Differential Regulation of D₂ and D₄ Dopamine Receptor mRNAs in the Primate Cerebral Cortex vs. Neostriatum: Effects of Chronic Treatment with Typical and Atypical Antipsychotic Drugs

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ABSTRACT

The RNase Protection Assay was used to examine the regulation of D₂ and D₄ dopamine receptor mRNAs in the cerebral cortex and neostriatum of nonhuman primates after chronic treatment with a wide spectrum of antipsychotic medications (chlorpromazine, clozapine, haloperidol, molindone, olanzapine, pimozide, remoxipride and risperidone). Tiapride, a D₂ antagonist that lacks antipsychotic activity, was also included. All drugs were administered orally for 6 months at doses recommended for humans. All antipsychotic drug treatments examined in this study caused a statistically significant up-regulation of both the long and short isoforms of the D₂ receptor mRNAs in the prefrontal and temporal cortex. Tiapride, in contrast, significantly up-regulated only the level of D₂-long mRNA in these areas. The same drug treatments produced less uniform effects in the neostriatum than in the cortex: clozapine and olanzapine failed to significantly elevate either D₂-long or D₂-short receptor messages in this structure unlike all other drugs, including tiapride. In both the cerebral cortex and striatum, D₄ receptor mRNA was upregulated by certain typical (chlorpromazine and haloperidol) and certain atypical (clozapine, olanzapine and risperidone) antipsychotic agents as well as by tiapride. Other drugs of the typical (molindone and pimozide) and atypical (remoxipride) classes had no effect on D₄ mRNA levels in either cortical or striatal tissue. The finding that up-regulation of D₂ dopamine receptor mRNAs was a consistently observed effect of a wide range of antipsychotic agents in the cerebral cortex but not in the neostriatum, coupled with the fact that the D₂-short isoforms in the cortex were not regulated by a non-antipsychotic D₂ antagonist, tiapride, draws attention to the importance of the D₂ dopamine receptor in the cerebral cortex as a potentially critical, common site of action of antipsychotic medications.

The D₂ dopamine receptor antagonism of antipsychotic drugs and their ability to up-regulate striatal D₂ sites are the cornerstones of the dopaminergic hypothesis of schizophrenia (Luchins, 1975; Meltzer and Stahl, 1976; Sayed and Garrison, 1983; Snyder, 1976; Van Kammen, 1979; Van Praag and Korf, 1975). The question of the role of D₂ receptors in schizophrenia, however, has been reopened by the development of clozapine, which, while being the best known antipsychotic medication, has low affinity for most subtypes of the D₂ receptor class (Roth, 1995; Seeman, 1992; VanTol et al., 1991), and there is one report that it may bind with high affinity to a sub-population of the D₂-short receptors lacking a sequence of 29 amino acids in its third cytoplasmic loop compared with D₂-long receptor isoform (Giros et al., 1989; Monsma et al., 1989) expressed in a mouse fibroblast (Ltk⁻) cell line (Malmberg et al., 1993). Nevertheless, the ability of clozapine to up-regulate these receptors is a matter of controversy (Balldessarinini, 1996; Kusumi et al., 1995; Seeman et al., 1993a). Although, in contrast to other antipsychotics, clozapine does not seem to regulate neostriatal dopaminergic receptors (Boyson et al., 1988; Lee and Tang, 1984; O’Dell et al., 1990; Rupniak et al., 1985; Wilmot and Szczepanik, 1989), several laboratories have found that it up-regulates D₂ sites in the cerebral cortex (Balldessarinini et al., 1996; Janowsky et al., 1992; Lidow and Goldman-Rakic, 1994). For example, we have observed that chronic treatment of rhesus monkeys with either haloperidol, remoxipride or clozapine up-regu-

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lates cerebral cortical D_2 receptors labeled with [^{32}P]Epidepride (Lidow and Goldman-Rakic, 1994). From these findings, we hypothesized that the D_2 receptors in the cerebral cortex may be among the common sites of therapeutic action of antipsychotic drugs (Lidow and Goldman-Rakic, 1994).

