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Long-Term Effects of S(+)N-*n*-Propylnorapomorphine Compared with Typical and Atypical Antipsychotics: Differential Increases of Cerebrocortical D_2-Like and Striatolimbic D_4-Like Dopamine Receptors

Frank I. Tarazi, Ph.D., Sylva K. Yeghiayan, Ph.D., Ross J. Baldessarini, M.D., Nora S. Kula, M.S., and John L. Neumeyer, Ph.D.

Changes in D_2-like dopamine (DA) receptor binding in rat brain regions were compared by quantitative in vitro receptor autoradiography after 21-d treatment with a typical (fluphenazine), atypical (clozapine), or candidate atypical antipsychotic (S[+]-N-n-propylnorapomorphine, [+]-NPA). Fluphenazine treatment significantly increased binding of the $D_{2,3,4}$ radioligands [^{3}H]nemonapride and [^{3}H]spiperone in caudate-putamen (CPu: 22%, 32%), nucleus accumbens (ACC: 67%, 52%), olfactory tubercle (OT: 53%, 43%), and medial prefrontal cerebral cortex (MPC: 46%, 47%) but not dorsolateral frontal cortex (DFC). D_2-like binding in MPC was also increased by (+)-NPA (49%, 39%) and clozapine (60%, 40%), but not in DFC, CPu, ACC, or OT. Binding of $D_{2,3}$-selective [^{3}H]raclopride increased less after fluphenazine in ACC (27%) and CPu (16%) than with the nonselective radioligands, and not after clozapine or (+)-NPA. D_3-selective binding of [^{3}H]R(+)-7-OH-DPAT was not changed with any treatment or region including islands of Calleja. Binding of [^{3}H]nemonapride or [^{3}H]spiperone under D_4-selective conditions (with 300 nM S[−]-raclopride and other masking agents, at sites occluded by D_4 ligand L-745,870), was increased by fluphenazine, (+)-NPA, clozapine in ACC (120%, 76%, 70%, respectively), and CPu (54%, 37%, 35%), but not in OT, DFC or MPC. These results support the hypothesis that cerebrocortical D_2-like and striatolimbic D_4-like receptors contribute to antipsychotic actions of both typical and atypical drugs and encourage further consideration of S(+)aporphines as potential atypical antipsychotics.
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From the Consolidated Department of Psychiatry and Neuroscience Program, Harvard Medical School, Boston, MA; the Mailman Research Center (FIT, SKY, RJB, NSK); the Alcohol and Drug Addiction Research Center (JLN), McLean Division of Massachusetts General Hospital, Belmont, MA; and the Bouvé College of Pharmacy and Allied Health Professions, Northeastern University, Boston, MA, USA (JLN).

Address correspondence to: Dr. Frank I. Tarazi, Mailman Research Center, McLean Hospital, Harvard Medical School, 115 Mill St., Belmont, MA 02178.

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Dopamine (DA) interacts with two groups of receptors, the D_1-like (D_1, D_5) and D_2-like (D_2, D_3, D_4, and their variants) DA receptors (Baldessarini and Tarazi 1996; Neve and Neve 1997). Interactions with DA receptors probably mediate beneficial and many adverse effects

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characteristic of antipsychotic drugs (Baldessarini 1996; Neve and Neve 1997). There is a keen interest in developing novel antipsychotics with less adverse effects but similar or even superior beneficial effects on standard agents. A major step in that direction was the prototype agent clozapine, which has an atypically limited risk of adverse extrapyramidal effects or hyperprolactinemia as well as superior antipsychotic effectiveness (Baldessarini and Frankenburg 1991). However, since use of clozapine is limited due to its high risk of potentially fatal bone marrow toxicity and other adverse effects, including excessive sedation and seizures, there is great interest in defining neuropharmacologic characteristics of clozapine that may lead to safer, improved antipsychotics.

Actions of clozapine that may contribute to its unique properties include antagonistic effects at serotonin 5-HT_2 and muscarinic acetylcholine receptors, balanced with moderate affinity for both D_1 and D_2 receptors (Baldessarini and Frankenburg 1991; Meltzer 1991; Brunello et al. 1995; Baldessarini 1996). Clozapine also shows somewhat greater affinity for D_4 than for other DA receptors (Van Tol et al. 1991; Roth et al. 1995; Baldessarini et al. 1997; Kebabian et al. 1997). Moreover, repeated treatment with clozapine results in selective upregulation of putative D_4-like, but not D_2, receptors in rat forebrain (Florijn et al. 1997; Tarazi et al. 1997). Interest in the D_4 receptor as a potential site of action of antipsychotic agents is further encouraged by observations suggesting that these sites may be upregulated in postmortem brain tissue of patients diagnosed with schizophrenia (Seeman et al. 1993; Murray et al. 1995; Sumiyoshi et al. 1995). Interpretation of these findings remains tentative, however, due to uncertainty about the localization of D_4 receptors in mammalian brain (Baldessarini and Tarazi 1996). Estimates of altered levels of D_4 receptors in studies cited have been limited to indirect methods involving subtraction of the number of binding sites defined with the $D_{2,3}$ receptor antagonist [^{3}H]raclopride from total binding defined with a nonselective inhibitor of D_2-like receptors such as [^{3}H]nemonapride. Presence of D_4 receptors in human caudate-putamen remains particularly uncertain (Reynolds and Mason 1994, 1995; Lahti et al. 1995), and there may be differences in relative abundance of D_4 protein and its mRNA between brain regions and across species (Van Tol et al. 1991; Schoots et al. 1995; Suzuki et al. 1995). Clarification awaits development of improved D_4-selective agents (Kebabian et al. 1997) or immunohistological techniques (Mrzljak et al. 1996).

