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Role of Dopamine D_4 Receptors in Motor Hyperactivity Induced by Neonatal 6-Hydroxydopamine Lesions in Rats

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The role of dopamine D_4 receptors in behavioral hyperactivity was investigated by assessing D_4 receptor expression in brain regions and behavioral effects of D_4 receptor-selective ligands in juvenile rats with neonatal 6-hydroxydopamine lesions, a laboratory model for attention deficit-hyperactivity disorder (ADHD). Autoradiographic analysis indicated that motor hyperactivity in lesioned rats was closely correlated with increases in D_4 but not D_2 receptor levels in caudate-putamen. D_4-selective antagonist CP-293,019 dose-dependently reversed lesion-induced hyperactivity, and D_4-agonist CP-226,269 increased it. These results indicate a physiological role of dopamine D_4 receptors in motor behavior, and may suggest much-needed innovative treatments for ADHD.
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KEY WORDS: *Attention deficit-hyperactivity disorder; Autoradiography; Dopamine; D_4; 6-hydroxydopamine; Motor activity*

Dopamine (DA) modulates physiological processes through activation of five G-protein coupled receptors of the D_1-like (D_1 and D_5) and D_2-like (D_2, D_3, and D_4) receptor families (Neve and Neve 1997). Since the cloning of their cDNA (Van Tol et al. 1991), D_4 receptors have received much attention, in part because some atypical antipsychotics, notably clozapine, bind to D_4 receptors with somewhat higher affinity than to the more prevalent D_2 receptors (Van Tol et al. 1991; Seeman et al. 1997; Tarazi et al. 1997; Tarazi and Baldessarini 1999).

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Human D_4 receptors occur in multiple forms with 2–11 copies of a 16-amino acid (48 base-pair) sequence in the putative third intracellular loop of the peptide (Van Tol et al. 1992; Lichter et al. 1993; Asghari et al. 1994). Several recent genetic studies indicate that the 7-repeat D_4 receptor allele ($D_{4.7}$), a relatively uncommon variant, is more prevalent in patients with attention deficit-hyperactivity disorder (ADHD) (La Hoste et al. 1996; Bailey et al. 1997; Rowe et al. 1998; Faraone et al. 1999). Haplotype relative-risk analysis to assess allele transmission also suggests that the $D_{4.7}$ receptor may be associated with ADHD (Swanson et al. 1998). The same $D_{4.7}$ phenotype is also linked to personality traits related to ADHD, notably, novelty-seeking and impulsivity (Benjamin et al. 1996; Ebstein et al. 1996; Comings et al. 1999). Biochemical analysis of these D_4 receptor variants indicates that $D_{4.7}$ receptors are less efficient in transducing extracellular DA signals to intracellular adenylyl cyclase than other forms with fewer repeat sequences (Asghari et al. 1995).

ADHD is a neuropsychiatric syndrome frequently found in school-aged children, especially boys, that is characterized by excesses of hyperactive, inattentive, and impulsive behavior (Barkley 1990). Salient features

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of ADHD are commonly modeled in juvenile rats following neonatal lesioning with 6-hydroxydopamine (6-OHDA) which destroys DA projections to forebrain (Shaywitz et al. 1976). Such rats exhibit several characteristics resembling core symptoms of ADHD, most notably, motor hyperactivity that occurs selectively during the periadolescent period, gradually declines as lesioned rats mature, and seems to represent deficient adaptation to environmental stimuli (Erinoff et al. 1979). The motor hyperactivity in this model can be dose-dependently antagonized by psychostimulants that are commonly used to alleviate symptoms of ADHD (Heffner and Seiden 1982; Luthman et al. 1989). Hyperactivity and attentional deficits that occur in ADHD are also modeled with genetic knockout mice that lack functional DA transporters (DAT) (Giros et al. 1996; Gainedtinov et al. 1999). However, the pathophysiology of these models may be dissimilar, and may or may not reflect mechanisms underlying clinical ADHD. Notably, the 6-OHDA lesioning model involves neonatal removal of DA with overgrowth of serotonin projections to forebrain (Kostrzewa et al. 1998), whereas the DAT knockout mouse involves a loss of the major mechanism for inactivating DA (Gainedtinov et al. 1999)—a mechanism that may be overexpressed in clinical ADHD (Dougherty et al. 1999; Dresel et al. 2000).

