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Selective Antagonism at Dopamine D_3 Receptors Enhances Monoaminergic and Cholinergic Neurotransmission in the Rat Anterior Cingulate Cortex

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Recent neuroanatomical and functional investigations focusing on dopamine (DA) D_3 receptors have suggested a potential role of this receptor in psychiatric diseases such as schizophrenia and drug dependence. In line with the key role of the prefrontal cortex in psychiatric disorders, the present study aimed at assessing the effects of the acute systemic administration of the selective DA D_3 receptor antagonist SB-277011-A on the *in vivo* extracellular levels of monoamines (DA, norepinephrine (NE), and serotonin (5-HT)) and acetylcholine (ACh) in the anterior cingulate subregion of the medial prefrontal cortex. The *in vivo* neurochemical profile of SB-277011-A (10 mg/kg, i.p.) in the anterior cingulate cortex was compared with both typical and atypical antipsychotics including clozapine (10 mg/kg, s.c.), olanzapine (10 mg/kg, s.c.), sulpiride (10 mg/kg, s.c.), and haloperidol (0.5 mg/kg, s.c.). The acute administration of SB-277011-A, clozapine, and olanzapine produced a significant increase in extracellular levels of DA, NE, and ACh without affecting levels of 5-HT. Sulpiride also significantly increased extracellular DA, but with a delayed onset over SB-277011-A, clozapine, and olanzapine. In contrast, haloperidol failed to alter any of the three monoamines and ACh in the anterior cingulate cortex. These findings add to a growing body of evidence suggesting a differentiation between typical and atypical antipsychotic drugs (APDs) in the anterior cingulate cortex and a role of DA D_3 receptors in desired antipsychotic drug profile. Similar to their effects on DA and NE, SB-277011-A, clozapine, and olanzapine increased extracellular levels of ACh, whereas haloperidol and sulpiride did not alter ACh. The results obtained in the present study provide evidence of the important role of DA D_3 receptors in the effect of pharmacotherapeutic agents that are used for the treatment of psychiatric disorders such as schizophrenia and drug dependence.
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INTRODUCTION

The efficacy of conventional typical antipsychotic drugs (APDs) such as haloperidol is limited mostly by their property to induce tardive dyskinesia and extrapyramidal side effects in addition to their therapeutic effects. In fact, the efficacy of neuroleptic agents has been associated with antagonism at dopamine (DA) D_2 receptors in mesolimbic and mesocortical brain areas, whereas extrapyramidal side effects have been related to antagonism at D_2 receptors in the dorsal striatum (Carlsson, 1978; Meltzer and Stahl, 1976; Seeman *et al*, 1976). The identification of novel D_2-like receptor subtypes, that is D_3 or D_4, has provided new tools to assess the mechanisms of action of APDs (for a review see Neve and Neve, 1997; Sokoloff and Schwartz, 1995) and develop new compounds that retain neuroleptic properties with reduced side effects. Atypical *vs* typical APDs can be differentiated by their effects on behavior in schizophrenic patients. While typical and atypical APDs are both effective in treating the positive symptoms of schizophrenia, atypical APDs show considerably greater efficacy in alleviating the negative symptoms (Kinon and Lieberman, 1996; Meltzer, 1996). Furthermore, atypical APDs produce less extrapyramidal motor side effects than typical APDs (Arnt and Skarsfeldt, 1998; Bunney, 1992; Casey, 1997). The etiology of negative symptoms and cognitive dysfunction of schizophrenia have been associated with dopaminergic hypofunction in the medial prefrontal cortex (mPFC) (Davis *et al*, 1991; Goldman-Rakic and Selemon, 1997; Weinberger and Lipska, 1995). It has been proposed that a correlation exists between the increase in extracellular DA in the mPFC *vs* striatum and the efficacy *vs* side effect profile of APDs (Kuroki *et al*, 1999; Moghaddam and Bunney, 1990;

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Nomikos *et al*, 1994; Pehek and Yamamoto, 1994; Volonte *et al*, 1997).

Recently, investigations focusing on DA D_3 receptors have suggested a potential role of this receptor in psychiatric disorders. This association was originally suggested from the following observations: (1) Contrary to DA D_1 and D_2 receptors, DA D_3 receptors are expressed preferentially in granule cells of the islands of Calleja and in medium-sized spiny neurons of the rostral and ventromedial shell of the nucleus accumbens, regions in which the D_2 receptors are scarcely expressed (Gurevich and Joyce, 1999; Landwehrmeyer *et al*, 1993; Murray *et al*, 1994; Sokoloff *et al*, 1990); (2) DA D_3 receptors have been functionally associated with cognitive and emotional behavior, in line with a possible role of this receptor in the negative symptoms of schizophrenia (Gurevich and Joyce, 1999; Herroelen *et al*, 1994; Suzuki *et al*, 1998); (3) the density of DA D_3 receptors is elevated in the brains of cocaine overdose fatalities (Staley and Mash, 1996); (4) D_3 receptors are overexpressed in the ventral striatum of drug-free schizophrenic patients (Gurevich *et al*, 1997), and (5) in contrast with haloperidol, the majority of clozapine-induced Fos-like immunoreactive neurons in the major island of Calleja, nucleus accumbens, and lateral septal nucleus express DA D_3 receptor mRNA (Guo *et al*, 1998). Thus, there is increased evidence to support the role of DA D_3 receptors in the pathophysiology of schizophrenia. As a result, new DA D_3 receptor antagonists with improved selectivity at D_3 over D_2 receptors have been developed, such as (+)-UH-232, (+)-A-J-76, U-991994, and l-nafadotride. However, their selectivity for D_3 over D_2 receptors is only 10- to 20-fold. In contrast, the DA D_3 receptor antagonist SB-277011-A (*trans-N*-[4-[2-(6-cyano-1,2,3,4-tetrahydroisoquinolin-2-yl)ethyl]cyclohexyl]-4-quinolininecarboxamide) shows high affinity and 100-fold selectivity for D_3 over D_2 receptors and 66 other receptors, enzymes, and ion channels (Reavill *et al*, 2000).

Accordingly, the present study aimed at assessing the effects of the acute systemic administration of the selective DA D_3 receptor antagonist, SB-277011-A, on the *in vivo* levels of monoamines and acetylcholine (ACh) in the anterior cingulate subregion of the mPFC. The *in vivo* neurochemical profile of SB-277011-A in the anterior cingulate cortex was compared against other classical APDs, including haloperidol, as a prototype typical, and clozapine, as a prototype atypical. In addition, SB-277011-A was compared with another atypical APD, olanzapine as well as the benzamide sulpiride. Benzamides such as sulpiride, amisulpiride, and remoxipride are considered as effective APD compounds that induce relatively few extrapyramidal side effects (Lewander, 1994; Peuskens *et al*, 1999). According to clinical and behavioral data, only little evidence distinguishes benzamides from the atypical APD compounds such as olanzapine, risperidone, or ziprasidone. Moreover, animal data suggest that benzamides can be classified as atypical APDs (Arnt and Skarsfeldt, 1998).

