Down-Regulation of the D1 and D5 Dopamine Receptors in the Primate Prefrontal Cortex by Chronic Treatment with Antipsychotic Drugs

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ABSTRACT

D2 dopamine receptor antagonism is postulated to be the key to antipsychotic efficacy in the treatment of schizophrenia. Yet the D1 dopamine family of receptors is far more prevalent in the cortical areas of the brain, such as the prefrontal cortex, which have frequently been implicated in schizophrenia. Moreover, the prefrontal cortical D1 sites have recently been shown to be down-regulated by chronic treatment with several commonly used antipsychotic drugs (Lidow and Goldman-Rakic, 1994). To provide further insight into the pharmacological regulation of the D1 class of dopaminergic receptors, we have now used ribonuclease protection assays to examine the regulation of D1 and D5 dopamine receptor mRNAs in the prefrontal cortex and the neostriatum of nonhuman primates after chronic treatment with eight different drugs representing a wide structural and pharmacological spectrum of antipsychotic medications. The medications were administered for 6 months twice daily at doses that fall within the therapeutic range recommended for human patients. The study also included a substituted benzamide, tiapride, which is a D2 antagonist like the eight aforementioned drugs but reportedly lacks antipsychotic activity. Remarkably, all drugs used in this study, including tiapride, down-regulated the levels of both D1 and D5 mRNAs in the prefrontal cortex by 30% to 60% compared with a vehicle control group, whereas mRNAs in the neostriatum were not affected. This observation indicates that a reduction in the levels of prefrontal cortical dopamine receptors of the D1 class may be an obligatory consequence of D2 receptor antagonism and thus may be a pharmacological property of antipsychotic drugs.

Since the late seventies, the efficacy of antipsychotic drugs in the treatment of schizophrenia has been generally attributable to their D2 dopamine receptor antagonism (Seeman et al., 1975; Creese et al., 1976), and new drug development has largely focused on this class of receptors and on their regulation in subcortical structures where they are present in the high density (Creese et al., 1990; Angulo et al., 1991; Matsunaga et al., 1991; Xu et al., 1991; Kopp et al., 1992; Egan et al., 1994; Fishburn et al., 1994; Fox et al., 1994). In light of the mounting evidence for the involvement of the cerebral cortex in schizophrenia (Weinberger, 1988; Davies et al., 1991; Goldman-Rakic, 1987; 1991; Goldman-Rakic et al., 1992; Selemon et al., 1995), we recently used quantitative autoradiography to examine the regulation of dopaminergic receptors in the cortex of nonhuman primates after chronic treatment with three different antipsychotic drugs: haloperidol, remoxipride and clozapine (Lidow and Goldman-Rakic, 1994). These drugs were found to have a common regulatory effect on dopaminergic receptors in the cortex but not on those in the caudate nucleus. In particular, 6 months of daily treatment with all three antipsychotics resulted in a substantial decrease in the density of dopaminergic receptors of the D1 class in the prefrontal and temporal cortices, the two regions often implicated in schizophrenia (Goldman-Rakic, 1991; Shenton et al., 1992; Selemon et al., 1995). On the basis of this finding we suggested that the down-regulation of cortical D1 sites may be an important component of response to antipsychotic drugs (Lidow and Goldman-Rakic, 1994).

In order to gain further insight into D1 receptor regulation by antipsychotic medications, we have expanded our analysis to establish whether down-regulation of the D1 receptor class is a characteristic of all antipsychotic agents or is specific to the three drugs previously examined. We also wished to determine whether both the D1 and the D5 subtypes of the D1 receptor class are equally affected. Finally, we were interested in learning whether drug-induced down-regulation of cortical D1 receptors reflects changes in the level of recep-

ABBREVIATIONS: mRNA, messenger ribonucleic acid; rRNA, ribosomal ribonucleic acid; cDNA, cloned deoxyribonucleic acid; UTP, uridine triphosphate; CTP, cytidine triphosphate; RNase, ribonuclease; DNase deoxyribonuclease, SDS, sodium dodecyl sulfate; HVA, homovanillic acid.
tor mRNAs. To achieve these goals, we used ribonuclease protection assays to measure levels of cortical and neostriatal mRNAs encoding the D1 and D5 receptors after chronic treatment with an array of drugs representing a wide structural and pharmacological spectrum of antipsychotic medications.