Association of D_4 receptor polymorphism with ADHD led us to study the expression of D_4 receptors in juvenile rats following neonatal 6-OHDA lesions using quantitative autoradiography. DA D_1-like and D_2-like receptor binding was examined for comparison. Effects of highly D_4-selective agents on hyperactivity were studied and compared to representative stimulants used to treat ADHD.

MATERIALS AND METHODS

Radioligands and Chemicals

[^{3}H]Nemonapride (R[+]-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine; 85.5 Ci/mmol) and [^{3}H]SCH-23390 (R[+]-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepin-7-ol; 81.4 Ci/mmol) were obtained from New England Nuclear (NEN; Boston, MA). [^{3}H]β-CIT ([–]-2-β-carbomethoxy-3-β-[4-iodophenyl]-tropane; 64.7Ci/mmol) was obtained from Tocris Cookson (Bristol, UK). Tritium-sensitive Hyperfilm and autoradiography standards were from Amersham (Arlington Heights, IL). D-19 developer and fixative were from Eastman-Kodak (Rochester, NY). (+)-Amphetamine sulfate, 1,3-ditolylguanidine (DTG), *cis*-flupenthixol dihydrochloride, 6-OHDA hydrobromide, ketanserin tartrate, S(–)-pindolol, and S(–)-sulpiride were from Sigma–Research Biochemicals International (RBI; Natick, MA). (+)-Methylphenidate hydrochloride was provided by Celgene (Warren, NJ). CP-226,269 and CP-293,019 were generously donated by Pfizer (Groton, CT). All other drugs and chemicals were purchased from Fisher Scientific (Dallas, TX) or Sigma Chemicals (St. Louis, MO).

Neonatal Lesioning

Sprague-Dawley rats (Charles River Labs., Wilmington, MA) were maintained under a 12-h artificial daylight/dark schedule (on, 7 A.M.-7 P.M.), with free access to tap-water and standard rat chow. On postnatal day (PD) 1, male pups were assigned to lactacting dam (10/each). On PD 5, pups received a subcutaneous injection of desipramine hydrochloride (25 mg/kg). 45 Minutes later, pups randomly received an intracisternal injection of vehicle (0.9% NaCl containing 0.1% ascorbic acid), or 6-OHDA hydrobromide (100 μg free base) under hypothermal anesthesia (Shaywitz et al. 1976). Pups were returned to nursing dams immediately after the intracranial injections. A total of 210 rat pups were included in the study. All procedures were approved by the McLean Hospital Institutional Animal Care and Use Committee, in compliance with applicable federal and local guidelines for experimental use of animals. The extent of lesioning was verified by quantifying DAT binding with [^{3}H]β-CIT (Kula et al. 1999) at the completion of behavioral experiments.

Behavioral Experiments

Motor activity was monitored individually for 90 min in the periadolescent period between PD 21 and 30, using an infrared photobeam activity monitoring system (San Diego Instruments, San Diego, CA) connected with a microcomputer. Behavioral testings were conducted in a novel environment (17×8×8 inch transparent plastic cages with 4×8 horizontal infrared beams), usually between 10:00 and 16:00 h in the absence of food and water, except for experiments involving nocturnal testing (at 22:00–04:00 h). Locomotor activity was defined as breaking of consecutive photobeams, and accumulated every 5 min.

Test agents were dissolved in 0.9% saline or 35% 2-hydroxypropyl-β-cyclodextrin, and given intraperitoneally (i.p.) immediately prior to testing. Each subject was evaluated in two behavioral testing sessions (one with vehicle, the other with a test drug), separated by at least three days, and in randomized order. Some rats given CP-293,019 were pretreated with methysergide (2 mg/kg, i.p.) 30 min before behavioral testing.

Receptor Autoradiography

Rats were sacrificed 48 h after the last behavioral testing session (on PD 32) by rapid decapitation, and brains

were quickly removed and frozen. Coronal brain sections (10 μm) were prepared in a cryostat at –17°C, thaw-mounted on gelatin-coated microscopic slides and stored at –80°C until quantitative autoradiographic assays.