MATERIALS AND METHODS

Subjects

Male Sprague–Dawley rats (Charles River, UK Ltd) weighing 250–300 g were housed in groups of six per cage in a temperature- and humidity-controlled environment with free access to food (restricted to 20 g/day after surgery) and water. Rats were kept on a 12 h light : dark cycle with lights on at 0700 h. All experimental procedures carried out in the present study were within the guidelines of the Animals (Scientific Procedures) Act 1986.

Surgical Procedures

The animals were anaesthetized using a mixture of medetomidine® (0.04 ml/100 g, s.c.) and fentanyl® (0.9 ml/kg, i.p.). Once deep anaesthesia was obtained, rats were transferred to a stereotaxic frame (David Kopf, Tujunga, CA) with the upper incisor bar set at −3.2 mm below the interaural line. Rats were placed on a homeothermic blanket set at 37°C throughout the surgery. An incision was made into the scalp to reveal bregma, and holes were then drilled for four anchor screws, and another for unilateral placement of an intracerebral cannula guide (CMA 11, Biotech, UK) into the anterior cingulate subregion of the mPFC. The coordinates with respect to bregma were: +2.7 mm anterior (A) to bregma; 0.5 mm lateral (L) to the midsagittal sinus; 2.0 mm ventral (V) to the dura surface (Paxinos and Watson, 1986). The dura directly beneath the guide was broken, and the guide implanted. Using dental cement, the guide and a tether screw (Presearch Limited, UK) placed posterior to the probe, were secured in place, and the wound sealed. Anaesthesia was reversed using a mixture of atipamezole® (0.02 ml/100 g, s.c.) and nalbuphine® (0.02 ml/100 g, s.c.). The rats were monitored until they regained their righting reflex. The animals were allowed to recover for 1 week before commencing the dialysis experiment. At 18 h prior to the start of experiment, the animals were randomly assigned to one of six circular polycarbonate microdialysis cages (∅ 285 mm; *H*: 355 mm) and left to acclimatise to their new environment.

Brain Microdialysis Procedure

Before implantation, microdialysis probes (CMA/11, 2 mm active cuprophane membrane length, Biotech, UK) were placed in 70% ethanol, and perfused at 2–5 μl/min with artificial cerebrospinal fluid (aCSF) containing 125 mM NaCl, 2.5 mM KCl, 1.18 mM $MgCl_2 \cdot 6H_2O$, 1.26 mM $CaCl_2 \cdot 2H_2O$, and 2.0 mM Na_2HPO_4, adjusted to pH 7.4 with 85% H_3PO_4 (HPLC grade). Both inlet and outlet tubings of the probe were attached to a dual quartz lined two-channel liquid swivel (Instech 375/D/22QE, Instech lab, PA, USA) on a low mass spring counterbalanced arm, which in turn was connected to a gas tight syringe (CMA Exmire 1 ml, Biotech, UK) on a microinfusion pump (Univentor 864, Biotech, UK). The acetylcholinesterase inhibitor neostigmine chloride (Sigma, Poole, UK) was prepared in aCSF at a concentration of 100 nM. This aCSF was used to reduce the activity of acetylcholinesterase and thus increase the extracellular concentration of ACh. The animals were briefly anaesthetized with isoflurane to allow removal of the guide pin and insertion of the microdialysis probe into the guide cannula. Probes were perfused at 1 μl/min for 2 h before samples were collected. After this equilibration period, three basal samples were collected at 30 min intervals, before the animals were

administered with SB-277011-A (10 mg/kg, i.p.), clozapine (10 mg/kg, s.c.), olanzapine (10 mg/kg s.c.), haloperidol (0.5 mg/kg s.c.), and sulpiride (10 mg/kg, s.c.) or their respective vehicles. Dialysate samples were collected into glass vials (Chromacol Ltd, Welwyn Garden city, UK) containing 5 μl of 0.03% v/v acetic acid for an additional 240 min period.

Chromatographic Analysis of Brain Microdialysates

The detection of monoamines was carried out as previously described (Heidbreder *et al*, 2001a, b) by using an HPLC system composed of a Jasco 1580 pump (Jasco, Great Dunmow, UK), a Gilson 231 XL autosampler fitted with a 10 μl loop (Anachem, Luton, UK), a SSI pulse dampener (Presearch), a Decade electrochemical detector fitted with a VT03 3 mm glassy carbon cell with an *in situ* Ag/AgCl (ISAAC) reference electrode and 25 μm spacer (Antec, Leyden, The Netherlands) and a noise filter Link unit (Antec). The recorder output of the electrochemical detector was connected via the noise filter unit to Millennium^{32} version 3.04 data acquisition system (Waters, Milford, MA). Data were acquired at a rate of 2 Hz. Separations were performed using a 150×1.5 mm i.d. Capcell Pak SCX UG80 5 μm column (Phenomenex, Macclesfield, UK). The column and detector cell were housed within the Faraday cage of the electrochemical detector that was set to 40°C. A mobile phase composed of 200 mM ammonium acetate buffer (pH 6.3), containing 0.1 mM EDTA and methanol (80 : 20%, v/v) was used at a flow rate of 0.16 ml/min. Eluates were detected at an oxidation potential of 0.5 V *vs in situ* Ag/AgCl reference electrode. The filter time on the Decade and Link unit were set to 5 and 0.046 s, respectively. The limits of detection (LOD) for DA, norepinephrine (NE), and serotonin (5-HT) were found to be in the range 0.05–0.1 pg/μl with a signal-to-noise (S/N) ratio of 3 : 1.

For the ACh assay, HPLC with tandem mass spectrometry (LC/MS-MS) was performed using an Agilent 1100 HPLC system (Agilent, Bracknell, UK) composed of a binary gradient pumping system, a degasser and an autosampler. Separations were carried out using a 50×1-mm i.d. PRP-X200 10 μm, column (Hamilton, Lutterworth, UK). A mobile phase composed of 25 mM ammonium acetate and 25 mM ammonium formate (pH 4.0) mixed with acetonitrile (20 : 80 v/v) was used at a flow rate of 0.16 ml/min. The column was thermostated to 50°C. The HPLC system was coupled to an LCQ ion trap mass spectrometer (ThermoFinnigan, Warrington, UK) equipped with an electrospray ionization source. The mass spectrometer was used in the electrospray positive ion mode. All the samples were analyzed using the following parameters: ion spray voltage 4.5 kV, source temperature 300°C, capillary voltage 15 V, tube lens offset −15 V, multipole offset 1–3 V, lens −26 V, and multipole 2–9 V. Nitrogen was used as the curtain gas and auxillary gas at a pressure of 80 and 10 units, respectively. Collision-associated dissociation of ACh with helium gas was performed at collision energy of 30%. ACh was monitored using single reaction monitoring (SRM) of the ion transition precursor ion *m/z* −146 to fragment ion *m/z* 87. The precursor ion *m/z* 146 is the molecular ion $[M+H]^+$ and the fragment ion *m/z* 87 is produced from loss of the acetic acid moiety of the molecule. Data were collected and analyzed by using Excalibur 1.1 software (ThermoFinnigan, Warrington, UK). The LOD of ACh was 2 fmol/μl with an S/N ratio of 3 : 1.