To gain further insight into D_2 receptor regulation by antipsychotic medications, the present study was designed to establish whether the up-regulation of the cerebral cortical D_2 receptors is characteristic for all antipsychotic agents or is specific only for the small selection of drugs used in previous studies. We also wished to determine whether the D_2-short, D_2-long and D_4 subtypes of the D_2 receptor class are equally affected. Finally, the present study examines whether drug-induced up-regulation of cortical D_2 receptors reflects changes in the level of receptor mRNAs. To achieve these goals, we have used the RNase Protection Assay to measure levels of cortical and neostriatal mRNAs encoding the D_2-short, D_2-long and D_4 receptors after chronic treatment with an array of eight drugs representing a wide structural and pharmacological spectrum of antipsychotic medications.

**Materials 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 e.g., haloperidol, chlorpromazine, molindone and pimozide (Physicians’ Desk Reference, 1996) and atypical e.g., clozapine, olanzapine, remoxipride and risperidone (Janssen et al., 1988; Moore et al., 1994; Physicians’ Desk Reference, 1996) profiles (table 1). In addition to the antipsychotic drugs, tiapride was included in the study as a drug with high affinity for D_2 receptors.

**Materials and Methods.**

**Drug treatment.** A total of 28 rhesus monkeys (*Macaca mulatta*), 3 to 5 years of age, were studied. Each of the nine drugs examined in this study was given to three monkeys. The antipsychotic drugs were administered at daily doses recommended for human schizophrenic patients [table 1 (Pflug et al., 1990; Physicians’ Desk Reference, 1996; Moore et al., 1994)], whereas tiapride was given at doses that are effective for the treatment of tardive dyskinesia (Burma et al., 1982) but are nonantipsychotic (Eggers et al., 1988). The drugs were given orally (in fruit treats) twice a day for 6 months to approximate maintenance regimens in clinical practice (Hyman and Arana, 1987). Four animals constituted a control group that received daily fruit treats only.

At 12 to 18 hr after the last treatment, the animals were anaesthetized with sodium pentobarbital and perfused intracardially with phosphate-buffered saline for 2 min to clear blood from the tissue. The brains were rapidly removed, and the prefrontal and temporal cortical regions and the neostriatum were dissected out and immersed in liquid nitrogen for storage.

**Synthesis of the riboprobes.** The human D_2 and D_4 [^{32}P]-ribo probes used in this study and the tests of their specificity are described in Lidow et al. (in press). As reported previously (Lidow et al., in press), mRNAs encoding human dopamine receptors are extremely similar in structure to corresponding monkey mRNAs and, therefore, human riboprobes can be successfully used in studies of nonhuman primate material. The 384-base-long D_2 riboprobe was complementary to the sequence 665 to 1049 of the D_2-long receptor mRNA (O’Dowd et al., 1994). This incorporates the sequence 724 to 810 encoding the portion of the third cytoplasmic loop absent in the short isoform of this receptors (O’Dowd et al., 1994). Therefore, in the present RNase Protection Assay, the mRNA encoding the D_2-long receptors protected the entire length of the riboprobe, whereas the largest fragment of the riboprobe protected by the mRNA encoding D_2-short receptor was a 239-base-long fragment complementary to the mRNA sequence 811 to 1049. The 138-base-long D_4 riboprobe was complementary to the nucleotide sequences 102 to 240 of the D_4 receptor mRNA (O’Dowd et al., 1994). This unique sequence encodes a portion of the amino terminus of the D_4 receptor.

The radiolabeled riboprobes were produced by *in vitro* transcription of linearized plasmids containing fragments of the D_2 and D_4 receptor cDNA using an Ambion MAXiscript Kit (Austin, TX). In brief, 1 μg of linearized plasmid DNA was added to a tube containing 2 μl of 10× transcription buffer, 1 μl of 200 mM diithiothreitol, 1 μl of RNase inhibitor (12.5 units/μl), 3 μl of nucleotide mix (10 mM concentration each of ATP, GTP and UTP) and 5 μl of [^{32}P]CTP (800 Ci/mM, 10 mCi/ml; DuPont, Wilmington, DE). RNase-free sterile water was added to bring the final volume to 20 μl. Finally, 1 μl (10 units) 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 to 90°C for 5 min and loaded onto a 5% polyacrylamide gel to separate the full-length riboprobes that were eluted from the gel at 37°C overnight with buffer containing 0.5 M ammonium acetate, 1 mM EDTA and 0.2% sodium dodecyl sulfate. Riboprobes for 183-base-long human β-actin mRNA used as loading standards were produced using vectors obtained from Ambion. The protocols for their synthesis and purification were similar to those described above. The only exceptions were that in this case, 1.5 μl of [^{32}P]CTP (800 Ci/mM, 10 mCi/ml) and 2 μl of 1 mM cold CTP were added to the synthetic mixture.