Densities of D_2-like and D_4 receptors were determined autoradiographically as previously detailed and characterized pharmacologically (Tarazi et al. 1997, 1998a,b). Briefly, tissue sections were preincubated for 60 min at room temp in 50 mM Tris-HCl buffer containing (mM): NaCl (120), KCl (5), $CaCl_2$ (2), and $MgCl_2$ (1). Sections were then transferred to fresh buffer containing 1 nM [^{3}H]nemonapride in the presence of 0.5 μM 1,3-ditolylguanidine and 0.1 μM pindolol to block σ and 5-HT_{1A} sites for D_2-like receptor assays. The D_2/D_3-selective antagonist raclopride (300 nM) was included in D_4 assays to fully occlude D_2 and D_3 sites. Nonspecific binding was determined in both assays with 10 μM S(–)-sulpiride. D_1-like receptors were assayed with 1.0 nM [^{3}H]SCH-23390 in the presence of 40 nM ketanserin to mask 5-$HT_{2A/2C}$ receptor sites, with nonspecific binding determined with 1 μM *cis*-flupenthixol (Tarazi et al. 1998a).

After incubation with a radioligand, brain sections were washed twice (5 min in ice-cold buffer), rinsed in deionized water and air-dried. Sections were exposed to tritium-sensitive Hyperfilm for 2–6 weeks before standard photographic processing. Radioligand binding was quantified with a computerized image analyzer (Image Research Inc., St. Catherines, Ontario), and converted to nCi/mg tissue using [^{3}H]reference standards, with specific binding expressed in fmol/mg tissue, as detailed previously (Tarazi et al. 1997, 1998a,b).

Data Analysis

Lesion effects on receptor density were initially analyzed by two-way analysis of variance (ANOVA; for overall effects of treatment by brain region), followed by *post-hoc* Dunnett's *t*-tests for planned comparisons. Two-tailed probability of $<.05$ indicated statistically significant differences. Behavioral effects of test agents were analyzed similarly. Relationships between lesion-induced motor hyperactivity and DA receptor changes were evaluated by Spearman Rank Correlation (r_s).

RESULTS

Lesion-induced Motor Hyperactivity and Effects of Psychostimulants

Behavioral effects of neonatal lesions were examined by monitoring motor activity between PD 21–30 in a novel environment, to provoke exploratory activity. Lesioned rats exhibited overall much greater and more prolonged spontaneous motor activity than sham-lesioned littermate controls during both daytime and nocturnal testing (Figure 1, Panels A and B). Notably, motor activity of lesioned rats did not differ significantly from controls for the first 5–10 min of testing session, but failed to decline throughout the 90 min session, long after arousal in control rats had greatly diminished. Hyperactivity in lesioned rats was reduced by both (+)-amphetamine and (+)-methylphenidate (Figure 1, Panel C). In contrast to their motor-inhibiting effects in lesioned rats, both psychostimulants greatly stimulated motor activity in sham-lesioned controls, as expected (Figure 1, Panel D).

Effects of Lesions on DA Transporters and Receptors

Rats were sacrificed 2 days after the last behavioral testing for autoradiographic analysis of DA transporters and receptors. Neonatal 6-OHDA lesions reduced DAT binding in caudate-putamen (CPu) by >80% (39.3 ± 3.5 vs. 218 ± 9.2 fmol/mg in lesioned vs. sham-control rats). The lesions significantly increased D_4 receptor binding in CPu (lateral: 40.3%; medial: 35.2%), but not in nucleus accumbens (NAc) or prefrontal cortex (PFC) (Table 1). D_2–like ($D_2/D_3/D_4$) receptor binding also was increased in CPu, and not in NAc and PFC by the lesions. The magnitude of increase of D_2-like receptors (16.6% and 18.3% in lateral and medial CPu, respectively) was about half of that of D_4 receptors. D_1-like receptor binding was unchanged in CPu, NAc and PFC by neonatal 6-OHDA lesions. Lesion-induced motor hyperactivity was strongly correlated with increases of D_4 receptor binding in CPu in individual rats (Figure 2; Spearman nonparametric $r_s = 0.657$, $p < .05$), but not with increases of D_2-like receptors.