Technical Note

While the three monoamines, NE, DA, and 5-HT, have been detected simultaneously using LC with electrochemical detection (Heidbreder *et al*, 2001b), ACh was quantified by using a newly developed analytical method based on LC-MS^2 detection (Hows *et al*, 2002). In comparison with the most commonly used LC methods coupled with electrochemical detection, the LC-MS assay method used in the present study is very specific, minimizing the need to separate ACh from other components present in dialysates. The limit of detection of ACh achieved by using LC-MS^2 was comparable to the detection that can be achieved using LC-ECD methods. However, obvious advantages of LC-MS^2 assays for the detection of ACh can be summarized as follows: (i) there is no enzymatic reactions needed in order to separate choline from ACh; (ii) the assay provides a means of confirming the identity of the analyte using the specific mass transition together with its chromatographic retention time, and (iii) although neostigmine was used in the present study, we recently showed (Hows *et al*, 2002) that LC-MS/MS allows ACh to be measured in dialysates without the need to add neostigmine or physostigmine to the perfusate.

Drugs

SB-277011-A (GlaxoSmithKline Pharmaceuticals, Harlow, UK) was dissolved in 10% hydoxypropyl-β-cyclodextrine (Sigma, St Louis, MO, USA) and administered in a volume of 1 ml/kg i.p. Clozapine (Tocris, Bristol, UK), olanzapine (GlaxoSmithKline Pharmaceuticals, Harlow, UK), and sulpiride (Sigma, St Louis, MO, USA) were dissolved in 0.9% saline containing a minimal amount of acetic acid, raised to pH 6.0 with NaOH, and administered in a volume of 1 ml/kg s.c. Haloperidol (GlaxoSmithKline Pharmaceuticals, Harlow, UK) was dissolved in deionized water with an equal weight of tartaric acid, then titrated to pH 6.5 using 0.5 M aqueous sodium hydroxide.

The dose of SB-277011-A has been chosen based on pharmacokinetic characteristics (Reavill *et al*, 2000; Austin *et al*, 2001) and behavioral properties reported in previous studies (Reavill *et al*, 2000; Di Ciano *et al*, 2001; Le Foll *et al*, 2002; Vorel *et al*, 2002). Doses of clozapine, olanzapine, haloperidol, and sulpiride were based on previous behavioral and *in vivo* neurochemical studies (Parada *et al*, 1997; Li *et al*, 1998; Kuroki *et al*, 1999; Heidbreder *et al*, 2001a; Ichikawa *et al*, 2002).

Histology

After completion of the final experiment, brains were removed and fixed in 4% paraformaldehyde in phosphate buffer. Histological verification of probe placement was made via serial coronal sections (40 μm thick) using a

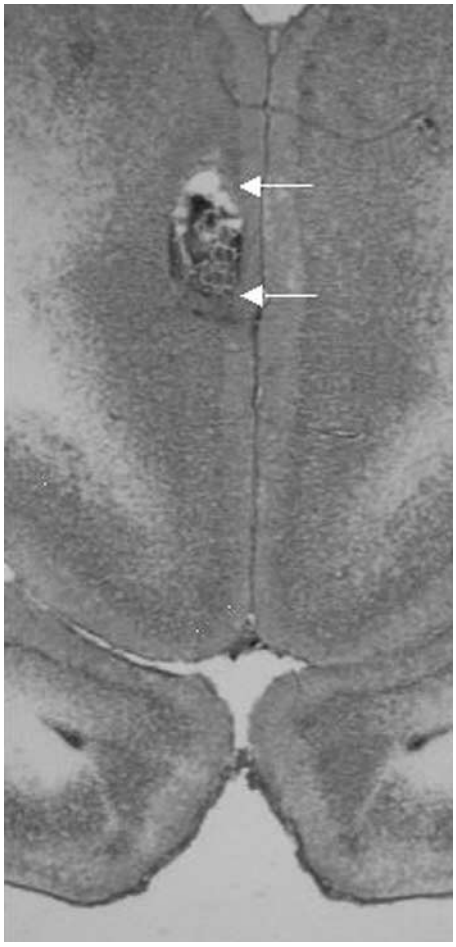

Figure 1 Representative photomicrograph of a coronal section at the level of the anterior cingulate subregion of the mPFC. The arrowheads indicate the segment of the microdialysis membrane.

cryostat. The sections were then processed for Fast cresyl violet stain (Figure 1).

Data Analysis

The data were analyzed by using analyses of variance (ANOVAs) followed by the *post hoc* Fisher's protected least significant difference pairwise comparison test when appropriate. Statistical significance was set at a probability level of $P<0.05$ for all tests. No significant differences were found between the vehicle of SB-277011-A and vehicle of clozapine (Cloz), olanzapine (Olanz), haloperidol (Hal), and sulpiride (Sulp). As a result, the data were collapsed into a single vehicle group (Veh). The average level of neurotransmitters was defined as basal dialysate levels, which were analyzed by means of one-way ANOVAs with repeated measures over time (three bins of 30 min each). The effect of drugs on extracellular levels of monoamines and ACh was analyzed by two-way ANOVAs consisting of a between-subjects factor of treatment (Veh, Hal, Sulp, Olanz, Cloz, and SB-277011-A) and a repeated measurements factor of time (eight bins of 30 min each). Finally, one-way ANOVAs with a main effect of drug treatment were used to assess the effect of drugs on the area under the curve (AUC) for each neurotransmitter.

RESULTS

Basal Extracellular Levels of Monoamines and ACh in the Anterior Cingulate Cortex

The mean ($\pm$ SEM) basal extracellular concentrations in the anterior cingulate cortex were 0.23 ± 0.02 fmol/μl for NE ($N=60$), 0.26 ± 0.04 fmol/μl for DA ($N=59$), 0.37 ± 0.12 fmol/μl for 5-HT ($N=47$), and 43.19 ± 0.54 fmol/μl for ACh ($N=37$). An ANOVA with a main factor of group and a repeated measurements factor of time (three bins of 30 min) was run to rule out any time effect as well as potential group artifact. Respective ANOVAs did not reveal any significant differences between groups (NE: $F_{5,52}=0.94$, $P=0.5$; DA: $F_{5,53}=1.15$, $P=0.34$; 5-HT: $F_{5,40}=0.2$, $P=0.97$, and ACh: $F_{5,30}=0.3$, $P=0.92$) and failed to yield any significant time × group interaction (NE: $F_{10,104}=1.4$, $P=0.2$; DA: $F_{[10,106]}=1.2$, $P=0.3$; 5-HT: $F_{[10,80]}=1.7$, $P=0.09$, and ACh: $F_{[15,90]}=0.9$, $P=0.6$), thus confirming stability of baseline over time and lack of pretreatment differences between groups.