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

**Extraction of the total RNA.** Total RNA was extracted from the prefrontal and temporal cortical and neostriatal tissue with an RNA STAT-60 reagent (TEL-TESTS, Friendswood, TX). For this purpose,
the tissue was homogenized in the above-mentioned reagent (1 ml/100 mg of tissue). Then, chloroform was added (0.2 ml of chloroform/1 ml of homogenate), and the mixture was 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/1 ml 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 quantified by measuring its absorbance at 260 nm. The ratios of 260/280 nm were usually >2.0.

Ribonuclease Protection Assay. The Ribonuclease Protection Assays were performed using an Ambion RPAII Kit. The general procedure was described previously in Lidow et al. (1997). The assay for D2 and D4 mRNAs was performed separately. For both the neostriatum and the cortex of every animal each assay was performed in triplicate. For the assay of the D2 mRNA in the neostriatum, 30,000 dpm of D2-32P-riboprobe and 3000 dpm of 32P-3b-actin riboprobe were added to 50 µg of total RNA in water, whereas for the assay of the D2 mRNA in the cerebral cortex, 60,000 dpm of D2-32P-riboprobe and 2000 dpm of 32P-3b-actin riboprobe were added to 80 µg of total RNA in water. For the assay of the D4 mRNA in either the neostriatum or cerebral cortex, 80,000 dpm of D4-32P-riboprobe, and 1,000 dpm of 32P-3b-actin riboprobe were added to 80 µg of total RNA in water. The mixtures were precipitated by adding 0.1 volume of 5.0 M ammonium acetate and 2.5 volumes of ethanol at −20°C (15 min) and centrifuged (15 min; 12,000 × g; 4°C). The pellets were washed twice with 75% ethanol and then dissolved in diethylpyrocarbonate-treated distilled water. Identical assays of yeast RNA resulted in gels with bands of appropriate size and intensity. Densitometry of the gels containing the results of the RNase Protection Assay of the D2 receptor mRNA in both the neostriatum and cerebral cortex was 14 hr. After that, bands containing riboprobes of ~138 bases long (protected by the β-actin message) were measured. Finally, the gels were opposed to X-Omat AR film (Eastman Kodak, Rochester, NY) with intensifying screen to obtain permanent visual record of the results.

Fig. 1. Typical autoradiogram of the polyacrylamide gel resulting from RNase Protection Assay of the D2 (three left columns) and D4 (three right columns) dopamine receptor mRNAs in the total RNA extract from the macaque prefrontal cortex. Each of the three columns are triplicate samples obtained from control animal receiving fruit treats only. β-Actin mRNA is the loading standard. Note that columns generated by the RNase Protection Assay of the D2 receptor mRNAs consist of only three major bands corresponding to riboprobe fragments, which should be protected by long and short isoforms of the D2 receptor mRNA and β-actin mRNA. In case of columns generated by the RNase Protection Assay of the D4 mRNA, only two major bands can be observed. They correspond to the length of the D4 and β-actin riboprobes.