Behavioral Effects of D_4 Receptor-selective Ligands

The highly D_4-selective antagonist CP-293,019 dose-dependently antagonized lesion-induced hyperactivity (Figure 3, Panel A). At a dose of 10 mg/kg (i.p.), motor activity in lesioned rats was inhibited by approximately 40%, and at 30 mg/kg, it was indistinguishable from sham-lesioned controls. Nocturnal hyperactivity (equivalent to daytime activity in humans) in lesioned rats also was completely reversed by CP-293,019 at 30 mg/kg (Figure 3, Panel B). In striking contrast to the effects of the D_4 antagonist CP-293,019, a highly D_4-selective agonist, CP-226,269, produced a dose-dependent exacerbation of lesion-induced hyperactivity (Figure 3, Panel C). However, neither D_4-agent affected motor activity in sham-lesioned controls (Figure 3, Panel D).

Pretreatment of 6-OHDA-lesioned rats with methysergide, a broad-spectrum 5-HT receptor antagonist, did not affect their motor responses to subsequent injection of CP-293,019 (Figure 4). Methysergide treatment alone did not alter motor activity. Eticlopride, a D_2 receptor antagonist, inhibited locomotor activity in sham-

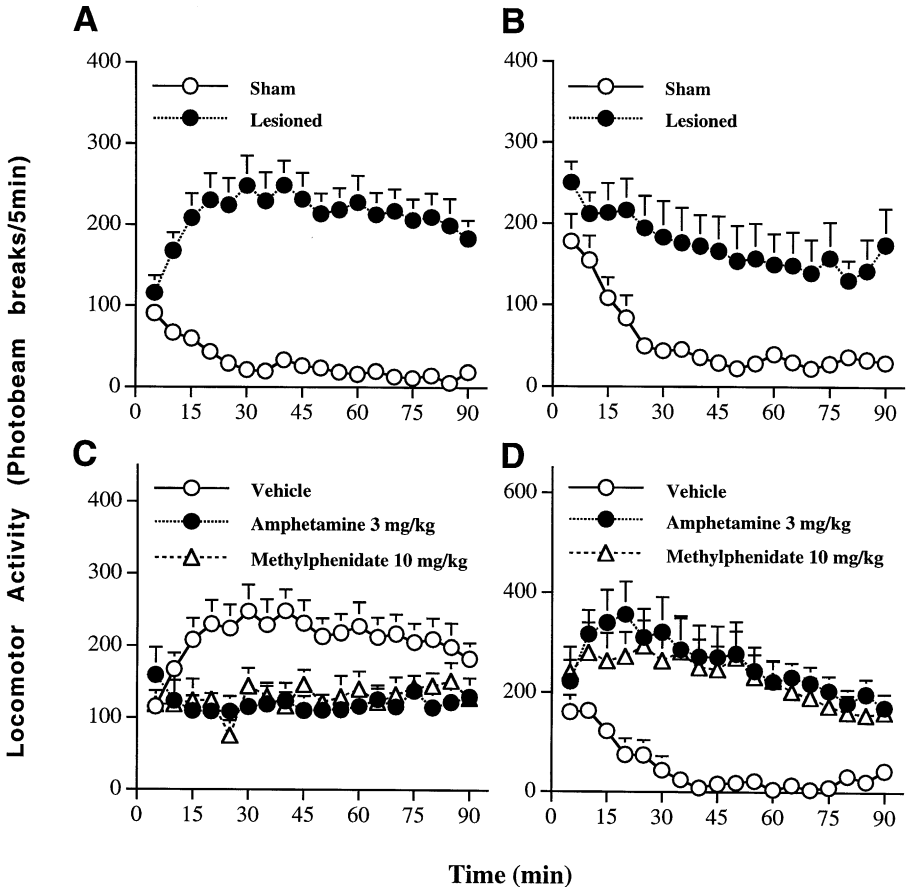

Figure 1. Hyperactivity induced by neonatal 6-OHDA lesions, and effects of psychostimulant drugs. **Panel A:** Daytime activity, 10 A.M.–4 P.M. (n = 23/group); **Panel B:** Nocturnal activity, 10 P.M.–4 A.M. (n = 17); **Panel C:** Effects of psychostimulants in 6-OHDA-lesioned rats, 10 A.M.–4 P.M. (n = 14–23); **Panel D:** Effects of psychostimulants in sham-lesioned rats, 10 A.M.–4 P.M. (n = 12).