Extracellular Levels of NE, DA, and 5-HT in the Anterior Cingulate Cortex of Vehicle-Treated Animals

The three vehicle solutions did not produce any significant changes in extracellular levels of NE, DA, 5-HT, and ACh in the anterior cingulate cortex. The overall ANOVA did not reveal any significant effect of vehicle treatment (NE: $F_{[2,22]}=0.08$, $P=0.9$; DA: $F_{[2,18]}=0.44$, $P=0.6$; 5-HT: $F_{[2,18]}=0.09$, $P=0.9$; ACh: $F_{[2,12]}=0.8$, $P=0.9$). Therefore, values from these animals were pooled for subsequent data analysis.

Effect of SB-277011-A (10 mg/kg, i.p.), Clozapine (10 mg/kg, s.c.), Olanzapine (10 mg/kg, s.c.), Haloperidol (0.5 mg/kg, s.c.), and Sulpiride (10 mg/kg, s.c.) on Extracellular Levels of NE in the Rat Anterior Cingulate Cortex

SB-277011-A, clozapine, and olanzapine induced a significant elevation in dialysate NE levels. Clozapine, olanzapine, and SB-277011-A produced their maximal increase within 60 min after drug administration. The effect was sustained for both clozapine and olanzapine, whereas the effect induced by SB-277011-A gradually decreased and reached baseline levels 180 min after the drug was administered. In contrast, neither sulpiride nor haloperidol altered dialysate NE levels (Figure 2).

Effect of SB-277011-A (10 mg/kg, i.p.), Clozapine (10 mg/kg, s.c.), Olanzapine (10 mg/kg, s.c.), Haloperidol (0.5 mg/kg, s.c.), and Sulpiride (10 mg/kg, s.c.) on Extracellular Levels of DA in the Rat Anterior Cingulate Cortex

SB-277011-A, clozapine, olanzapine, and sulpiride induced a significant elevation in dialysate levels of DA, whereas haloperidol did not alter dialysate levels of DA (Figure 3). Clozapine produced an asymptotic increase within 60 min postadministration that lasted to the end of the experiment. Both olanzapine and SB-277011-A produced their maximal increase at 60 min post-treatment and then gradually decreased to baseline levels by the end of the experiment. Finally, sulpiride produced a delayed increase in DA, which

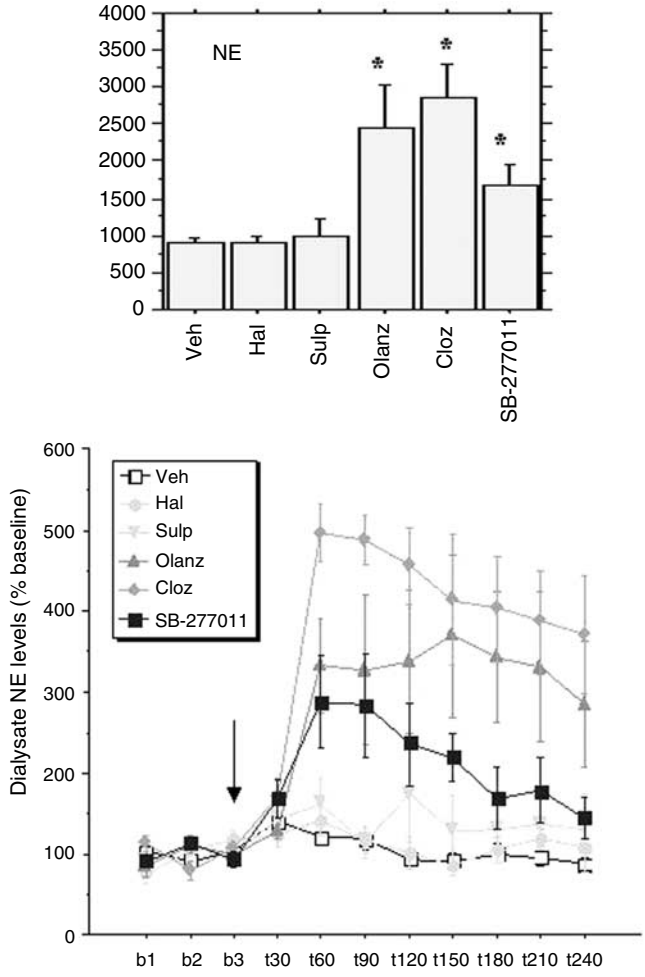

Figure 2 Time-dependent effect of SB-277011-A (10 mg/kg, i.p.; $N=7$), clozapine (Cloz) (10 mg/kg, s.c.; $N=8$), olanzapine (Olanz) (10 mg/kg, s.c.; $N=5$), haloperidol (Hal) (0.5 mg/kg, s.c.; $N=6$), and sulpiride (Sulp) (10 mg/kg, s.c.; $N=8$) on extracellular levels of NE in the rat anterior cingulate cortex (lower panel). The overall ANOVA applied to the NE data revealed a significant main effect of treatment ($F_{[5,52]}=12.91$; $P<0.001$) as well as a significant treatment × time interaction ($F_{[35,364]}=4.05$; $P<0.001$). *Post hoc* analysis revealed significant differences between Veh and Cloz ($P<0.01$), Olanz ($P<0.01$), and SB-277011-A ($P<0.01$), but no significant differences between Hal and Sulp. The upper panel represents the cumulative increase (%AUC (± SEM)) following drug administration. ANOVA applied to AUC revealed a significant main effect of treatment ($F_{[5,51]}=17.27$; $P<0.001$); *post hoc* analysis confirmed that Olanz ($P<0.001$), Cloz ($P<0.001$), and SB-277011-A ($P<0.01$) increased significantly NE release compared with both Veh and Hal. The arrow indicates time at which the drug was administered.