**Results**

Levels of mRNAs encoding D2-long and D2-short dopamine receptors. As shown in figure 1, the gels generated by the RNase Protection Assay of the striatal and neocortical D2 dopamine receptor mRNAs contained three major bands. The upper band was formed by 32P-RNA similar in length to the entire D2 riboprobe, which should be protected by the D2-long isoform of the D2 receptor mRNA. The middle band was formed by 32P-RNA corresponding in length to the fragment of the D2 riboprobe, which should be protected by the D2-short isoform of the D2 receptor mRNA. And the lower band was formed by 32P-RNA with length of the β-actin riboprobe. Identical assays of yeast RNA resulted in gels with bands of appropriate size and intensity. Densitometry of the gels containing the results of the RNase Protection Assay of the D4 receptor mRNA in both the neostriatum and cerebral cortex was 14 hr. After that, bands containing riboprobes of ~138 bases long (protected by the β-actin message) were measured. Finally, the gels were opposed to X-Omat AR film (Eastman Kodak, Rochester, NY) with intensifying screen to obtain permanent visual record of the results.
study were from the prefrontal cortex (areas 9, 46 and 12 of Walker (1940)) and temporal lobe (area 21 (Brodmann, 1994)), areas that have often been implicated in schizophrenia (Goldman-Rakic, 1991; Shenton et al., 1992; Weinberger, 1988). In contrast to the heterogeneous effects of the antipsychotic medications in the neostriatum, Tukey’s multiple-comparison of the levels of the D2 receptor mRNAs in the cortex of the control and drug-exposed monkeys showed that the effect of drugs in the cortex was remarkably uniform. Each drug produced statistically significant (25–40%; P < .05) increases in the levels of D2-long and D2-short dopamine receptor mRNAs in both the frontal and temporal areas (fig. 3). Furthermore, there was a notable difference in the regulatory activity of the antipsychotic drugs and the noneffective D2 antagonist, tiapride (fig. 3). The statistically significant effect (P < .05) of this drug was restricted only to the D2-long message (fig. 3).

**Evaluation of the levels of mRNAs encoding D4 dopamine receptors.** The gels generated by the RNase Protection Assay of the striatal and the cerebral cortical D4 dopamine receptor mRNA contained two major bands formed by 32P-RNAs corresponding in length to D4 and β-actin riboprobes (fig. 1). In addition, the negative control conducted by substitution of the brain total mRNA by the yeast RNA generated no bands (not shown). Therefore, our riboprobes are appropriate for the RNase Protection Assay of the D4 receptor mRNA in the monkey tissue.

The comparison of the levels of the D4 receptor mRNA in the control and drug-exposed monkeys showed a statistically significant up-regulation of both cortical and neostriatal D4 receptor messages in animals treated only with certain drugs (fig. 4). The typical antipsychotics, chlorpromazine and haloperidol, the atypical antipsychotics, clozapine, olanzapine and risperidone as well as tiapride all produced up-regulation (25–40%; P < .05). Neither the typical antipsychotics molindone and pimozide nor the atypical drug remoxipride significantly altered D4 receptor mRNA levels in either the cortex or neostriatum (P > .05).

**Discussion**

**Effect of antipsychotic medications on the D2 receptor in the primate cerebral cortex.** The present study provides evidence that chronic treatment with a wide spectrum of antipsychotic drugs uniformly up-regulates D2 dopamine receptor mRNAs in the prefrontal and temporal regions of the primate cerebral cortex but has variable effects in the neostriatum. This finding strongly supports the hypothesis that interaction with cortical D2 receptors may be a common site of therapeutic activity of drugs effective in the treatment of schizophrenia (Lidow and Goldman-Rakic, 1994). This hypothesis is further supported by the fact that treatment with the D2 antagonist tiapride, which reportedly does not benefit schizophrenic patients (Eggers et al., 1988), produced much less impact on the D2-short dopamine receptor mRNAs in the cerebral cortex than in the striatum.

At the present time, the mechanisms by which interaction of antipsychotic drugs with cortical D2 sites may lead to beneficial effects in schizophrenic patients are unclear. Some light on this mechanism may be shed by the finding that disconnection between the prefrontal and temporal cortical regions produces symptoms identical to those observed during florid schizophrenic episodes that are particularly responsive to treatment with contemporary antipsychotic medications (for review, see Weinberger, 1991). It is perhaps relevant that in humans, D2 receptor message has been reported to be most concentrated in layers III and V of the
prefrontal cortex and layer III of the temporal cortex (Meador-Woodruff et al., 1996), layers that contain the majority of corticocortical projection neurons (Jones, 1981; Schwartz and Goldman-Rakic, 1984). Therefore, the D₂ receptors may produce their beneficial effects by stabilizing prefrontal-temporal interactions through modulation of corticocortical communication.