lesioned controls, but not in 6-OHDA-lesioned rats at a moderate dose of 0.5 mg/kg (Figure 5).

DISCUSSION

In accord with previous studies (Shaywitz et al. 1976; Heffner and Seiden 1982; Luthman et al. 1989), neonatal 6-OHDA lesions resulted in spontaneous motor hyperactivity when rats were tested at a later, periadolescent, developmental stage in a novel environment. Also consistent with previous findings (Heffner and Seiden 1982; Luthman et al. 1989), lesion-induced hyperactivity was antagonized by both amphetamine and methylphenidate (Figure 1). Neonatal 6-OHDA lesions significantly increased D_4 receptor levels selectively in CPu. D_2-like receptor binding in the same brain region was also increased, but to a lesser extent. Motor hyperactivity induced by the lesions was significantly correlated with increased D_4 receptor levels but not D_2 levels (Figure 2).

Previous studies of postnatal development of DA receptors in rat brain indicated that D_4 receptors undergo extensive pruning at 4–5 weeks after birth (Tarazi et al. 1998b). The finding that D_4 receptor binding in CPu was significantly increased in lesioned rats above levels

Table 1. Effects of Neonatal 6-OHDA Lesions on Dopamine Receptor Binding in Rat Forebrain. Data are Specific Binding, As Mean fmol/mg Tissue ± SEM. By ANOVA: (*) $p < 0.05$, (**) $p < 0.01$, Significantly Different (Boldface) From Sham-Lesioned Control Littermates (N = 12)

	D_1-like		D_2-like		D_4	
	Sham	**6-OHDA**	**Sham**	**6-OHDA**	**Sham**	**6-OHDA**
CPu/Lateral	253.6 ± 13.7	228.5 ± 11.6	194.3 ± 9.8	**226.5 ± 7.2***	20.6 ± 2.2	**28.9 ± 1.4****
CPu/Medial	244.1 ± 14.3	233.4 ± 11.8	133.3 ± 6.8	**157.7 ± 6.0***	14.2 ± 1.5	**19.2 ± 1.7****
NAc	167.9 ± 10.4	173.9 ± 9.7	114.6 ± 8.2	102.2 ± 6.5	14.0 ± 1.2	15.0 ± 1.6
PFC	36.3 ± 2.4	39.0 ± 2.3	9.8 ± 0.4	10.3 ± 0.5	2.0 ± 0.4	2.0 ± 0.2

Brain regions are caudate-putamen (CPu), nucleus accumbens septi (NAc), and prefrontal cerebral cortex (PFC).

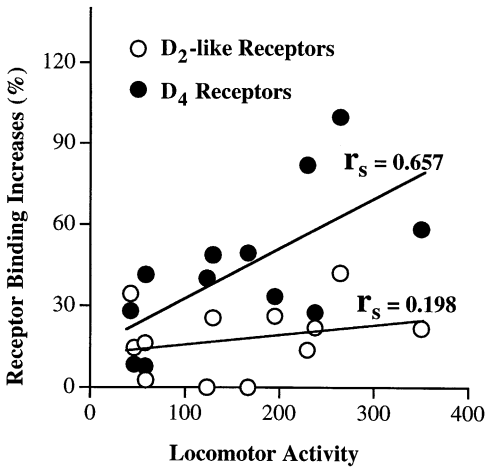

Figure 2. Relationship between motor hyperactivity and changes of dopamine receptor binding in CPu of 6-OHDA-lesioned rats (n = 12), analyzed by Spearman Rank Correlation (r_s).

in controls (Table 1) at a comparable age (PD 32) suggests that this pruning process may be hampered by neonatal 6-OHDA lesions. In addition, the severity of lesion-induced hyperactivity was highly correlated with the extent of the apparent up-regulation of D_4, but not D_2 receptors (Figure 2), further implicating impaired development of D_4 receptors in abnormal behaviors induced by the lesions.