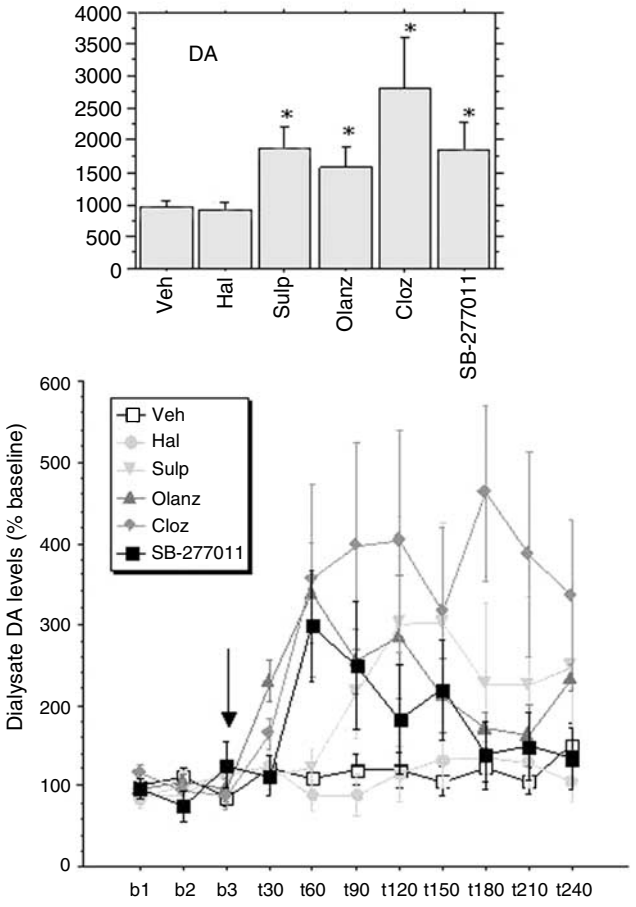

Figure 3 Time-dependent effect of SB-277011-A (10 mg/kg, i.p.; $N=7$), Cloz (10 mg/kg, s.c.; $N=8$), Olanz (10 mg/kg, s.c.; $N=5$), Hal (0.5 mg/kg, s.c.; $N=6$), and Sulp (10 mg/kg, s.c.; $N=8$) on extracellular levels of DA in the rat anterior cingulate cortex (lower panel). The overall ANOVA applied to the DA data revealed a significant main effect of treatment ($F_{[5,49]}=5.21$; $P<0.01$) as well as a significant treatment × time interaction ($F_{[35,343]}=2.65$; $P<0.001$). *Post hoc* analysis revealed significant differences between Veh and SB-277011-A ($P<0.05$), Cloz ($P<0.01$), Olanz ($P<0.05$), and Sulp ($P<0.05$), but no significant difference with Hal. The upper panel represents the cumulative increase (%AUC (± SEM)) following drug administration. ANOVA applied to AUC revealed a significant main effect of treatment ($F_{[5,53]}=4.92$; $P<0.001$); *post hoc* analysis confirmed that Sulp ($P<0.05$), Olanz ($P<0.05$), Cloz ($P<0.001$), and SB-277011-A ($P<0.05$) increased significantly DA release compared with Veh. The arrow indicates time at which the drug was administered.

started 90-min postadministration and lasted to the end of the experiment.

Effect of SB-277011-A (10 mg/kg, i.p.), Clozapine (10 mg/kg, s.c.), Olanzapine (10 mg/kg, s.c.), Haloperidol (0.5 mg/kg, s.c.), and Sulpiride (10 mg/kg, s.c.) on Extracellular Levels of 5-HT in the Rat Anterior Cingulate Cortex

There were no significant differences between dialysate levels of 5-HT with vehicle, SB-277011-A, clozapine, olanzapine, haloperidol, and sulpiride groups (Figure 4).

Effect of SB-277011-A (10 mg/kg, i.p.), Clozapine (10 mg/kg, s.c.), Olanzapine (10 mg/kg, s.c.), Haloperidol (0.5 mg/kg, s.c.), and Sulpiride (10 mg/kg, s.c.) on Extracellular Levels of ACh in the Rat Anterior Cingulate Cortex

SB-277011-A, clozapine, and olanzapine produced a significant elevation in dialysate levels of ACh, whereas neither haloperidol nor sulpiride significantly altered dialysate levels of ACh (Figure 5). Clozapine, olanzapine, and SB-271011-A produced their maximal effects on extracellular levels of ACh within 60 min postadministration and then gradually decreased by the end of the experiment.

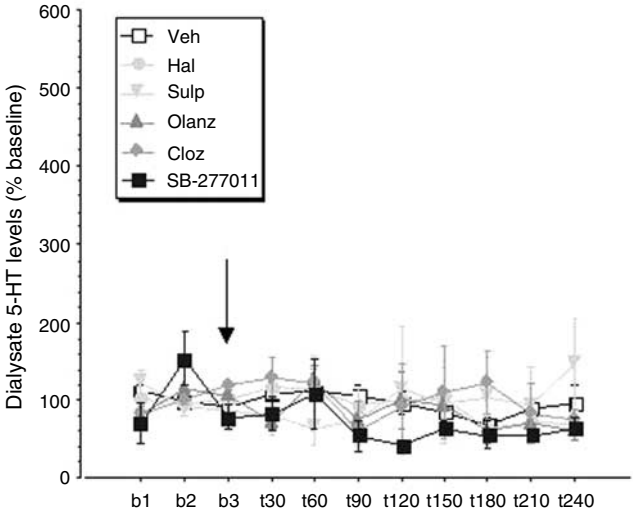

Figure 4 Time-dependent effect of SB-277011-A (10 mg/kg, i.p.; $N=4$), Cloz (10 mg/kg, s.c.; $N=4$), Olanz (10 mg/kg, s.c.; $N=6$), Hal (0.5 mg/kg, s.c.; $N=7$), and Sulp (10 mg/kg, s.c.; $N=4$) on extracellular levels of 5-HT in the rat anterior cingulate cortex (lower panel). The arrow indicates time at which the drug was administered.