The DA partial-agonist derivatives, S[+]-N-*n*-propylnorapomorphine ([+]-NPA; Campbell et al. 1985, 1986, 1991), its orally active methylenedioxy prodrug, S(+)-10,11-methylenedioxy-N-*n*-propylnoraporphine (Campbell et al. 1987) and 11-monohydroxy analog, S(+)-11-hydroxy-N-*n*-propylnoraporphine (Gao et al. 1988; Campbell et al. 1990) have properties suggestive of atypical antipsychotics. These include inhibition of spontaneous or DA-induced locomotor arousal without catalepsy, weak antagonism of stereotyped gnawing behaviors induced by R(−)-aporphines, regional changes in brain concentrations of neurotensin similar to those of clozapine, and lack of hyperprolactinemia, tolerance, or induction of supersensitivity to DA agonists after repeated administration (Campbell et al. 1985, 1986, 1987, 1991, 1993; Nemeroff et al. 1991; Baldessarini et al. 1994). Mechanisms underlying highly regionally selective behavioral actions of S(+)-aporphines (Campbell et al. 1991) are not well defined, but selective interactions with D_4 receptors might be involved (Seeman and Van Tol 1993; Lahti et al. 1993, 1996; Kebabian et al. 1997).

The present study was undertaken to verify D_4 selectivity of [+]-NPA, and to test the hypothesis that it induces regionally selective changes in levels of different D_2-like DA receptors resembling those of clozapine but not typical antipsychotics, and specifically to test for its long-term upregulation of D_4-like sites. Effects of three weeks of daily administration of [+]-NPA on D_2-like receptor levels were compared to a typical neuroleptic, fluphenazine, and the atypical antipsychotic, clozapine, using quantitative *in vitro* receptor autoradiography.

METHODS

Materials and Animal Subjects

Radioligands were from New England Nuclear (Boston, MA): R,S(±)-[N-methyl-^{3}H]nemonapride (YM-09151–2: 86 Ci/mmol) and [methoxy-^{3}H]S(−)-raclopride (74 Ci/mmol); or Amersham (Arlington Heights, IL): [2,3-^{3}H] R(+)-7-hydroxy-N,N-di-*n*-propyl-2-amino-1,2,3,4-tetrahydronaphthalene (7-OH-DPAT: 116 Ci/mmol) and [2,3,4-^{3}H]spiperone (114 Ci/mmol).

DTG (1,3-ditolylguanidine), S(−)-eticlopride-HCl, *cis*-flupenthixol-di-HCl, fluphenazine-di-HCl, ketanserin tartrate, L-745,870, pindolol, and S(−)-sulpiride were from Research Biochemicals International (RBI, Natick, MA). S[+]-N-*n*-propylnorapomorphine-HCl ([+]-NPA), as well as frozen transfected cell membranes expressing human D_2 (human D_{2L} in Sf-9 cells) and D_4 receptors (human $D_{4.2}$ receptors in CHO cells) also were provided by an NIMH-RBI program. Drug gifts included clozapine from Sandoz Research (Berne, Switzerland) and haloperidol from McNeil Labs. (Ft. Washington, PA). Cation hydrochlorides, guanosine-5′-triphosphate sodium (GTP), leupeptin, phenylmethylsulfonyl fluoride (PMSF), polyethyleneimine (PEI), and *tris*-(hydroxymethyl)-aminomethane-HCL (Tris), were purchased from Sigma Chemicals (St. Louis, MO).

Animals were male Sprague-Dawley rats (Charles River Labs, Wilmington, MA) initially weighing 200–225 g, maintained under controlled light, temperature,

and humidity with free access to standard rat chow and tapwater in a USDA-inspected, veterinarian-supervised, small-animal research facility of the Mailman Research Center, with approval by the McLean Hospital Institutional Animal Care and Use Committee (Campbell et al. 1993).

In Vitro Receptor Affinities

Frozen membranes of genetically transfected cells were thawed and diluted as recommended by the supplier to provide 6 (D_2) or 50 μg (D_4) membrane protein/assay in Tris-HCl (50 mM, pH 7.4) containing for D_2 assays: 150 mM NaCl (D_2); or D_4 assays: (mM): NaCl (120), KCl (5.0), $CaCl_2$ (1.5), EDTA (5.0), and protease inhibitors PMSF (1.0) and leupeptin (0.002). [^{3}H]spiperone (free concentration = F = 0.1 nM, [D_2] or 0.5 nM [D_4]) was incubated with haloperidol (1 μM, [D_2] or 10 μM [D_4]) to define nonspecific binding; its difference from total binding was considered specific binding (B). In other preliminary experiments, homogenates of rat corpus striatum were incubated with [^{3}H]spiperone or [^{3}H]nemonapride (D_2 buffer, 60 min, 25°C; blank = 10 μM haloperidol), with a wide range of 12 concentrations (0.1 nM–10 μM) of S(−)-raclopride (Baldessarini et al. 1992). D_2 assays with cell membranes were conducted for 60 minutes at 27°C, and D_4 assays for 150 minutes at 25°C. Agents were tested at six or more concentrations in duplicate. Incubations were terminated by immersion in an ice bath, followed by rapid filtration in a cell harvester (Brandel, Gaithersburg, MD) on SS34 (for D_2) or SS32 (D_4) glass fiber filter sheets (Schleicher-Schuell, Keene, NH) impregnated with 0.3% (w/v) PEI; samples were counted in 3.5 ml Polyfluor (Packard, Downers Grove, IL) in a scintillation spectrophotometer (Wallace, Gaithersburg, MD) at ca. 50% efficiency. $IC_{50} \pm SE$ was obtained with the ALLFIT program to fit percent inhibition of specific binding vs. test agent concentration, and converted to Ki from the Cheng-Prusoff relationship, $Ki = IC_{50}/(1 + F/Kd)$, where F is free radioligand concentration and Kd is the independently determined radioligand affinity (Kd = 0.22 nM in the D_2, and 0.21 nM in the D_4 assay), all as described previously (Kula et al. 1994).