The role of up-regulated D_4 receptors in motor hyperactivity was further investigated by assessing the behavioral effects of a D_4-selective antagonist and agonist (Sanner et al. 1998; Zorn et al. 1997). The D_4 antagonist CP-293,019 dose-dependently reversed lesion-induced motor hyperactivity without affecting activity in control littermates that received sham lesions (Figure 3). The D_4 agonist CP-226,269, in contrast, dose-dependently exacerbated motor hyperactivity in lesioned rats without affecting motor activity in unlesioned control rats. The finding that lesion-induced motor hyperactivity can be dose-dependently reversed by a D_4 antagonist rather than agonist suggests that stimulant properties in normal animals or agonistic properties at DA receptors are poor indicators for behavioral effects in ADHD models. These observations also indicate that psychostimulants do not antagonize motor hyperactivity by stimulating D_4 receptors.

In addition to blocking or reversing neuronal transport of DA, stimulant drugs also release serotonin (5-hydroxytryptamine, 5-HT) (Ritz et al. 1990). Increased innervation of forebrain structures, especially neostriatum, by serotonergic neurons is well documented in

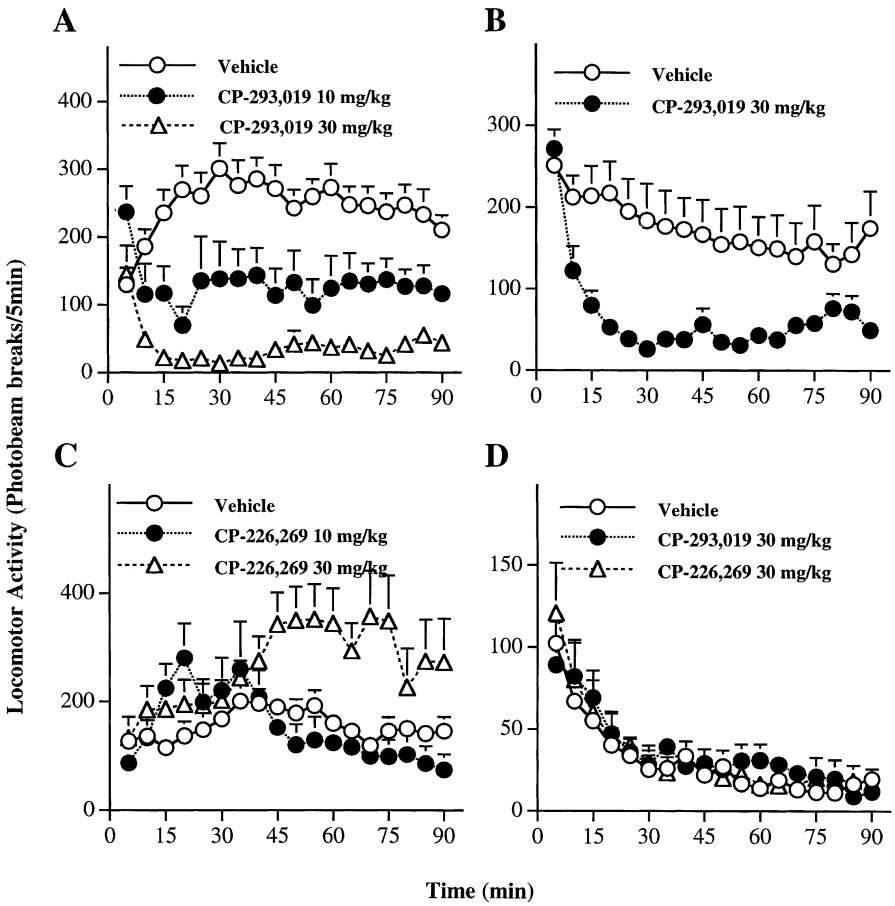

Figure 3. Effects of D_4 receptor-selective agents on hyperactivity induced by neonatal 6-OHDA lesions. **Panel A:** Effects of CP-293,019 in 6-OHDA-lesioned rats, 10 A.M.–4 P.M. (n = 23/group); **Panel B:** Effects of CP-293,019 in 6-OHDA-lesioned rats, 10 P.M.–4 A.M. (n = 13); **Panel C:** Effects of CP-226,269 in 6-OHDA-lesioned rats, 10 A.M.–4 P.M. (n = 17); **Panel D:** Effects of CP-293,019 and CP-226,269 in sham-lesioned rats, 10 A.M.–4 P.M. (n = 10).

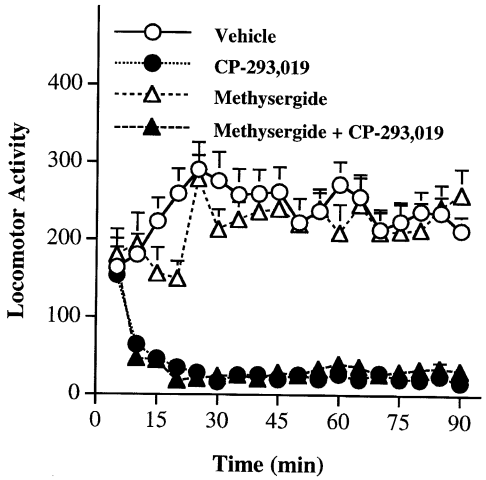

Figure 4. Effects of methysergide pretreatment on motor-inhibiting effects of CP-293,019 in 6-OHDA-lesioned rats (n = 14/group). Methysergide (2 mg/kg, i.p.) was administered 30 min prior to testing.