The present report may help to resolve the controversy concerning possible preferential interactions of several well known atypical antipsychotics, including clozapine and remoxipride, with the short isoform of the D₂ receptor (Malmberg et al., 1993). Our results clearly demonstrate that chronic treatments with all the antipsychotic drugs evaluated in this study, including clozapine and remoxipride, produce similar regulatory effects on both D₂-long and D₂-short receptor mRNAs. Therefore, the absence of extrapyramidal side effects of a drug does not appear to reflect drug selectivity for the D₂-short dopamine receptor. On the other hand, it may be significant that the nonantipsychotic D₂ antagonist tiapride was the only drug that while strongly affecting the cortical D₂-long dopamine receptor mRNA did not up-regulate the short isoform of the D₂ receptor message. This finding may indicate that interaction with this particular D₂ receptor isoform in the cortex is of special importance for antipsychotic rather than extrapyramidal activity.

The present findings further extend previous receptor binding studies that indicate that chronic clozapine treatment significantly up-regulates cortical but not striatal dopamine receptors of the D₂ class (Baldessarini et al., 1996; Janowsky et al., 1992; Lidow and Goldman-Rakic, 1994). Our results are the first to demonstrate that the differential effect of clozapine on cortical and striatal dopamine receptors is specifically associated with regulation of the D₂-long and D₂-short receptor subtypes. One possible mechanism responsible for this preferential effect of clozapine treatment on the cortical D₂ receptors may be related to the well-documented unique regulatory and adaptive properties of the cortical dopamine system (for review, see Bannon and Roth, 1983). An alternative explanation is that pharmacodynamics of clozapine may allow a higher concentration and/or a longer half-life of this drug in the cortex, providing for a more potent effect in this brain region compared with the striatum. Conversely, the lack of antipsychotic activity by tiapride at conventional doses may be due to its insufficiently high concentrations and/or half-life in the cerebral cortex.

Although the mechanisms underlying a stronger interaction of clozapine with D₂ receptors in the cerebral cortex compared with the striatum are unknown, this selectivity may be the basis of the reported ability of this drug to preferentially increase of dopamine turnover in the cortex (Karoum and Egan, 1992; Mefford et al., 1988). This regional specificity distinguishes clozapine from other antipsychotics,
like haloperidol, which increases dopamine turnover in both cortical and subcortical regions (Karoum and Egan, 1992; Mefford et al., 1988). Clozapine does not block D$_2$ sites as effectively as other antipsychotic agents in the striatum and thus, in contrast to these agents, does not elicit a significant compensatory response in this structure as it does in the cortex. It is, of course, possible that the lack of compensatory response to clozapine in the neostriatum may be related to some additional activities of this drug.

In the present study, several atypical as well as typical antipsychotic chronic drug treatments simultaneously up-regulated both striatal and cortical D$_2$ receptor mRNAs. This finding indicates that the selectivity to the cortical D$_2$ receptors is not an obligatory attribute of all atypical antipsychotic medications; rather, atypicality is probably a function of D$_2$/5-hydroxytryptamine$_2$ affinity ratios, which have, so far, been a reliable predictor of this property in known antipsychotics (Meltzer et al., 1989; Roth et al., 1995). Nevertheless, our observation that only clozapine among the well-established drugs displays a clear preference for the cerebral cortical D$_2$ receptors leads us to speculate that this action is the feature of clozapine that gives it an edge over other antipsychotic D$_2$ antagonist medications currently prescribed for the treatment of schizophrenia (for review, see Daniel, 1996; Fitton and Benfield, 1993; Meltzer, 1990). Only the recently developed antipsychotic drug olanzepine, the chemical structure of which closely resembles that of clozapine (Moore, 1994), possesses a similar selectivity for cerebral cortical D$_2$ receptors.