Material and Methods

Drugs. The eight antipsychotic drugs examined in this study were selected to represent the major chemical classes of antipsychotic drugs, including those with both typical [such as haloperidol, chlorpromazine, molindone (Physicians’ Desk Reference., 1996)] and atypical [such as clozapine, olanzapine, risperidone (Physicians’ Desk Reference., 1996; Moore et al., 1994; Janssen et al., 1988)] profiles. Whereas all of these drugs have high affinities for the dopamine receptor subtypes that belong to the D2 class (Janssen et al., 1994; Seeman, 1992), some of them, such as olanzapine, also have high affinities for the receptors of the D1 class (Moore et al., 1994). In addition to the above-mentioned antipsychotic drugs, tiapride was selected to represent the major chemical classes of antipsychotic drugs, including those with both typical [such as haloperidol, chlorpromazine, molindone (Physicians’ Desk Reference., 1996; Moore et al., 1994; Janssen et al., 1988; Pflug et al., 1994)]. The drugs were given p.o. (in fruit treats) twice a day for 6 months to approximate maintenance regimens in clinical practice (Hyman and Arana, 1987). The same treatment period was used in our previous study (Lidow and Goldman-Rakic, 1994). Four animals constituted a control group that received daily fruit treats only. During the entire 6 months of treatment, none of the animals displayed behavioral abnormalities attributable to the actions of the drugs. Neither did we observe drug effects on blood hematocrit, total white cell count or hemoglobin level.

Between 12 and 18 hr after the last treatment, the animals were anesthetized with sodium pentobarbital and perfused intracardially with phosphate buffered saline for 2 min (1 min of room-temperature solution and 1 min of ice-cold solution) to clear blood from the tissue. The brains were rapidly removed, and the prefrontal cortex [areas 9, 46 and 12 of Walker (Walker, 1940)] and the neostriatum (head and body of the caudate nucleus and putamen) were dissected out and immersed in liquid nitrogen for storage.

Synthesis of the riboprobes. The D1 and D5 riboprobes employed in this study and the tests of their specificity are described in detail in Lidow et al. (in press). They were fragments of the 32P-RNA complementary to the 228-and 335-base-long sequences from the third cytoplasmic loop of D1 and D5 dopamine receptor mRNAs, respectively.

The radiolabeled riboprobes were produced by in vitro transcription of linearized plasmids containing fragments of D1 or D5 receptor cDNA using an Ambion MAXiscript Kit (Ambion, Inc., Austin, TX). In brief, 1 μg of linearized plasmid DNA was added to a tube containing 2 μl 10X transcription buffer, 1 μl 200 mM dithiothreitol, 1 μl RNase inhibitor (12.5 U/μl), 3 μl nucleotide mix (10 mM each of ATP, GTP and UTP), 5 μl 32P-CTP (800 Ci/mM, 10 mCi/ml; Du Pont, Inc., Wilmington, DE). RNase-free sterile water was added to bring the final volume to 20 μl. Finally, 1 μl (10 U) of T7 RNA polymerase was added. The mixture was vortexed, incubated for 1 hr at 37°C and then added to an equal volume of solution containing 80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue and 2 mM EDTA. The resulting solution was heated at 90°C for 5 min and loaded on a 5% polyacrylamide gel in order to separate the full-length riboprobes, which were eluted from the gel at 37°C overnight with buffer containing 0.5 M ammonium acetate, 1 mM EDTA and 0.2% SDS. Riboprobes for human β-actin mRNA and 18S rRNA (183 bases long and 82 bases long, respectively), employed as loading standards, were produced using vectors obtained from Ambion, Inc. (Austin, TX). The protocols for their synthesis and purification were similar to those described above. The only exceptions were that in the case of the β-actin riboprobe, 1.5 μl 32P-CTP (800 Ci/mM, 10 mCi/ml) and 1 μl 1 mM cold CTP, and in the case of the 18S riboprobe, 4 μl 32P-CTP (800 Ci/μl, 100 μCi/ml) and 1 μl 10 mM cold CTP, were added to the synthetic mixture.

32P-RNA molecular weight markers were synthesized using a Century Marker Template Set (Ambion, Inc.) according to the protocol described in the instruction supplied with this set. Upon completion of the synthetic reaction, the marker template DNA was digested with 2 U DNase at 37°C (15 min), and 32P-RNA markers were extracted with phenol-chloroform and precipitated with ethanol.