Drug Treatment and Tissue Preparation

Four groups of rats (N = 7/group) received intraperitoneal (i.p.) injections at 1 ml/kg body wt daily for 21 days with doses defined as mg of salts. Two groups were injected once daily (08:00 h) with physiological saline (0.9% w/v) or fluphenazine-$(HCl)_2$ (1 mg/kg); a third received clozapine base (20 mg/kg, twice daily: 08:00 and 20:00 h); and a fourth received (+)-NPA-HCl (2 mg/kg, thrice daily: 08:00, 14:00, and 20:00 h). At 24 h after final injections, rats were decapitated and their brains quickly removed, frozen in chilled isopentane, and stored at −80°C until use. Coronal sections (10 μm) were cut from ca. 0.2–4.2 mm anterior to the bregma (Paxinos and Watson 1982) in a cryostat at −20°C, mounted on gelatin-coated microscopic slides, and stored at −20°C until thawed at room temperature (rt) for autoradiography.

Receptor Labeling for Autoradiography

To reduce variance, brain samples from subjects exposed to all treatment conditions were evaluated at one time in each radioreceptor assay, following preincubation of brain sections for 1 h at rt in 50 mM Tris-HCl buffer (pH 7.4) containing (mM): NaCl (120), KCl (5), $CaCl_2$ (2), and $MgCl_2$ (1), for the D_2-like and D_4-like assays, or a modification for the D_3 assay (0.3 mM GTP, 40 mM NaCl, no $MgCl_2$).

For assays of *D_2-like receptors*, sections were incubated in the same buffer with one of three combinations of radioligand and masking agents: [1] 1.0 nM [^{3}H]nemonapride ($D_{2,3,4}$) with 0.5 μM 1,3-ditolylguanidine (DTG) and 0.1 μM pindolol to mask sigma ($\sigma_{1,2}$) and $5HT_{1A}$ sites, respectively (Lahti et al. 1995); [2] 1.2 nM [^{3}H]spiperone ($D_{2,3,4}$) with 40 nM ketanserin (to block 5-HT_2-like sites); or [3] 5 nM [^{3}H]raclopride ($D_{2,3}$) alone (Tarazi et al. 1997). Incubation continued for 1 h at rt. Nonspecific binding was determined with 10 μM S(−)-sulpiride with [^{3}H]nemonapride or 1 μM *cis*-flupenthixol with [^{3}H]spiperone and [^{3}H]raclopride.

For *D_3 receptor binding*, sections were preincubated for 1 h in a modified Tris buffer, already described. Sections were then incubated in the same buffer for 1 h, with 3 nM [^{3}H]7-OH-DPAT and 5 μM DTG added to mask sigma sites (Wallace and Booze 1995). Nonspecific binding was determined with 1 μM S(−)-eticlopride. After incubation, slides were washed twice for 3 minutes in ice-cold, fresh buffer and dried under a stream of air. With the partially D_3-selective agonist radioligand, [^{3}H]R(+)-7-OH-DPAT, Na^+ (40 mM) and GTP (0.3 mM) were included and Mg^{2+} excluded, to avoid labeling the high-affinity agonist binding state of D_2 receptors by driving D_2 receptors into their agonist low-affinity state (Gonzalez and Sibley 1995).

For *D_4-like assays*, an optimal concentration of S(−)-raclopride to fully block D_2 and D_3 receptors selectively was determined with a range of concentrations of unlabeled raclopride to compete vs. [^{3}H]nemonapride in rat forebrain sections (Florijn et al. 1997; Tarazi et al. 1997). Curve-fitting optimized with a two site model indicated that 300 mM raclopride fully occupied a high-affinity binding component (presumably D_2 and D_3 receptors), but not residual specific binding (ca. 20%) that may represent D_4-like sites (Seeman et al. 1995). Similar evidence of two components of binding defined with a range of concentrations of S(−)-raclopride vs. [^{3}H]-nemonapride and [^{3}H]spiperone was found preliminar-

ily with rat striatal homogenates; the first component represented ca. 80% of all specific binding with both radioligands. The D_4 assays employed: [1] 1.0 nM [^{3}H]nemonapride with 0.5 μM DTG, 0.1 μM pindolol, and excess S(−)-raclopride (0.3 μM), or [2] 1.2 nM [^{3}H]spiperone with 40 nM ketanserin and same concentration of S(−)-raclopride to mask D_2, D_3, and other nonspecific binding sites, and reveal D_4-like receptors (Florijn et al. 1997; Tarazi et al. 1997). Non-specific binding determined with 10 μM S(−)-sulpiride with [^{3}H]nemonapride and 1 μM *cis*-flupenthixol with [^{3}H]spiperone. After each radioligand assay, slides were washed twice for 5 min in ice-cold buffer, dipped in ice-cold deionized water, and then dried in a stream of air. The D_4-selective ligand L-745,870 (Kulagowski et al. 1996; Kebabian et al. 1997) was used to characterize the raclopride-insensitive component of binding with both radioligands.

Autoradiography and Image Analysis

Radiolabeled slides and calibrated [^{3}H]standards (Amersham) were exposed to Hyperfilm (Eastman-Kodak, Rochester, NY) for 2–5 weeks at 4°C. [^{3}H]Nemonapride-labeled brain sections were exposed for 2 weeks (caudate-putamen [CPu] and nucleus accumbens[ACC]) for 5 weeks (cerebral cortex) to compensate for lower receptor abundance; [^{3}H]spiperone was exposed for 2 weeks; [^{3}H]raclopride and [^{3}H]7-OH-DPAT, for 4 weeks. These exposures provided optical densities within the linear range of the tritium-sensitive film, which was developed in Kodak D-19 developer and fixative. Optical density (OD) in brain regions of interest was measured with a computerized densitometric image analyzer (MCID-M4, Imaging Research, St. Catharines, Ontario). Brain regions of interest were outlined (Figure 1) and their OD was measured. Left and right sides of two contiguous sections (4 measurements/subject-brain) represented total binding and two other sections represented nonspecific binding; the four determinations were averaged for each subject (N = 7 subjects for each treatment condition). OD was converted to nCi/mg of tissue with calibrated [^{3}H]standards and, after subtracting non-specific from total binding to compute specific binding, expressed as fmol/mg tissue.