rats following neonatal lesioning with 6-OHDA (Kostrzewa et al. 1998). In hyperactive juvenile rats with neonatal 6-OHDA lesions, as well as hyperactive DAT knockout mice, the motor-inhibiting effects of stimulants seem to be mediated by enhanced release of 5-HT (Heffner and Seiden 1982; Gainedtinov et al. 1999). Therefore, we tested the possibility of interaction of D_4 antagonist CP-293,019 with 5-HT neurotransmission with methysergide, a broad-spectrum 5-HT receptor antagonist. Pretreatment of 6-OHDA-lesioned rats with methysergide did not affect their motor responses to subsequent injection of CP-293,019 (Figure 4), suggesting that the behavioral effects of the D_4 antagonist were not mediated by increased release of 5-HT. Methysergide alone failed to affect lesion-induced hyperactivity, further indicating that the motor-inhibiting effects of CP-293,019 in lesioned rats were not related to its moderate potency at 5-HT_{1A} and 5-HT_{2A} receptors (Ki = 150 and 500 nM, respectively; Sanner et al. 1998). Instead, these findings indicate that CP-293,019 antagonized lesion-induced hyperactivity by a mechanism distinct from that of stimulants.

A contribution of D_2 receptor blockade to behavioral effects of CP-293,019 seems very unlikely since this agent interacts very weakly at D_2 receptors (Ki >3.0 μM; Sanner et al. 1998). In addition, our recent studies indicated that CP-293,019 did not alter rotational behavior induced by D_2 receptor agonists in adult rats with unilateral nigrostriatal DA lesions (Zhang et al. 2001). To further rule out involvement of D_2 receptors, behavioral effects of eticlopride were investigated. At a moderate dose (0.5 mg/kg, i.p.), eticlopride markedly inhibited motor activity in sham-lesioned controls, but did not alter such behavior in 6-OHDA-lesioned rats (Figure 5). These observations accord with previous findings that adult rats with neonatal 6-OHDA lesions were less sensitive to D_2 receptor antagonists, in association with increased D_2 receptor binding (Bruno et al. 1985). Although eticlopride blocks D_2 receptors with only limited selectivity (D_2 vs. D_4 Ki-ratio ⩾20; Van Tol et al. 1991), the finding that it did not affect motor hyperactivity in lesioned rats at a dose that was effectively motor-inhibitory and produced some catalepsy in intact rats indicates that the effect of CP-293,019 is unlikely to be mediated by D_2 receptors.