Extraction of the total RNA. Total RNA was extracted from the prefrontal cortical and the neostriatal tissue with an RNA STAT-60 reagent (TEL-TEST® B’, Inc., Friendswood, TX). For this purpose, the tissue was homogenized in the above-mentioned reagent (1 ml per 100 mg tissue). Then chloroform was added (0.2 ml of chloroform per milliliter of homogenate), and the mixture centrifuged for 15 min at 12,000 × g and 4°C. After the centrifugation, the upper aqueous phase was precipitated with isopropanol (0.5 ml of isopropanol per milliliter of the aqueous phase) at room temperature for 5 min. After another centrifugation (15 min, 12,000 × g; 4°C), the RNA pellet was washed with 75% ethanol and dissolved in diethylpyrocarbonate-treated distilled water. The RNA was quantitated by measuring its absorbance at 260 nm. The ratios of 260/280 nm were usually over 2.0.

| TABLE 1 |
| List of chronically administered drugs |
| | Chlorpromazine | Clozapine | Haloperidol | Molindone | Olanzapine | Pimozide | Risperidone | Tiapride |
| Drug | | | | | | | | |
| Antipsychotic Drugs | | | | | | | | |
| Chemical Class | Phenothiazines | Dibenzodiazepines | Butyrophenons | Indoles | Thienobenzodiazepines | Diphenylbutylpiperidines | Substituted benzamides | Substituted benzamides |
| Daily Dose (mg/Kg) | 2.80 | 5.20 | 0.20 | 1.25 | 0.35 | 0.20 | 3.70 | 0.20 | 3.50 |
**Ribonuclease protection assay.** The ribonuclease protection assay was performed using an Ambion RPAII Kit. For both the neostriatum and the cortex of every animal, the assay was performed in triplicate. For the assay, 80,000 cpm of D1 $^{32}$P-riboprobe, 80,000 cpm of D5 $^{32}$P, 10,000 cpm of $^{32}$P-$\beta$-actin riboprobe and 10,000 cpm 18S $^{32}$P-riboprobe were added to 80 $\mu$g of total RNA in water. The mixture was precipitated by adding 0.1 volume of 5.0 M ammonium acetate and 2.5 volumes of ethanol at $-20^\circ$C (15 min) and centrifuged (15 min; 12,000 $\times$ g; 4°C). The pellet was hybridized in 20 $\mu$l of buffer containing 80% deionized formamide, 100 mM sodium citrate, 330 mM sodium acetate and 1 mM EDTA (pH 6.4) for 16 hr at 45°C. After hybridization, 0.5 U RNase A and 20 U RNase T1 were added. Samples were incubated at 37°C for 30 min to digest unhybridized RNA. The protected RNA fragments were precipitated, denatured by heating at 90°C for 5 min and separated on a 5% polyacrylamide gel. The 3000 cpm of $^{32}$P-RNA marker was loaded on the same gel. To make possible a comparison of the data obtained from different runs, one of the samples on each gel was a repeat from another gel. The negative control consisted of 80 $\mu$g of yeast RNA processed as described for total brain RNA. The gels were dried for 1 hr at 70°C on a Drygel Sr. Vacuum Gel Drier (Hoefer Scientific Instruments, San Francisco, CA). They were then placed in a PhosphorImager SI (Molecular Dynamics, Sunnyvale, CA) for 8 hr, and the radioactivity of each line containing D1, D5, $\beta$-actin and 18S riboprobes was measured. After that, the gels were apposed for 2 days at $-70^\circ$C to X-OMAT AR film (Eastman Kodak Co., Rochester, NY) with intensifying screen in order to obtain a permanent visual record of the results.

The data collected were expressed as radioactivity produced by D1 and D5 riboprobes per radioactivity produced by $\beta$-actin and 18S riboprobes. The final results were the mean values $\pm$ S.E.M. of all repeats within each animal group ($n = 6$ for drug-treated groups and $n = 12$ for control group). The data for control and for each of the treated groups were compared with two-tailed Student’s t tests.

**HPLC.** A single tissue sample from the neostriatum and a single tissue sample from the prefrontal cortex of each animal were sonicated in ice-cold 0.1 M perchloric acid containing 0.13 mM EDTA and dihydroxybenzylamine as internal standard. They were then centrifuged at 23,000 $\times$ g for 20 min at 4°C. The pellets were saved for protein determination by the method of Lowry et al. (1951). The supernatants were removed and analyzed by HPLC. An aliquot (50 $\mu$l) of each sample was separated on reverse-phase column (10 cm $\times$ 3.2 mm Phase II ODS-3, Bioanalytical Systems Inc., West Lafayette, IN) under isocratic conditions. The mobile phase, delivered at 0.3 ml/min, comprised sodium citrate (30 mM), sodium dihydrogen phosphate (14 mM), sodium octanesulphonate (2.3 mM), EDTA (0.025 mM), acetonitrile (6.5%), tetrahydrofuran (0.6%) and diethylamine (0.1%) at pH 3.1. An electrochemical detector (Bioanalytical Systems Inc.) was used at a potential of 0.7 V, and the retention time was 20 min to resolve dopamine and HVA. Quantification was achieved by dividing the peak height of the unknown by that of the internal standard and referring this ratio to an external standard.