Statistical Analysis

Two-way analysis of variance (ANOVA) with each autoradiographic assay initially tested for effects of drug treatments in preselected brain regions; given overall significance, post-hoc Dunnett *t*-tests identified significant differences between treatment groups. Unless stated otherwise, data are presented as means ± SEM. Comparisons were considered significant at $p < 0.05$ in two-tailed tests, with degrees of freedom (df) based on N subjects/treatment group.

RESULTS

Preliminary experiments with transfected cell membranes (Table 1) confirmed that the new D_4 ligand L-745,870 (Kulagowski et al. 1996; Kebabian et al. 1997) showed expected high D_4 affinity and selectivity, whereas clozapine and (+)-NPA had similar and more moderate D_4 affinity, though (+)-NPA had somewhat higher D_4-

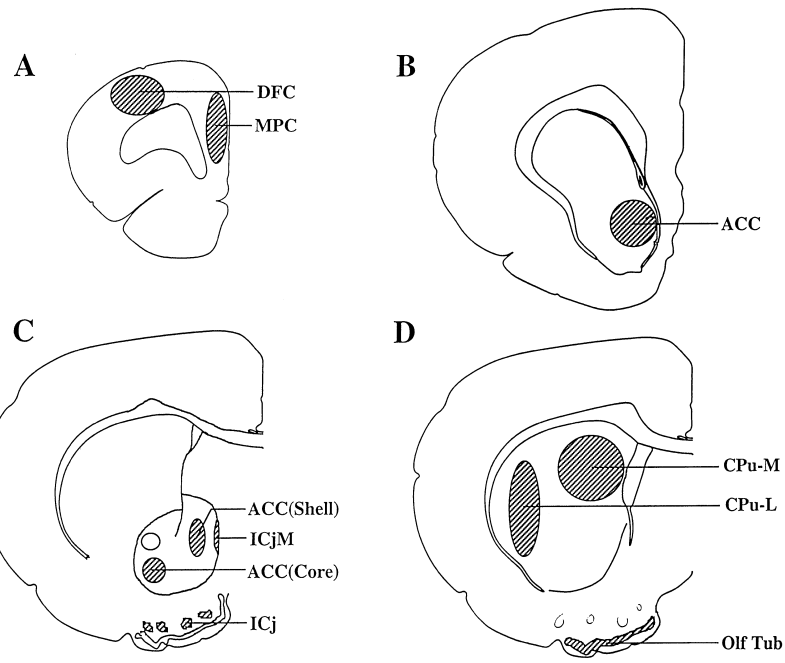

Figure 1. Sites for autoradiographic analyses of rat brain regions. Samples included 10 μm coronal sections from: **A** (A 3.2–4.2), **B** (A 1.7–2.2), **C** (A 0.7–1.2), and **D** (A 0.2−0.7 mm anterior to bregma according to Paxinos and Watson, 1982). *ACC*, nucleus accumbens septi (core and shell subdivisions); *CPu*, caudate-putamen (*L*, lateral and *M*, medial); *DFC*, dorsolateral frontal cerebral cortex; *ICj*, islands of Calleja; *ICjM*, major island of Calleja; *MPC*, "medial prefrontal" (cingulate or anterior limbic) cerebral cortex; *Olf Tub*, olfactory tubercle.

Table 1. Affinities to Dopamine Receptors in Membranes of Transfected Cells

Agent	Ki (nM 6 SE) D_{2L}	Ki (nM 6 SE) $D_{4.2}$	Affinity Ratio
Nemonapride	0.015 ± 0.012	0.280 ± 0.020	0.05
Spiperon	0.250 ± 0.001	0.073 ± 0.006	3.4
Clozapine	262 ± 25	43.6 ± 8.5	6.0
S(+)-NPA	774 ± 118	56.4 ± 9.5	13.7
L-745,870	>10,000	0.59 ± 0.07	>10,000

Assays and analyses are described in Methods, using [^{3}H]spiperone as radioligand. The affinity ratio (Ki D_2/Ki D_4) inidicates D_4 preference.

over-D_2 selectivity than clozapine. In addition, 1 μM L-745,870 reduced the raclopride-insensitive component of [^{3}H]spiperone and [^{3}H]nemonapride binding to D_4-like sites in sections of rat forebrain from 40.7 ± 2.1 and 15.9 ± 0.8 to 10.5 ± 0.8 and 3.2 ± 0.2 fmol/mg tissue respectively, reflecting a displacement of 74–80% of D_4-like binding sites in the CPu, but had no significant effect on [^{3}H]nemonapride binding to mainly D_2-like receptors (106.8 ± 2.9 vs. 117.4 ± 3.2 fmol/mg, with or without L-745,870 added). These observations indicate that the great majority of the raclopride-insensitive D_4-like binding sites reported here are probably D_4 receptors.

Three weeks of single daily injections of fluphenazine significantly increased binding of the non-selective $D_{2,3,4}$ radioligands [^{3}H]nemonapride and [^{3}H]spiperone in the limbic ACC (67% and 52%, respectively), olfactory tubercle (OT: 53% and 43%), and CPu (average 22%, 32%) (Tables 2 and 3). In contrast, fluphenazine induced less increase in binding of the $D_{2,3}$ radioligand [^{3}H]raclopride in both ACC (27%) and CPu (average, 16%) (Table 4). Repeated daily treatment with (+)-NPA and clozapine did not significantly change binding of any of the three radioligands in ACC or CPu (Tables 2–4). However, significant increases in [^{3}H]nemonapride and [^{3}H]spiperone binding were observed in "medial prefrontal" cerebral cortex (MPC, containing cingulate or anterior limbic cortex), but not dorsolateral frontal cortex (DFC), following treatment with fluphenazine (46% and 47%, respectively), (+)-NPA (48%, 39%), or clozapine (59%, 40%) (Tables 2 and 3). Repeated treatment with all three drugs also did not significantly affect binding of [^{3}H]7-OH-DPAT to D_3 receptors in the islands of Calleja, where it was most abundant, nor in ACC shell or core subregions, OT, or CPu (Table 5).