Dysfunction of the frontal cerebral cortex is critically involved in the pathophysiology of ADHD (Barkley 1997; Faraone and Biederman 1998). Inhibition of behavioral responses, a primary function of the PFC in humans, has been found consistently to be deficient in patients with ADHD, based on neuropsychological testing (Barkley et al. 1992). Abnormal PFC structure or function is also strongly suggested by brain imaging studies using various techniques (Ernst and Zametkin 1995). The PFC sends glutamatergic projections to many subcortical structures, including CPu, NAc, and midbrain areas (Fonnum et al. 1981; Walaas 1981). Removal of these excitatory corticofugal efferents by PFC

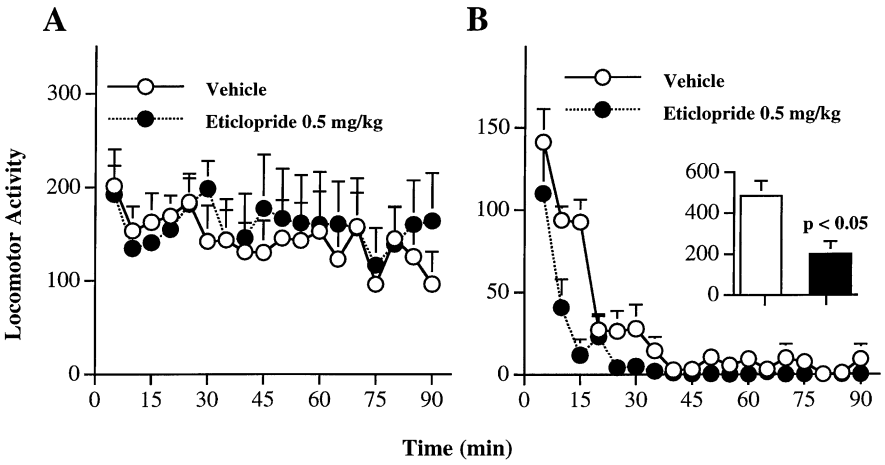

Figure 5. Effects of eticlopride (0.5 mg/kg, i.p.) on locomotor activity (n = 12/group) in: Panel A: 6-OHDA-lesioned rats; Panel B: sham-lesioned rats; inset: cumulative score for the entire session in sham controls.

ablation results in hyperactivity in response to environmental stimuli in primates (Fuster 1989; Wilkinson et al. 1997), as well as supersensitive behavioral responses to both external and internal stimuli in rats (Bubser and Schmidt 1990; Flores et al. 1996; Lacroix et al. 1998). Relatively high levels of D_4 receptors are expressed in glutamatergic pyramidal neurons and GABAergic interneurons in PFC, as well as in the nerve terminals of glutamatergic projections from PFC to CPu (Ariano et al. 1997; Mrzljak et al. 1996; Tarazi et al. 1998a; De la Garza and Madras 2000), suggesting that the excitatory output from PFC to subcortical structures may be inhibited by D_4 receptors. We did not find altered D_4 receptor binding in PFC or NAc of 6-OHDA-lesioned rats (Table 1). However, a substantial increase in D_4 binding was detected in CPu, and this increase correlated closely with motor hyperactivity (Figure 2). Up-regulated, and possibly supersensitive, D_4 receptors may contribute to hyperactivity in lesioned rats by selectively inhibiting excitatory output from PFC to CPu. Further studies on *in vivo* regulation of glutamate release by D_4 receptors in normal and lesioned subjects are required to test this hypothesis.

Finally, the treatment of ADHD has for many years been based on the ability of stimulant drugs to attenuate hyperactivity and improve cognition (Barkley 1990; Swanson 1993; Goldman et al. 1998). These drugs, however, are far from satisfactory, owing to their short-lived benefits, tendency to impair appetite and sleep and to induce abnormal movements, as well as their potential for abuse or illicit distribution (Goldman et al. 1998). The effectiveness of CP-293,019 in antagonizing motor hyperactivity induced by neonatal DA lesions encourages clinical testing of innovative treatments of ADHD with D_4 receptor selective agents.

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