than for D_2 receptors (Schotte et al., 1996; Roth et al., 1998). On the basis of radioligand binding studies, it has been proposed that a high ratio between the affinity for 5-HT_{2A} and D_2 receptors is characteristic of atypical antipsychotic drugs (Meltzer et al., 1989; Meltzer and Nash, 1991; Roth et al., 1992, 1998). However, this proposition is only based on a phenomenological observation, and it is not clear by which mechanism this peculiar combination between 5-HT_{2A} and D_2 receptor blockade could lead to atypical antipsychotic activity. It is arguable that the unique clinical profile of atypical antipsychotic drug might result from the combination of several mechanisms involving the blockade of various receptors. In this respect, it is important to point out that atypical antipsychotic drugs, including clozapine and risperidone, bind with submicromolar affinity to rodent and human 5-HT_{2C} receptors (Canton et al., 1990; Kuoppamäki et al., 1993b; Schotte et al., 1993, 1996; Roth et al., 1992, 1998; Herrick-Davis et al., 2000). Interestingly, a number of atypical antipsychotic drugs, including clozapine and risperidone, were recently found to decrease the basal formation of inositol phosphate in mammalian cells expressing rat or human 5-HT_{2C} receptors (Herrick-Davis et al., 2000). This effect was taken as evidence of inverse agonist activity of atypical antipsychotic at 5-HT_{2C} receptors (Herrick-Davis et al., 2000). These findings prompted us to study the effect of clozapine and risperidone on in vivo DA release in the nucleus accumbens, because a number of studies carried out in our laboratory have shown that selective blockade of 5-HT_{2C} receptors enhances DA function in the mesocorticolimbic system (Di Matteo et al., 2001), a brain system which might be relevant in the pathophysiology of schizophrenia (Deutch et al., 1991). To carry out our studies, we choose the 5-HT_{2C} receptor agonist RO 60-0175 which shows nanomolar affinity for 5-HT_{2C} receptors (Martin et al., 1998). However, in vitro studies have shown that RO 60-0175 binds also to 5-HT_{2A} and 5-HT_{2B} receptors with submicromolar affinity (Martin et al., 1998; Porter et al., 1999). Nevertheless, there are several in vivo studies showing that the behavioral and biochemical effects of this drug are specifically blocked by the potent and selective 5-HT_{2C} receptor antagonist SB 242084 (Kennett et al., 1997). Thus, the discriminative stimulus properties and the hypolocomotor effect of RO 60-0175 were completely blocked by SB 242084 (Dekeyne et al., 1999; Higgins et al., 2001). Moreover, SB 242084 fully antagonized the decrease in mesolimbic dopaminergic function elicited by RO 60-0175 (Di Matteo et al., 1999; 2000a,b). It is also unlikely that 5-HT_{2B} receptors might contribute to this biochemical effect because the 5-HT_{2B} agonist BW 723C86 did not cause any significant alteration in accumbal DA release (Di Matteo et al., 2000a). The present study shows that both clozapine and risperidone completely block the decrease in accumbal DA release induced by RO 60-0175, whereas haloperidol was without any effect. Since this neurochemical effect of RO 60-0175 is mediated by 5-HT_{2C} receptors (Di Matteo et al., 1999, 2000a,b), it is possible to maintain that clozapine and risperidone, in our experimental conditions, block 5-HT_{2C} receptors. In contrast, haloperidol does not cause any modification of RO 60-0175-induced decrease in DA release, which is consistent with its low affinity for 5-HT_{2C} receptors (Canton et al., 1990; Schotte et al., 1996; Roth et al., 1992, 1998) and with its lack of inverse agonist activity at these receptors (Herrick-Davis et al., 2000). Thus, RO 60-0175-induced decrease in DA release in the nucleus accumbens might represent a simple and useful in vivo model to test the effect on central 5-HT_{2C} receptors of new antipsychotics with a putative atypical profile. However, other atypical and typical antipsychotic drugs need to be tested in these experimental conditions, in order to validate this biochemical model. Behavioral assays have already shown that clozapine can block 5-HT_{2C} receptors in vivo as indicated by the evidence that this drug attenuates hypolocomotion induced by the unselective 5-HT_{2C} receptor agonist mCPP (*m*-chlorophenylpiperazine) (Prinssen et al., 2000). However, the atypical antipsychotics risperidone and loxapine were inactive in this behavioral test (Prinssen et al., 2000), thus questioning the general validity of this model in assaying antipsychotic drugs with atypical characteristics.

It has recently been reported that atypical antipsychotics, including clozapine and risperidone cause a robust increase in DA release in the medial prefrontal cortex of freely moving rats (Kuroki et al., 1999). In contrast, typical antipsychotic drugs such a *S*-(−)-sulpiride and haloperidol were more effective in enhancing DA release in the nucleus accumbens as compared to medial prefrontal cortex (Kuroki et al., 1999). Another study carried out on awake rats, found that clozapine was much more effective in increasing DA release in the medial prefrontal cortex as compared with haloperidol, which caused a more marked increase of DA efflux in the

striatum (Westerink et al., 2001). Moreover, clozapine preferentially increased DA release in the dorsolateral prefrontal cortex of anesthesized rhesus monkeys (Youngren et al., 1999). Therefore, a preferential increase of DA release in medial prefrontal cortex seem to be a common mechanism of action of atypical antipsychotic drugs, an effect which might be relevant for their therapeutic action on negative symptoms of schizophrenia (Kuroki et al., 1999). In this respect, it is important to note that the selective 5-HT_{2C} receptor antagonist SB 242084 (Kennett et al., 1997), markedly increases DA release in the frontal cortex of awake rats (Millan et al., 1998; Gobert et al., 2000). Thus, it is possible to argue that blockade of 5-HT_{2C} receptors might contribute to the preferential effect of atypical antipsychotics on DA release in the prefrontal cortex. However, this hypothesis awaits experimental confirmation. Another important point to consider is that both typical and atypical antipsychotics exert their clinical effect only after chronic administration. Therefore, it would be of interest to test whether repeated administration of clozapine and risperidone would potentiate their inhibitory effect on central 5-HT_{2C} receptors. In this respect, it is important to report that chronic clozapine administration was found to down-regulate 5-HT_{2C} receptors in the rat choroid plexus (Kuoppamäki et al., 1993a; 1995).

In conclusion, this study shows that clozapine and risperidone, unlike haloperidol, block 5-HT_{2C} receptors in vivo. These findings are consistent with in vitro studies showing the inverse agonistic activity of clozapine and risperidone at 5-HT_{2C} receptors, and may partly explain the well-known preferential effect of atypical antipsychotic drugs on the mesocorticolimbic dopaminergic system. Although further studies are needed to confirm and extend these findings to other typical and atypical antipsychotic drugs, we propose this biochemical study as an in vivo model to test the putative effect on antipsychotic drugs on 5-HT_{2C} receptors.

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