D_4-like sites were labeled with [^{3}H]nemonapride or [^{3}H]spiperone in the presence of masking agents to occlude additional binding sites. Labeling with [^{3}H]nemonapride plus unlabeled raclopride (Table 6) indicated that repeated treatment with fluphenazine, (+)-NPA, and clozapine all induced large increases in D_4-like binding in ACC (124%, 69%, and 71%, respectively) and CPu (average, 62%, 47%, and 43%). Similarly, [^{3}H]spiperone binding (Table 7) was increased significantly by fluphenazine, (+)-NPA, and clozapine in ACC (115%, 71%, and 80%) and CPu (average, 46%, 24%, and 32%). Percent changes in D_4-like binding in the 18 comparisons with [^{3}H]nemonapride vs. [^{3}H]spiperone (Tables 6 and 7) were highly correlated ($r = 0.93$; slope = 0.95, $p < 0.0001$).

To summarize, when [^{3}H]nemonapride and [^{3}H]spiperone labeled mainly the highly prevalent D_2 receptors, fluphenazine as well as (+)-NPA and clozapine all produced substantial increases of D_2-like receptor binding in the rat MP × C, but only the typical antipsychotic fluphenazine produced significant increases of these receptors in ACC or CPu (Tables 2 and 3, Figure 2A). In contrast, in the presence of raclopride to occlude D_2 and D_3 sites and reveal D_4-like sites selectively, all three agents had only minor effects in MPC, but produced large increases of D_4-like abundance in CPu and especially in ACC (Tables 6 and 7, Figure 2B).

DISCUSSION

The present autoradiographic findings indicate that in the rat's ACC and CPu tissues, fluphenazine pretreat-

Table 2. [^{3}H]Nemonapride Binding to D_2-Like ($D_{2,3,4}$) Receptors after Three Weeks of Daily Antipsychotic Treatment

Brain Region	Controls	Fluphenazine	(+)-NPA	Clozapine
Cerebral cortex				
Medial-prefrontal	14.2 ± 0.9 (100)	20.8 ± 0.4 (**146**)*	21.1 ± 1.5 (**149**)*	22.7 ± 1.7 (**160**)*
Dorsolateral	12.2 ± 1.0 (100)	11.2 ± 1.0 (92)	12.2 ± 1.0 (100)	13.5 ± 0.6 (111)
Nucleus accumbens	83.0 ± 2.6 (100)	138.4 ± 4.3 (**167**)*	91.7 ± 4.6 (110)	85.3 ± 4.4 (103)
Caudate-putamen				
Lateral	134.9 ± 3.1 (100)	158.3 ± 4.6 (**117**)*	138.6 ± 3.9 (103)	147.2 ± 3.5 (109)
Medial	99.8 ± 3.2 (100)	125.8 ± 4.4 (**126**)*	101.8 ± 5.0 (102)	104.2 ± 4.3 (104)
Olfactory tubercle	53.6 ± 2.1 (100)	82.1 ± 5.2 (**153**)*	53.3 ± 4.2 (99)	56.9 ± 4.1 (106)

Data are mean ± SEM values for binding (fmol/mg tissue and % of control), determined by quantitative autoradiography following antipsychotic or control injections daily for 3 weeks, with significant differences from controls indicated ([*], bold: ($p < 0.05$, N = 7 rats/group), all as described in Methods.

Table 3. [^{3}H]Spiperone Binding to D_2-Like ($D_{2,3,4}$) Receptors after Three Weeks of Daily Antipsychotic Treatment

Brain Region	Controls	Fluphenazine	(+)-NPA	Clozapine
Cerebral cortex				
Medial-prefrontal	33.2 ± 2.0 (100)	48.9 ± 1.7 (**147**)*	46.2 ± 2.1 (**139**)*	46.4 ± 1.0 (**140**)*
Dorsolateral	26.9 ± 2.4 (100)	26.2 ± 3.5 (97)	25.4 ± 3.0 (94)	27.6 ± 1.3 (102)
Nucleus accumbens	80.5 ± 4.2 (100)	122.2 ± 4.1 (**152**)*	84.6 ± 3.6 (105)	82.9 ± 4.0 (115)
Caudate-putamen				
Lateral	123.5 ± 4.2 (100)	159.5 ± 3.4 (**129**)*	130.6 ± 3.9 (106)	135.9 ± 2.5 (110)
Medial	89.3 ± 2.8 (100)	120.1 ± 3.3 (**134**)*	98.3 ± 4.4 (110)	101.4 ± 2.3 (114)
Olfactory tubercle	83.7 ± 2.5 (100)	119.9 ± 4.3 (**143**)*	77.5 ± 4.0 (93)	74.0 ± 5.3 (88)

Data are mean ± SEM values for binding (fmol/mg tissue and % of control), determined by quantitative autoradiography following antipsychotic or control injections daily for 3 weeks, with significant differences from controls indicated ([*], bold: $p < 0.05$, N = 7 rats/group), all as described in Methods.