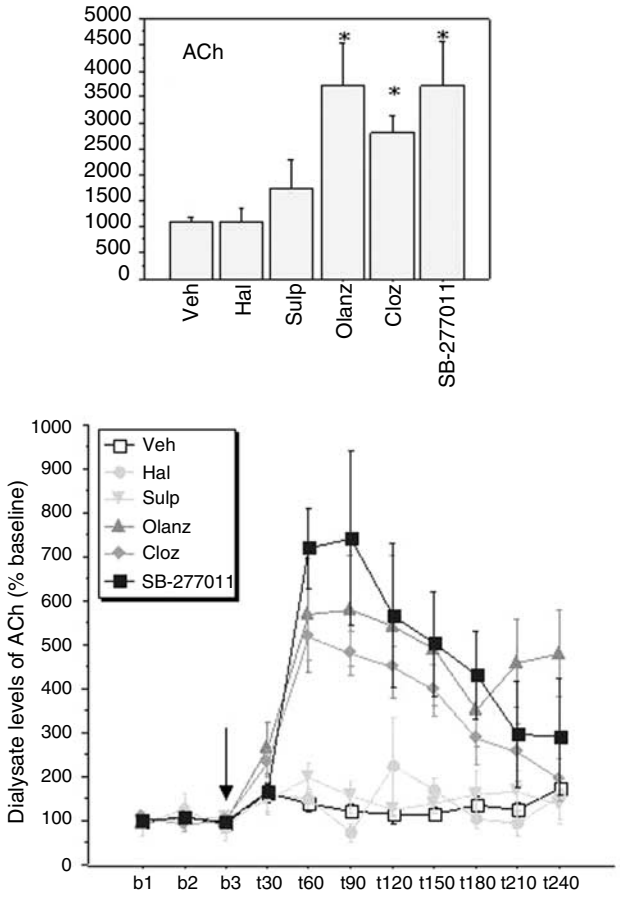

Figure 5 Time-dependent effect of SB-277011-A (10 mg/kg, i.p.; $N=5$), Cloz (10 mg/kg, s.c.; $N=5$), Olanz (10 mg/kg, s.c.; $N=5$), Hal (0.5 mg/kg, s.c.; $N=4$), and Sulp (10 mg/kg, s.c.; $N=5$) on extracellular levels of ACh in the rat anterior cingulate cortex (lower panel). The overall ANOVA applied to the ACh data revealed a significant main effect of treatment ($F_{[5,28]}=8.07$; $P<0.01$) as well as a significant treatment × time interaction ($F_{[35,196]}=5.59$; $P<0.01$). *Post hoc* analysis revealed significant differences between Veh and SB-277011-A ($P<0.01$), Cloz ($P<0.01$), and Olanz ($P<0.01$) but no significant differences with Hal and Sulp, which did not differ from each other. The upper panel represents the cumulative increase (%AUC (± SEM)) following drug administration. ANOVA applied to AUC revealed a significant main effect of treatment ($F_{[5,31]}=7.38$; $P<0.001$); *post hoc* analysis confirmed that Olanz ($P<0.001$), Cloz ($P<0.001$), and SB-277011-A ($P<0.01$) increased significantly ACh release. The arrow indicates time at which the drug was administered.

DISCUSSION

The present study aimed at investigating the profile of systemic administration of the selective D_3 receptor antagonist SB-277011-A on *in vivo* extracellular levels of monoamines and ACh in the rat anterior cingulate subregion of the mPFC. In addition, the effect of SB-277011-A was compared with the respective neurochemical profiles of both typical and atypical APDs including clozapine, olanzapine, sulpiride, and haloperidol.

Electrochemical Detection of Extracellular ACh and Monoamine Levels in the Presence of an Acetylcholinesterase Inhibitor

One controversial methodological issue related to the electrochemical detection of ACh and monoamines in microdialysates from the rat brain is the addition of acetylcholinesterase inhibitors to the perfusion fluid to improve basal recovery of ACh by hindering its enzymatic degradation (Ichikawa *et al*, 2000). The argument is that artificially increased amounts of ACh in the extracellular space are likely to increase activation of inhibitory presynaptic autoreceptors, thus decreasing subsequent ACh release from nerve terminals and possibly dampening the responsiveness of cortical cholinergic neurons to pharmacological or behavioral stimulation. Furthermore, the presence of a local acetylcholinesterase inhibitor would reduce the efficiency with which extracellular ACh is removed from the synaptic environment, potentially resulting in artificially elevated levels of cortical ACh that persist beyond the real time frame of the neuronal response. Thus, one may argue that manipulations that are associated with transient increases in ACh efflux in a physiological system may appear to elicit more long-lasting increases in the presence of a local acetylcholinesterase inhibitor. In the most recent study by Ichikawa *et al* (2002), results show that in the presence of neostigmine (0.3 μM), clozapine (20 mg/kg), but not haloperidol (1 mg/kg), produced an enhanced increased outflow of ACh compared with the no-neostigmine design. Thus, the effect of clozapine (20 mg/kg, s.c.) on dialysate ACh concentrations was potentiated two- to three-fold in the presence of 0.3 μM neostigmine compared with the increase observed in the absence of the acetylcholinesterase inhibitor. In contrast, neostigmine 0.3 μM given in the perfusion medium did not affect the inability of haloperidol (1 mg/kg, s.c.) to increase dialysate ACh concentrations in the mPFC in the absence of neostigmine. Two relevant observations can be made with regard to these results: (1) only a high concentration of neostigmine (0.3 μM) was shown to increase basal ACh levels in the mPFC (616 ± 55 *vs* 19.5 ± 0.7 fmol) and to potentiate the

effect of clozapine on ACh outflow in the mPFC up to a two- to three-fold increase; (2) in the present study, neostigmine was perfused at a low concentration of 0.1 μM that is three times lower than the concentration used in the Ichikawa study (Ichikawa *et al*, 2002). Thus, although it is reasonable to suggest that, in the present study, the presence of neostigmine in the perfusion medium produced an overestimation of basal dialysate levels of ACh (see also DeBoer and Abercrombie, 1996; Acquas and Di Chiara, 1999), it is rather unlikely that 0.1 μM neostigmine modified the dynamics and temporal pattern of drugs in a significant manner. This is further supported by evidence showing that although basal levels of ACh in the mPFC are dependent on the dose of neostigmine (0.05 μM: 0.053 ± 0.009 pmol/min *vs* 0.5 μM: 0.170 ± 0.023 pmol/min) added to the perfusion fluid, cortical ACh efflux during and following tactile stimulation is increased relative to baseline in a similar manner at these two neostigmine concentrations (0.5 *vs* 0.05 μM), suggesting that the responsiveness of cortical neurons to this tactile stimulation procedure is not compromised by artificially increased occupation of presynaptic inhibitory autoreceptors resulting from the inclusion of up to 0.5 μM of neostigmine in the perfusion fluid (Himmelheber *et al*, 1998). Furthermore, the temporal pattern of the increases in ACh efflux elicited by tactile stimulation is similar following perfusion of neostigmine at both 0.5 and 0.05 μM (Himmelheber *et al*, 1998).

It has also been argued that the presence of an acetylcholinesterase inhibitor in the perfusion fluid may affect the release of monoamines in general, DA in particular, and that the pharmacological response of striatal cholinergic neurons may be altered under such conditions. For example, it has been suggested that the stimulatory influence of DA D_1 receptors on striatal ACh release is a function of the concentration of neostigmine (0, 10, and 100 nM) in the perfusion fluid (DeBoer and Abercrombie, 1996). Furthermore, continuous perfusion with neostigmine (0, 10, 50, and 100 nM) seems to attenuate the effect of L-dopa on striatal DA release in a dose-dependent manner (Izurieta-Sanchez *et al*, 2000). However, these findings can be challenged by the observation that, in both the DeBoer and Abercrombie (1996) and Izurieta-Sanchez *et al* (2000) studies, changes in either the amount of D_1-stimulated release or in the effect of L-dopa on DA release are associated with significant changes in basal values. Thus, recalculation of these data as percent changes from basal release shows that apparent changes in the release of either ACh or DA are, in fact, independent from neostigmine concentrations in the perfusion fluid (see for example Di Chiara *et al*, 1996; Acquas and Di Chiara, 1999). These conclusions are also consistent with those of Acquas and Fibiger (1998), which showed that DA regulation of striatal ACh release is independent from neostigmine concentrations when data are expressed as percent values of basal release. That said, in order to avoid these methodological issues and potential confounding variables, there is a growing body of evidence supporting the rationale for the use of new sensitive analytical methods for the detection of ACh and choline without the use of acetylcholinesterase inhibitors in the perfusion medium (see for example Hows *et al*, 2002; Ichikawa *et al*, 2000, 2002).