### Results

**Evaluation of the levels of mRNAs encoding D1 and D5 dopamine receptors with ribonuclease protection assay.** Observation of the film autoradiograms and of images generated by the PhosphorImager revealed that the ribonuclease protection assay of the brain RNAs employed in this study generated gels with four major bands with molecular weights corresponding to those of the D1, D5, $\beta$-actin and 18S riboprobes (fig. 1). Identical assays of yeast RNA resulted in gels without bands (the negative control is not shown). These results indicate that our riboprobes are appropriate for the ribonuclease protection assay of macaque RNA and can be used for quantitative analysis of their specific targets in tissue.

Levels of cortical D1 and D5 dopamine receptor mRNAs were calculated as ratios of these receptor mRNAs to $\beta$-actin mRNA as well as to 18S rRNA. The data obtained by both of these methods showed that all drug-treated animals expressed 30% to 60% lower levels of D1 receptor mRNA than animals in the control group (fig. 2A). Similar decrements were also observed for D5 receptor mRNA (fig. 2B). Among the drugs examined, risperidone produced the greatest decrease in both dopamine receptor mRNAs, and olanzepine produced the least reduction (fig. 2). The nonantipsychotic D2 antagonist tiapride down-regulated D1 and D5 mRNAs to levels comparable to those in remoxipride- and molindone-treated animals (fig. 2). For all drugs, the decrease in the levels of cortical D1 and D5 receptor mRNAs was statistically significant ($P < .05$).

In contrast to decreased levels of the mRNAs encoding the D1 class of dopamine receptors in the prefrontal cortex, the ribonuclease protection analysis of the neostriatal RNA did not show a common regulatory effect of antipsychotic drugs on either D1 or D5 mRNAs (fig. 3). The levels of these mes-
Sages in all drug-treated groups were very similar to those observed in the control group (fig. 3). The nonantipsychotic, tiapride, also did not significantly influence the levels of the neostriatal mRNAs encoding D1 and D5 dopamine receptor subtypes (fig. 3).

Evaluation of the dopamine turnover with HPLC. In order to evaluate dopamine turnover, we calculated levels of HVA and HVA/dopamine ratios in the neostriatum and prefrontal cortex (Scatton, 1977; Bacopoulos et al., 1979; Essig et al., 1991). Table 2 shows the data obtained from each animal used in this study. It is clear from this table that the drugs examined in the present study did not produce similar changes in either HVA levels or HVA/dopamine ratios. Rather, the effects on basal dopamine turnover in both the neostriatum and the prefrontal cortex were variable. For example, in the neostriatum, molindone increased the HVA level and the HVA/dopamine ratio in both treated animals compared with controls, whereas exposure to pimozide decreased these parameters. In the cortex, all the animals of the chlorpromazine-treated group had increased dopamine
clozapine can up-regulate neostriatal D1 sites (Rupniak et al., 1994; MacKenzie and Zigmond, 1985; Hess et al., 1985; O’Dell et al., 1990). There are several reports that clozapine can up-regulate neostriatal D1 sites (Rupniak et al., 1985; O’Dell et al., 1990; See et al., 1990). Our results support the studies that show no clozapine-related effects at these sites (Ashby et al., 1989; Jiang et al., 1990). However, it is possible that clozapine up-regulates subcortical D1 sites under specific conditions not fulfilled in our study. Furthermore, the possibility that clozapine regulates the synthesis and degradation of D1 and D5 proteins in the basal ganglia without affecting mRNA levels cannot be ruled out; such an effect would not be detected by the ribonuclease protection assay.