ment induced large increases in D_2-like receptor binding of [^{3}H]nemonapride and [^{3}H]spiperone (ligands for $D_{2,3,4}$ receptors), with smaller increases in [^{3}H]raclopride binding (selective for $D_{2,3}$ receptors) and no changes in the binding of [^{3}H]R[+]-7-OH-DPAT under D_3-selective assay conditions (Tables 2–5). Similarly, greater increases in the binding of [^{3}H]nemonapride and [^{3}H]spiperone, than [^{3}H]raclopride, have been reported in CPu tissue in mainly neuroleptic-treated patients diagnosed with schizophrenia, in both postmortem radioreceptor assays and clinical brain scanning (Wong et al. 1986; Farde et al. 1990; Seeman et al. 1993; Murray et al. 1995; Sumiyoshi et al. 1995; Neve and Neve 1997). Nemonapride and spiperone have relatively high affinity for cloned D_2, D_3, and D_4 receptors expressed in genetically transfected cells (Ki = 0.07–0.61 nM) (Sokoloff et al. 1990; Van Tol et al. 1991), whereas raclopride has high affinity (1.8 and 3.5 nM) for cloned D_2 and D_3 receptors, but much lower affinity for cloned D_4 receptors (2.0 μM) (Seeman et al. 1993). The difference in radioligand binding defined by nemonapride or spiperone compared to raclopride may correspond to D_4-like receptors or binding sites (Seeman et al. 1993, 1995).

The differential affinity of DA antagonists to specific types of D_2-like receptors led to the present assays based on including a saturating concentration of raclopride to occlude D_2 and D_3 receptors selectively, so that the remaining binding of [^{3}H]nemonapride or [^{3}H]spiperone would predominantly represent D_4 or D_4-like binding sites (Florijn et al. 1997; Tarazi et al. 1997). This conclusion is supported by finding that the highly D_4-selective ligand L-745,870 occluded most (74–80%) of the raclopride-insensitive binding of [^{3}H]nemonapride or [^{3}H]spiperone to rat striatal sections, in the presence of other agents used to mask other relevant non-DA receptor sites. These included pindolol to occlude 5-HT_{1A} serotonin receptors and DTG to mask sigma sites in the presence of [^{3}H]nemonapride, and with ketanserin added to block 5-HT_2-like receptors in the presence of [^{3}H]spiperone.

Consistently higher estimates of D_4-like binding (fmol/mg) were obtained with [^{3}H]spiperone than with [^{3}H]nemonapride in all brain regions studied with [^{3}H]spiperone vs. [^{3}H]nemonapride (in 24 comparisons based on data in Tables 6 and 7: slope = 2.41, r = 0.99, $p < 0.0001$). These radioligands were assayed at similar concentrations (1.0–1.2 nM) but spiperone has 38-fold greater D_4 affinity and is much more D_4-over-D_2 selective than nemonapride. Thus, spiperone showed much higher affinity for $D_{4.2}$ than D_{2L} receptors (3.42-fold) in transfected cell membranes assayed with [^{3}H]spiperone,

Table 4. [^{3}H]Raclopride Binding to D_2 and D_3 Receptors after Three Weeks of Daily Antipsychotic Treatment

Brain Region	Controls	Fluphenazine	(+)-NPA	Clozapine
Nucleus accumbens	35.5 ± 2.0 (100)	45.2 ± 1.8 (**127**)*	37.3 ± 1.4 (105)	39.0 ± 2.7 (110)
Caudate-putamen				
Lateral	57.3 ± 1.1 (100)	64.8 ± 0.6 (**113**)*	57.6 ± 2.2 (101)	60.1 ± 2.1 (105)
Medial	40.6 ± 1.9 (100)	47.8 ± 0.6 (**118**)*	40.3 ± 1.4 (99)	41.9 ± 1.4 (103)

Data are mean ± SEM values for binding (fmol/mg tissue and % of control), determined by quantitative autoradiography following antipsychotic or control injections daily for 3 weeks, with significant differences from controls indicated ([*], bold: $p < 0.05$, N = 7 rats/group), all as described in Methods.

Table 5. [^{3}H]7-OH-DPAT Binding to D_3-Like Receptors after Three Weeks of Daily Antipsychotic Treatment

Brain Region	Controls	Fluphenazine	(+)-NPA	Clozapine
Islands of Calleja	17.6 ± 1.3 (100)	17.4 ± 1.4 (100)	18.2 ± 1.6 (105)	16.7 ± 0.9 (97)
Olfactory tubercle	4.7 ± 0.3 (100)	4.8 ± 0.5 (102)	5.2 ± 0.3 (110)	4.8 ± 0.2 (102)
Nucleus accumbens				
Shell	7.3 ± 1.2 (100)	7.9 ± 1.4 (108)	8.0 ± 1.4 (109)	6.8 ± 0.6 (93)
Core	3.7 ± 0.5 (100)	3.5 ± 0.5 (95)	3.7 ± 0.4 (100)	3.6 ± 0.3 (97)
Caudate-putamen	1.6 ± 0.3 (100)	1.9 ± 0.4 (118)	1.4 ± 0.1 (88)	1.6 ± 0.2 (100)

Data are mean ± SEM values for binding (fmol/mg tissue and % of control), determined by quantitative autoradiography following antipsychotic or control injections daily for 3 weeks, (N = 7 rats/group), all as described in Methods.

whereas nemonapride was less selective for $D_{4.2}$ than D_{2L} sites (by 18.5-fold; Table 1). In addition, [^{3}H]spiperone may have labeled some nonspecific sites not as effectively excluded with masking agents as with [^{3}H]nemonapride. While the present indirect assays may not provide quantitative measures of D_4 levels in brain tissue, both radioligands yielded similar displacement of D_4-like binding with L-745,870 and similar percentage changes with the drugs tested, supporting at least the qualitative reliability of the findings reported (Tables 6 and 7).