**Regulation of the D$_4$ dopamine receptors by typical and atypical antipsychotic drugs.** Particular attention has recently been paid to the D$_4$ subtype of the D$_2$ receptor class as a possible candidate for the major dopaminergic site affected in schizophrenia (Brunello, 1995; Seeman, 1992; Seeman et al., 1993a). This assumption is, in part, based on the observation that clozapine has a high affinity for this receptor (Seeman, 1992; VanTol et al., 1991). The present study showed that in nonhuman primates, chronic treatment with this drug can indeed up-regulate D$_4$ receptor mRNA in both the striatum and neocortex. The clozapine up-regulation is in contrast with previous attempts that failed to detect clozapine-induced up-regulation of D$_4$ receptor mRNA in both the striatum and cerebral cortex. The clozapine up-regulation is in contrast with previous attempts that failed to detect clozapine-induced up-regulation of the striatal D$_4$ sites by using an indirect receptor binding technique (Seeman et al., 1993a; Kusumi et al., 1995). This discrepancy can possibly be accounted for by the fact that D$_4$ receptors constitute an extremely small percentage of the striatal receptors belonging to the D$_4$ class (Reynolds and Mason, 1995; Seeman et al., 1993b; Sumiyoshi et al., 1995) and, as a consequence, even a statistically significant change in the density of D$_4$
sites would be below the detection threshold of a relatively insensitive indirect receptor binding assay.

Although treatments with several typical and atypical antipsychotic agents up-regulated D₂ receptor mRNAs in both the cerebral cortex and the striatum, treatments with other typical and atypical drugs had no significant effect on the D₄ message in either cortical or striatal tissue. Furthermore, the nonneuroleptic tiapride produced a statistically significant up-regulation of cortical and striatal D₄ receptor mRNA. Our observations thus indicate that the interaction with D₂ receptors neither confers the atypical profile nor is necessarily relevant for the antipsychotic effectiveness of the drugs. These results add to other findings that call into question the critical importance of D₁ receptors in the treatment of schizophrenia. For example, many typical and atypical drugs have a very low affinity for D₂ sites (Roth et al., 1995). In addition, earlier reports of increased D₁ receptor density in the striatum of schizophrenic patients (Murray et al., 1995; Seeman et al., 1995a, Sumiyoshi et al., 1995) have not been confirmed by more recent studies (Lahti et al., 1996; Reynolds and Mason, 1994, 1995). A critical role for D₁ receptors in normal brain function activity has also been challenged by the observation that in 2500 Germans and 4 in 100 Afro-Caribbeans who completely lack functional D₁ receptors due to gene mutations (Northen et al., 1994; Seeman et al., 1994) appear to have no detectable psychiatric abnormalities. These various findings suggest that ligands directed exclusively at D₂ sites may not be particularly effective as antipsychotic agents. The possibility should not be excluded, however, that action at the D₄ receptor (or other receptors not examined in this study) that are embedded in the same integrated cortical circuits as the D₂ receptor subtype could achieve comparable clinical results by acting through adjuvant mechanisms (Goldman-Rakic and Selemon, in press).

D₁ and D₂ receptor interaction. Although the present study has revealed a consistent up-regulation of D₂ receptors by a variety of antipsychotic medications, it should be noted that prolongation treatment with the same group of drugs also have a common but opposite regulatory effect on the D₁ and D₂ subtypes of dopamine receptor (Lidow et al., 1997). In addition, a recent positron emission tomography imaging study has shown that the D₁ receptor in the prefrontal cortex is down-regulated in never-medicated schizophrenic patients compared with control subjects and further is correlated with poor performance on the Wisconsin Card Sort Test of prefrontal function (Okubo et al., 1997). At the same time, Williams and Goldman-Rakic (1995) have shown that when D₁ receptor stimulation falls below an optimal level, single cell neuronal activity related to working memory function becomes depressed. In light of these several findings, we are led to speculate that the therapeutic effects of antipsychotics may be related to their up-regulation of D₂ receptors in the cortex, whereas the obligatory D₁ down-regulation produced by prolonged treatment with the same drugs may be less helpful in alleviation of negative and cognitive symptomology because they may exacerbate an endogenous D₁ insufficiency.

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