A Potential Role of DA D_3 Receptors in the Effect of APDs on Anterior Cingulate DA and NE Neurotransmission Systems

The primary findings of the present study are that acute administration of SB-277011-A, clozapine, and olanzapine produced a significant increase of DA, NE, and ACh extracellular levels without affecting 5-HT levels in the anterior cingulate cortex of freely moving rats. The acute administration of sulpiride also significantly increased extracellular levels of DA, but with a delayed onset of action compared with SB-277011-A, clozapine, and olanzapine. Finally, haloperidol did not alter any of the three monoamines in the anterior cingulate cortex. These results add to a growing body of evidence suggesting a differentiation between typical and atypical APD drugs in the anterior cingulate cortex and a role of D_3 receptors in the APD drug profile.

In a previous study, Reavill *et al* (2000) showed that SB-277011-A does not affect *ex vivo* DA levels in the nucleus accumbens, striatum, or frontal cortex, but can reverse the *in vivo* quinelorane-induced decrease in DA levels in the nucleus accumbens in a dose-dependent manner. The present study extends these results by demonstrating that SB-277011-A can increase both DA and NE levels without affecting serotonergic neurotransmission in the anterior cingulate cortex. In addition, SB-277011-A displayed a so-called atypical APD profile as the effects were similar to the ones observed for both clozapine and olanzapine.

The preferential increase of DA (Kuroki *et al*, 1999; Moghaddam and Bunney, 1990; Nomikos *et al*, 1994; Pehek and Yamamoto, 1994; Volonte *et al*, 1997) and NE (Li *et al*, 1998; Westerink *et al*, 2001) following both clozapine and olanzapine, but not haloperidol treatment is in line with data from the prelimbic/infralimbic subregion of the mPFC (Li *et al*, 1998; Moghaddam and Bunney, 1990; Nomikos *et al*, 1994; Westerink *et al*, 2001). The results obtained with sulpiride are also in agreement with findings reporting that benzamides such as sulpiride or raclopride stimulate DA release in the striatum but have little effect on DA release in the mPFC (Ichikawa and Meltzer, 1999; Kuroki *et al*, 1999; Moghaddam and Bunney, 1990). In addition, consistent with the lack of effects of benzamides on NE release in the prefrontal cortex (Westerink *et al*, 2001), sulpiride did not alter levels of NE in the anterior cingulate cortex in the present study. These results are also in agreement with data obtained using inducible immediate-early gene approach to mark activated neurons and extended circuits in response to typical and atypical APDs (Kovacs *et al*, 2001; Miller, 1990; Nguyen *et al*, 1992; Robertson and Fibiger, 1992). Both typical and atypical APDs activate neurons in the nucleus accumbens. However, whereas haloperidol induces *c-fos* expression in the dorsal striatum, clozapine, and olanzapine induce *c-fos* expression in the prefrontal cortex and some limbic structures (eg lateral septal nucleus, islands of Calleja). In addition, the *c-fos* response to clozapine in the islands of Calleja and prefrontal cortex seems to be selectively mediated by the DA D_3 receptor (Guo *et al*, 1998).

The mechanisms by which selective antagonism at DA D_3 receptors can increase DA outflow in the mPFC are unknown. Haloperidol, clozapine, and olanzapine have

been shown to stimulate the percentage of burst firing and spikes per burst of VTA DA neurons antidromically identified from the mPFC (Gessa *et al*, 2000). Conversely, the acute administration of SB-277011-A (3 and 10 mg/kg) has been shown to preferentially decrease bursting activity (fewer spikes per burst) and decrease firing rate of spontaneously active VTA DA neurons over A9 DA neurons (Ashby *et al*, 2000). Altogether, these results contrast with the ability of these compounds to modify DA outflow in the mPFC. Thus, the effects of clozapine and olanzapine on extracellular levels of DA in the mPFC do not seem to depend on their stimulating effect on mesocortical DA neurons. Furthermore, the findings after the acute administration of SB-277011-A suggest that haloperidol, chlorpromazine, and clozapine do not increase the firing rate of spontaneously active DA neurons via blockade of D_3 receptors. Alternatively, the effects of these drugs may be mediated through a local action in the mPFC as suggested by the finding that local perfusion of both clozapine and olanzapine can increase DA outflow in the mPFC, whereas microinfusion of haloperidol slightly decreases DA levels (Gessa *et al*, 2000). The question of whether or not local application of SB-277011-A can modify DA efflux in the mPFC warrants further investigations.

It has been suggested that both DA and NE neurons are interacting closely in the mPFC (Carboni *et al*, 1990; Gresch *et al*, 1995; Tassin, 1992; Yamamoto and Novotney, 1998). However, data on benzamides (Westerink *et al*, 2001) together with the present results with sulpiride suggest that DA D_2 receptors are not involved in the regulation of cortical NE release. In addition, observations that the release of DA and NE in the mPFC have been observed to change independently, which suggests that modifications in the levels of the two neurotransmitter systems are correlated rather than coupled. For instance, whereas the α1-adrenoceptor antagonist prazosin induces only increases in NE levels, the β-adrenoceptor antagonist propanolol produces specific increase in DA in the mPFC (Kawahara *et al*, 2001). To date, investigations attempting to understand the mechanism of interactions between NE and DA have not yielded conclusive answers. Reuptake mechanisms have been proposed to play a critical role in DA–NE interactions in the mPFC. This idea is based on the similar affinity displayed by the noradrenaline transporter for NE and DA, which may contribute to the removal of DA from the extracellular fluid (Carboni *et al*, 1990; Tanda *et al*, 1997). Another hypothesis suggests that the anatomical connections between the locus coereleus and the ventral tegmental area (VTA) and the α1-adrenoceptors at the level of the VTA may be involved in these DA–NE interactions (Grenhoff *et al*, 1993; Tassin, 1992). Further investigations are needed to clarify this issue.