With both radioligands, D_4-like binding density (fmol/mg) in untreated brain tissue was less in cerebral cortex than in ACC and CPu (Tables 6 and 7) but represented a somewhat higher proportion of all D_2-like receptor binding in the cortex than in ACC or CPu (Tables 2, 3, 6, and 7). Higher levels of D_4-like binding in ACC and CPu than in frontal cortex contrast with more abundant expression of D_4 mRNA in cerebral cortex than in subcortical regions of rat and human forebrain (Van Tol et al. 1991; Meador-Woodruff 1994, 1996). Some D_4-like sites in ACC and CPu may arise on afferent axons with cell bodies in cerebral cortex as well as on intrinsic local neurons (Tarazi et al. 1997) and expression of D_4 receptor immunoreactivity has been found in glutamatergic pyramidal cells as well as intrinsic GABAergic neurons in primate cortex (Mrzljak et al. 1996). Failure of typical and atypical antipsychotics to significantly upregulate cortical D_4-like sites (Tables 6 and 7) may suggest regional differences in the regulation of D_4 genes or protein synthesis and turnover. This suggestion is consistent with a reported lack of increase of both D_4 mRNA and receptor protein in rat cortex after treatment with haloperidol, in contrast to a coordinated rise of both message and product in rat striatum (Schoots et al. 1995).

Increased binding of [^{3}H]nemonapride and [^{3}H]spiperone to D_2-like receptors in MPC, and not DFC, by both typical and atypical antipsychotic agents in the present study (Tables 2 and 3) probably reflects a selective increase in D_2 receptors, since cortical D_4-like binding was not altered by these drugs (Tables 6 and 7) and D_3 receptors are infrequent in rat cortex (Sokoloff et al. 1990). Some upregulation of D_2 receptors in cerebral cortex in response to repeated treatment with both atypical and typical antipsychotic agents has also been

Table 6. Binding of [^{3}H]Nemonapride with Raclopride to D_4-Like Receptors after Three Weeks of Daily Antipsychotic Treatment

Brain Region	Controls	Fluphenazine	(+)-NPA	Clozapine
Cerebral cortex				
Medial-prefrontal	8.62 ± 0.73 (100)	9.18 ± 1.10 (106)	9.90 ± 0.92 (115)	10.1 ± 0.9 (117)
Dorsolateral	7.68 ± 0.54 (100)	5.73 ± 0.73 (75)	6.08 ± 0.69 (79)	7.43 ± 0.72 (97)
Nucleus accumbens	13.4 ± 0.9 (100)	30.0 ± 2.2 (**224**)*	22.6 ± 2.7 (**169**)*	22.9 ± 2.0 (**171**)*
Caudate-putamen				
Lateral	18.2 ± 1.3 (100)	29.2 ± 1.8 (**160**)*	26.0 ± 1.6 (**143**)*	26.0 ± 2.3 (**143**)*
Medial	13.5 ± 0.6 (100)	22.0 ± 4.4 (**163**)*	20.4 ± 2.3 (**151**)*	19.3 ± 0.9 (**143**)*
Olfactory tubercle	6.86 ± 1.70 (100)	7.67 ± 0.35 (112)	8.63 ± 1.15 (126)	6.41 ± 0.46 (93)

Data are mean ± SEM values for binding (fmol/mg tissue and % of control), determined by quantitative autoradiography following antipsychotic or control injections daily for 3 weeks, with significant differences from controls indicated ([*], bold: $p < 0.05$, N = 7 rats/group), all as described in Methods.

Table 7. Binding of [^{3}H]Spiperone with Raclopride to D_4-Like Receptors after Three Weeks of Daily Antipsychotic Treatment

Brain Region	Controls	Fluphenazine	(+)-NPA	Clozapine
Cerebral cortex				
Medial-prefrontal	17.9 ± 1.98 (100)	17.8 ± 2.17 (99)	18.0 ± 1.89 (101)	16.1 ± 2.09 (90)
Dorsolateral	15.9 ± 2.13 (100)	13.5 ± 1.62 (85)	14.4 ± 2.06 (91)	14.0 ± 1.05 (88)
Nucleus accumbens	3.12 ± 24 (100)	67.2 ± 2.8 (**215**)*	53.3 ± 2.8 (**171**)*	56.1 ± 1.6 (**180**)*
Caudate-putamen				
Lateral	44.9 ± 2.1 (100)	67.6 ± 2.7 (**151**)*	56.8 ± 1.2 (**127**)*	60.5 ± 1.1 (**135**)*
Medial	35.6 ± 2.0 (100)	50.6 ± 1.8 (**142**)*	42.6 ± 1.4 (**120**)*	45.5 ± 1.3 (**128**)*
Olfactory tubercle	14.3 ± 1.5 (100)	12.1 ± 0.9 (85)	15.5 ± 1.2 (108)	15.9 ± 1.1 (111)

Data are mean ± SEM values for binding (fmol/mg tissue and % of control), determined by quantitative autoradiography following antipsychotic or control injections daily for 3 weeks, with significant differences from controls indicated ([*], bold: $p < 0.05$, N = 7 rats/group), all as described in Methods.

found in rats and primates (Janowsky et al. 1992; Lidow and Goldman-Rakic 1994). Selective increases in MPC, but not CPu or ACC, D_2 receptors after treatment with the atypical agents clozapine and (+)-NPA (Tables 2 and 3) may reflect differences in the types of D_2-like receptors in these brain regions or different regulatory responses of cells which express them.

Neither D_2 nor D_3 receptor upregulation was detected in CPu or ACC after long-term treatment with clozapine or (+)-NPA, and fluphenazine evidently increased D_2 but not D_3 binding (Florijn et al. 1997; Tarazi et al. 1997) (Tables 4 and 5). Increased [^{3}H]raclopride binding in striatum following fluphenazine probably represents D_2 receptors because D_3 receptors are found in low levels there, and binding of [^{3}H]R(+)-7-OH-DPAT under D_3-selective conditions (Gonzales and Sibley 1995) was not increased by any of the treatments tested (Table 5), consistent with previous findings after antipsychotic treatment (Levésque et al. 1995; Florijn et al. 1997; Tarazi et al. 1997). Lack of a response of D_3 receptors to repeated treatment with DA antagonists may reflect their inability to upregulate in response to prolonged blockade, perhaps paralleling their limited or inconsistent interactions with G-proteins and second-