The marked similarity between the nonhuman primate brain and the human brain suggests that the results obtained in the present study may be relevant to chronic antipsychotic treatment of patients suffering from schizophrenia. Indeed, the somewhat faster metabolism and excretion of antipsychotic drugs in monkeys than in humans (Stafford et al., 1981; Lidow and Goldman-Rakic, unpublished) suggest that the same doses of drugs may produce greater changes in the dopamine receptor mRNA levels in humans than in monkeys. Additionally, it should be emphasized that down-regulation of D1 and D5 mRNA was produced not only by the antipsychotically active drugs but also by the presumably nonantipsychotic D2 antagonist tiapride. This finding indicates that the regulation of mRNAs encoding the D1 dopamine receptor class in the prefrontal cortex may be more a function of D2 antagonism than of the ability of these drugs to counteract the florid symptoms of schizophrenia. This down-regulatory activity is apparently quite powerful, because it is capable of overriding the D1 up-regulatory potential of drugs such as olanzapine that have a high affinity not only for the D2 but also for the D1 receptor class (Moore et al., 1994). It is of interest that the observed decrease in the level of cortical D1 sites appears to require prolonged treatment with D2 antagonists; we have not observed down-regulation of these receptors after only 1 month of exposure to haloperidol, remoxipride and clozapine (Lidow and Goldman-Rakic, in preparation).

**Possible mechanisms of drug-induced down-regulation of cortical D1 receptors.** All drugs used in this study are antagonists of the D2 receptor class and have been shown to up-regulate cortical D2 sites (Janssen et al., 1988; Seeman, 1992; Lidow and Goldman-Rakic, 1994). A plausible mechanism by which D2 antagonists used in this study could reduce the levels of the prefrontal cortical D1 and D5 receptors is a compensatory reaction of these receptors to an increase in cortical dopamine release resulting from the blockade of D2 pre- and postsynaptic sites (Lidow and Goldman-Rakic, 1994). The neostriatal D1 and D5 receptors may not be down-regulated, because, in contrast to the cortical dopaminergic system, the neostriatal dopaminergic innervation quickly develops tolerance to chronic D2 antagonist treatment (Scatton, 1977; Bacopoulous et al., 1979; Lappalainen et al., 1990; Essig et al., 1997).
et al., 1991; Parsons et al., 1993). However, we failed to find a consistent pattern of change in either cortical or neostriatal basal dopamine turnover resulting from chronic treatment with the drugs used in this study. This may indicate that in monkey, both neostriatal and cortical dopaminergic systems develop tolerance to prolonged drug treatment (Bacopoulous et al., 1979) and that the down-regulation of cortical D1 sites may not be a response to an increase in dopamine levels in this tissue. This conclusion must be tempered, however, by the fact that animals sacrificed 18 hr after the last drug administration can provide only a measure of basal dopamine turnover (Grace, 1991). An in vivo examination of changes in levels of dopamine and its metabolites within several hours after daily drug treatments may be more relevant for understanding whether D2 antagonists can affect cortical D1 sites through regulation of dopamine release.

It is also possible that the down-regulation of the D1 receptor class may be secondary to a drug-induced increase in the level of responsiveness of D2-mediated adenylate cyclase to dopamine (Lidow and Goldman-Rakic, 1994; Creese and Hess, 1986). A strong interaction between D1 and D2 second messenger systems is well established (Seeman et al., 1989; Strange, 1991), so it is conceivable that an increase in the sensitivity of D2-associated adenylate cyclase would be accompanied by an increase in the sensitivity of the D1-coupled enzyme, which, in turn, would constitute a signal for down-regulation of the D1 receptor class (Lidow and Goldman-Rakic, 1994; Creese and Hess, 1986).

**D1 receptor regulation and cognition.** The present findings may be pertinent to understanding the effect of antipsychotic drugs on the cognitive impairments that are characteristic of schizophrenia (Taylor and Abrams, 1984; Goldman-Rakic, 1987; 1991; Weinberger, 1988; King, 1990). It is widely believed that antipsychotic drugs have little impact on cognitive function in schizophrenia (Berman et al., 1985; McKenna et al., 1985; Moller, 1993; Lindenmayer, 1995). Furthermore, D1-specific drugs have been reported to affect the negative symptoms without having any significant influence on the positive symptoms of the disease (Davidson, et al., 1990; De Boer, 1995; Karle et al., 1985). Altogether, these findings suggest that, although the ability to down-regulate cortical D1 receptors may not be a unique feature of antipsychotic drugs, but rather an effect associated with D2 antagonism, this ability is nevertheless common to all presently effective antipsychotic agents and so may have important implications for their therapeutic effects. In evaluating the regulatory actions of antipsychotic treatments, investigators should therefore give greater consideration to changes in prefrontal cortical D1 receptors, and future antipsychotic treatments might be designed to provide optimal stimulation of cortical D1 sites as well as to antagonize D2 receptors.

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**References**