A Potential Role of DA D_3 Receptors in the Effect of APDs on Cholinergic Function in the Anterior Cingulate Cortex

Similar to their effects on DA and NE, SB-277011-A, clozapine, and olanzapine increased extracellular ACh, whereas haloperidol and sulpiride did not alter ACh levels in the anterior cingulate subregion of the mPFC. It has been shown that clozapine can increase ACh release in the mPFC, nucleus accumbens, and dorsal striatum by using microdialysis with acetylcholinesterase inhibition to increase basal ACh to detectable levels (Parada *et al*, 1997). More recently, Ichikawa *et al* (2002) have shown that atypical APDs such as clozapine (2.5–20 mg/kg), olanzapine (10 mg/kg), risperidone (1 mg/kg), and ziprasidone (3 mg/kg) can increase ACh levels in the mPFC in contrast with the typical APDs haloperidol (0.1–1 mg/kg), S(−)-sulpiride (10–25 mg/kg), and thioridazine (5–20 mg/kg), which failed to modify extracellular ACh levels in the mPFC.

Several mechanisms may account for the effects of APDs on extracellular levels of ACh in the mPFC. For example, muscarinic M_2 autoreceptor antagonism and muscarinic M_1 receptor stimulation by clozapine or muscarinic M_2 receptor antagonism by olanzapine (Bymaster *et al*, 1996) may be involved in this effect. This, however, remains unclear as thioridazine, which has affinity for M_1 and M_2 receptors comparable to that of olanzapine, fails to increase ACh levels in the mPFC (Ichikawa *et al*, 2002). Furthermore, both risperidone and ziprasidone can increase ACh levels in the mPFC (Ichikawa *et al*, 2002) despite their lack of affinity for M_1 or M_2 receptors. Finally, SB-277011-A has been shown to produce less than 40% inhibition at both M_1 and M_2 receptors (Reavill *et al*, 2000). Beyond direct effects of APDs on muscarinic cholinergic receptors, antagonism at 5-HT_{2A} receptors may be involved. A higher 5-HT_{2A}/D_2 receptor antagonism ratio, which is a common feature of atypical APDs compared with typical APDs (Kuroki *et al*, 1999; Heidbreder *et al*, 2001a), may contribute to the ability of APDs to increase ACh release in the mPFC. Recent studies have also shown a positive relation between the potency of clozapine and olanzapine to increase extracellular levels of DA in the mPFC and their respective affinities for 5-HT_{1A} over DA D_2 receptors (Heidbreder *et al*, 2001a). Interestingly, clozapine (pK_i values for D_2 and 5-HT_{1A} are 7.0 and 6.7, respectively) and SB-277011-A (pK_i values for D_2 and 5-HT_{1A} are 5.55 and 5.53, respectively) have a similar 5-HT_{1A}/D_2 ratio of 0.96 and 0.99, respectively, in contrast with haloperidol (5-HT_{1A}/D_2 ratio equivalent to 0.61). Thus, a higher 5-HT_{1A}/D_2 ratio may contribute to both increased DA and ACh outflow in the mPFC via direct and/or indirect 5-HT_{1A} receptor stimulation (Kuroki *et al*, 1999; Heidbreder *et al*, 2001a; Ichikawa *et al*, 2002).

Like other brain neurotransmitters such as DA and NE that have been implicated in cognitive functions, cortical ACh has been suggested to play an important role in cognition. More specifically, prefrontal ACh has been implicated in working memory processes as demonstrated by behavioral tasks including delayed nonmatching to sample (Broersen *et al*, 1995) and object recognition tests (Aigner *et al*, 1987; Giovannini *et al*, 1998; Scali *et al*, 1994). Furthermore, cognitive-enhancing drugs can increase ACh release in the prefrontal cortex (Scali *et al*, 1994; Yamamoto *et al*, 1994) whereas direct application of the muscarinic cholinergic receptor antagonist scopolamine into the frontal cortex (Mouton *et al*, 1988) or the hippocampus (Messer *et al*, 1991) impairs working memory. Consistent with these findings, increased ACh release has been demonstrated in the prefrontal cortex during and after performance in a delayed alternation task (Hironaka *et al*, 2001). Thus, release of ACh in the anterior cingulate cortex heightens

arousal, which in turn is required for the processing of sensory and motor information as well as spatial working memory. Taken together, these findings suggest that the increase in prefrontal ACh levels produced by SB-277011-A and both clozapine and olanzapine indicates a potential involvement of these drugs in improvement of cognitive performance. The potential role of DA D_3 receptor antagonism on memory processes has already been suggested by the finding that the relatively selective DA D_3 receptor antagonist nafadotride blocks scopolamine-induced memory disruption (Sigala *et al*, 1997). In line with these data, R(+)-7-OH-DPAT has been shown to impair passive avoidance learning through DA D_3 receptor, but not D_1 or D_2 receptors (Ukai *et al*, 1997). Furthermore, the involvement of atypical APDs in cognitive functions is suggested by clinical data reporting cognitive improvements, especially attention and verbal fluency, in schizophrenic patients treated with clozapine (Lee *et al*, 1994; Manschreck *et al*, 1999). In contrast, typical neuroleptic treatment produces only minor improvements in cognitive function (Lee *et al*, 1994). The increase in ACh outflow following atypical APD treatment is also consistent with data demonstrating the antimuscarinic properties of both clozapine and olanzapine, but not haloperidol (Bymaster *et al*, 1996). Such properties have been suggested by the antagonism of clozapine, but not haloperidol, pretreatment on oxotremorine-induced elevation in striatal ACh (Sethy *et al*, 1996). Finally, olanzapine can reduce muscarinic receptor availability in a dose-dependent manner (Raedler *et al*, 2000).

CONCLUSIONS

The results obtained in the present study support the possible implication of DA D_3 receptors in the mechanism of action of atypical APD drugs at the level of the anterior cingulate cortex. These results further support clinical data reporting overexpression of the D_3 receptor in the ventral striatum of schizophrenic patients who were free of APD medication for at least 1 month prior to death (Gurevich *et al*, 1997). Furthermore, D_3 receptor overexpression has been proposed to be responsible for the sensitization to DA agonists. Consistent with these observations, a growing body of evidence also involves the D_3 receptor in mechanisms of drug dependence and abuse: (1) DA D_3 receptors are implicated in cue-controlled modulation of cocaine seeking behavior (Pilla *et al*, 1999; Di Ciano *et al*, 2001; Vorel *et al*, 2002) and cocaine cue-conditioned hyperactivity (Le Foll *et al*, 2002); (2) DA D_3 receptor-preferring agonists generalize from the discriminative stimulus effects of cocaine (Acri *et al*, 1995); (3) DA D_3 receptor-preferring agonists can be self-administered (Caine *et al*, 1997; Caine and Koob, 1993), and (4) DA D_3 receptor-preferring agonists can produce conditioned place preference (Khroyan *et al*, 1997). Altogether these findings suggest an important role of DA D_3 receptors in the mechanisms by which atypical APDs enhance DA, NE, and ACh in the mPFC. Furthermore, the potential use of selective DA D_3 receptor antagonists as a new pharmacotherapeutic approach for the treatment of drug dependence is warranted.

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