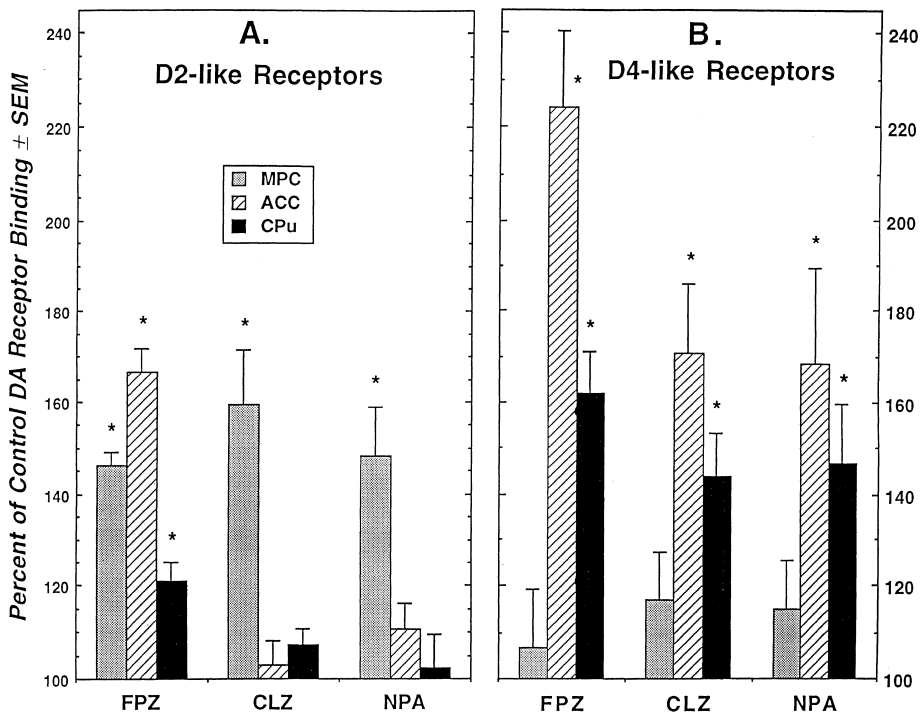

Figure 2. Percent of control DA receptor binding (± SEM) after 3 weeks of daily treatment of rats with fluphenazine (*FPZ*), clozapine (*CLZ*), or S(±)-N-*n*-propylnorapomorphine (*NPA*), comparing changes in medial prefrontal cortex (*MPC*; shaded bars), accumbens (*ACC*; striped bars), and caudate-putamen (*CPu*; black bars). **A.** Binding of [^{3}H]nemonapride alone (mainly to highly prevalent D_2, little D_3, and perhaps D_4 receptors; Cf. Table 1). **B.** Binding of the same radioligand with unlabeled raclopride (300 nM) to mask D_2 and D_3 sites selectively (with other masking agents to occlude sigma and 5-HT binding sites as defined in Methods) and reveal D_4 receptors more clearly (Cf. Table 5). (*) Indicates statistically significantly greater than in vehicle controls, as described in Methods (N = 7 rats; $p < 0.05$).

messenger systems (Sokoloff et al. 1990; Baldessarini and Tarazi 1996).

Regarding D_4 receptors, three weeks of treatment with a typical antipsychotic (fluphenazine), as well as a prototype (clozapine) and a candidate atypical antipsychotic agent ([+]-NPA), all produced consistent increases in abundance of D_4-like binding in some subcortical regions of rat forebrain (CPu and ACC), but not in OT or cerebral cortex (Tables 6 and 7), in accord with results obtained with another typical neuroleptic, haloperidol (Shoots et al. 1995; Florijn et al. 1997; Tarazi et al. 1997). All three test agents used in this study have high affinity to cloned D_4 receptors expressed in transfected cells (Ki = 10–60 nM) (Van Tol et al. 1991; Seeman and Van Tol 1993) (Table 1). Increases in D_4-like binding in CPu and ACC following all three treatments evidently reflect increased tissue density of D_4 receptors (upregulation) and perhaps supersensitivity, as occurs with D_2 receptors after treatment with D_2 antagonists and other anti-DA treatments (Creese et al. 1977; Campbell et al. 1993; Baldessarini and Tarazi 1996).

Apparent increases of D_4-like binding by both typical and atypical antipsychotic agents suggests that D_4 receptors may be a common site of mediation of drug antipsychotic effects. Moreover, clozapine-induced increases, and possibly supersensitivity, of D_4 receptors may contribute to a high risk of psychotic exacerbation after discontinuing clozapine treatment of chronically psychotic patients (Baldessarini et al. 1997). In addition, reported elevations of D_4-like receptors in postmortem brain tissue from patients with schizophrenia (Seeman et al. 1993; Murray et al. 1995; Sumiyoshi et al. 1995) may include adaptations to antipsychotic drug exposure, although some studies failed to detect D_4-like receptor upregulation in such patients (Reynolds and Mason 1994, 1995; Lahti et al. 1996). Clarification of a role of D_4 receptors in the pathophysiology and treatment of psychotic disorders, and details of their regional regulation following long-term antipsychotic treatment in various species await further improvements in the assay of D_4 receptor proteins, including selective radioligands or antibodies (Kebabian et al. 1997).

In conclusion, striatolimbic D_4-like binding sites that appear to be accounted for mainly by D_4 receptors, as well as medioprefrontal cortical D_2-like receptors, may constitute attractive targets for guiding development of novel psychotropic drugs. Lower occupancy of D_2 receptors in neostriatum (CPu) by atypical antipsychotic agents may contribute to their low risk of neurological side effects. Current findings of similar long-term effects of (+)-NPA and clozapine on different D_2-like DA receptor subtypes in specific regions of mammalian brain add evidence of the unique properties of such drugs and specifically encourage further consideration of S(+)-aporphines as potential atypical antipsychotic agents.

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