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Behavioral effects of dopamine agonists and antagonists in MPTP-lesioned D_3 receptor knockout mice

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Abstract

To test the modulatory role of D_3 receptors in normal and dopamine-depleted mice, D_3 receptor KO mice and wild-type (WT) littermates were administered saline, L-dopa/carbidopa (20/2 mg/kg ip), a preferential D_3>D_2 agonist S32504, a D1 + D_2/D_3 agonist apomorphine, a selective D_3 antagonist S33084, or apomorphine with S33084 prior to and after administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). We monitored lines crossed in a 55-min session, average number of rears, and average number of grooming bouts. MPTP treatment produced equivalent 70% losses of dopamine fibers in the caudate putamen (CPu) and nucleus accumbens (NAC) of WT and D_3 KO mice as compared to their control (vehicle injected) counterparts. D_3 receptors were absent in KO mice, and the number of D_3 receptors was unaffected by MPTP-induced loss of DA terminals in WT mice. The results support a lack of involvement of the D_3 receptor for D1:D2 receptor-mediated behavioral activity (synergy). First, S32504 inhibited all behaviors and to a similar degree in D_3 KO and WT mice. Second, S33084 at the higher concentration increased number of lines crossed in response to high dose apomorphine in both D_3 KO and WT mice. Third, in nonlesioned mice, apomorphine-induced gnawing stereotypies were inhibited by S33084 in both D_3 KO and WT mice. Interestingly, the inhibition of apomorphine-induced gnawing was not apparent in MPTP-lesioned mice, and this stereotypy was elevated in D_3 KO-MPTP-lesioned mice. Thus, the suppressive effects of S32504 could be via D2 autoreceptor inhibition of DA release, and D2 receptor blockade by S33084 leads to release of that inhibition. This may be more apparent in MPTP-lesioned partially DA denervated mice.

Keywords: Parkinson's disease; Caudate putamen; Substantia nigra; Dopamine transporter; S33084; S32504

1. Introduction

The DA D_3 receptor's pattern of expression in brain would indicate that behaviors mediated via this receptor should be closely related to the functions of the mesolimbic DA system (Joyce, 2001b). Because of that fact and its close relationship in structure and coupling to intracellular transduction systems of the D_2 receptor, the focus of the analysis of the behavioral functions of the D_3 receptor has paralleled studies of the D_2 receptor. Thus, the research has been largely limited to analysis of D_3 receptor mediation of changes in locomotion, although a few other behavioral paradigms have been explored. In addition, the agonists used to test these behavioral effects (e.g., 7-OH-DPAT and PD128907) show pronounced D_3 to D_2 receptor selectivity in vitro, but this is far more difficult to establish in vivo (Joyce, 2001b). Nonetheless, certain concepts have emerged with respect to the functional effects of D_3 receptor stimulation that are important to consider. The D_3-preferring agonists 7-OH-DPAT and PD128907 have been reported to decrease locomotor activity at low doses in rats and increase locomotor activity at higher doses (Damsma et al., 1993; Daly and Waddington, 1993; Depoortere et al., 1996; Khroyan et al., 1995; Gilbert and Cooper, 1995). The behavioral effects of the low dose of these agonists are not associated with decreased release of DA, suggesting postsynaptic effects of these compounds (Svensson et al., 1994; Thorn et al., 1997), and the same effects occur upon systemic or intraaccumbens injections (Gilbert and Cooper, 1995). It is hypothesized that the low dose effects of D_3-preferring agonists occur through the D_3 receptor and higher doses through D_2 and D_3 receptors, and that the D_3 receptor within NA exerts inhibitory activity on the behavioral

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activation to DA agonists (Damsma et al., 1993; Gilbert and Cooper, 1995; Waters et al., 1994). However, the agonists used to test these behavioral effects (e.g., 7-OH-DPAT and PD128907) show pronounced D_3 to D_2 receptor selectivity in vitro, but this is far more difficult to establish in vivo (Joyce, 2001b; Levant, 1997). For example, testing of the selectivity of these compounds often does not take into account the multiple affinity states of the D_2 receptor, which may be a better prediction of their in vivo selectivity (Burris et al., 1995; Levant, 1997).

Interestingly, recent data suggest that the behavioral action of D_3 receptor-preferring agonists may be altered in animals depleted of DA. For example, locomotor stimulatory activity is observed in 6-hydroxydopamine (6-OHDA)-lesioned rats at the same doses of D_3-preferring agonists that are inhibitory in normosensitive rats (Van den Buuse, 1993). The D_3-preferring agonist pramipexole can reverse muscle rigidity produced by the combination of reserpine and α-methyl-p-tyrosine to deplete DA or haloperidol to block DA receptors (Lorenc-Koci and Wolfarth, 1999). This suggests that animals depleted of DA might respond to D_3-preferring agonists differently from normosensitive animals. This has been further explored in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinsonism in monkeys. In monkeys treated with MPTP, to produce severe akinesia, the D_3-preferring agonist PD 128,907 was found to be equally potent as apomorphine in reversing the hypoactivity, and this effect was blocked by the D_3 antagonist U-99194A (Blanchet et al., 1997). Similarly, L-dopa reversal of MPTP-induced hypoactivity in monkeys is dose dependently inhibited by the D_3 antagonist nafadotride (Hadjtahar et al., 1999) or ST 198 (Bézard et al., 2003). In normosensitive mice, low doses of apomorphine induce hypolocomotion but at higher doses induce stereotyped locomotor activity and gnawing (Geter-Douglass et al., 1997). Similarly, apomorphine at low doses inhibits climbing activity in mice but increases it at higher doses (Spooren et al., 1998). In mice depleted of DA by MPTP, apomorphine shows a behavioral supersensitivity represented by a shift in dose–response curve to lower doses to induce the increase in climbing activity (Lau and Fung, 1986; Spooren et al., 1998). It is possible that following extensive DA depletion D_3-preferring agonists might also elevate locomotor activity in mice through stimulation of D_3 receptors.

To test this more specifically, we examined the behavioral responses of D_3 receptor KO (−/−) and wild-type (WT) (+/+) mice to the nonselective agonist apomorphine in the presence and absence of the selective D_3 receptor antagonist S33084 (Millan et al., 2000a,b) and in unlesioned and MPTP-treated mice. We also examined in the same mice the behavioral response to the D_3 receptor-preferring agonist S32504 (Brocco et al., 2001; Dekeyne et al., 2001). S32504 displays marked affinity for cloned human (h) dopamine D_3 receptors (0.1 nM), with 100-fold selectivity or greater for the long (hD_{2L}) and short (hD_{2S}) isoforms of D_2 receptors and hD_4, hD_1, and hD_5 receptors, and very low affinity for non-DAergic receptors (Millan et al., 1999). In functional assays, S32504 is a potent and efficacious agonist at hD_3 receptors, actions blocked by haloperidol and by S33084; but at higher concentrations, its actions are blocked by the selective D_2 antagonist, L741,626 (Millan et al., 1999, 2000b) (Cussac and Millan, Institut de Servier, Croissy, France, unpublished observation). Here we report that both S32504 and S33084 had behavioral actions by themselves and S33084 modified the behavioral responses to the high dose of apomorphine. However, as the behavioral responses did not differ between D_3 KO and WT mice, this suggests a lack of involvement of the D_3 receptor for D1:D2 receptor-mediated behavioral activity (synergy).

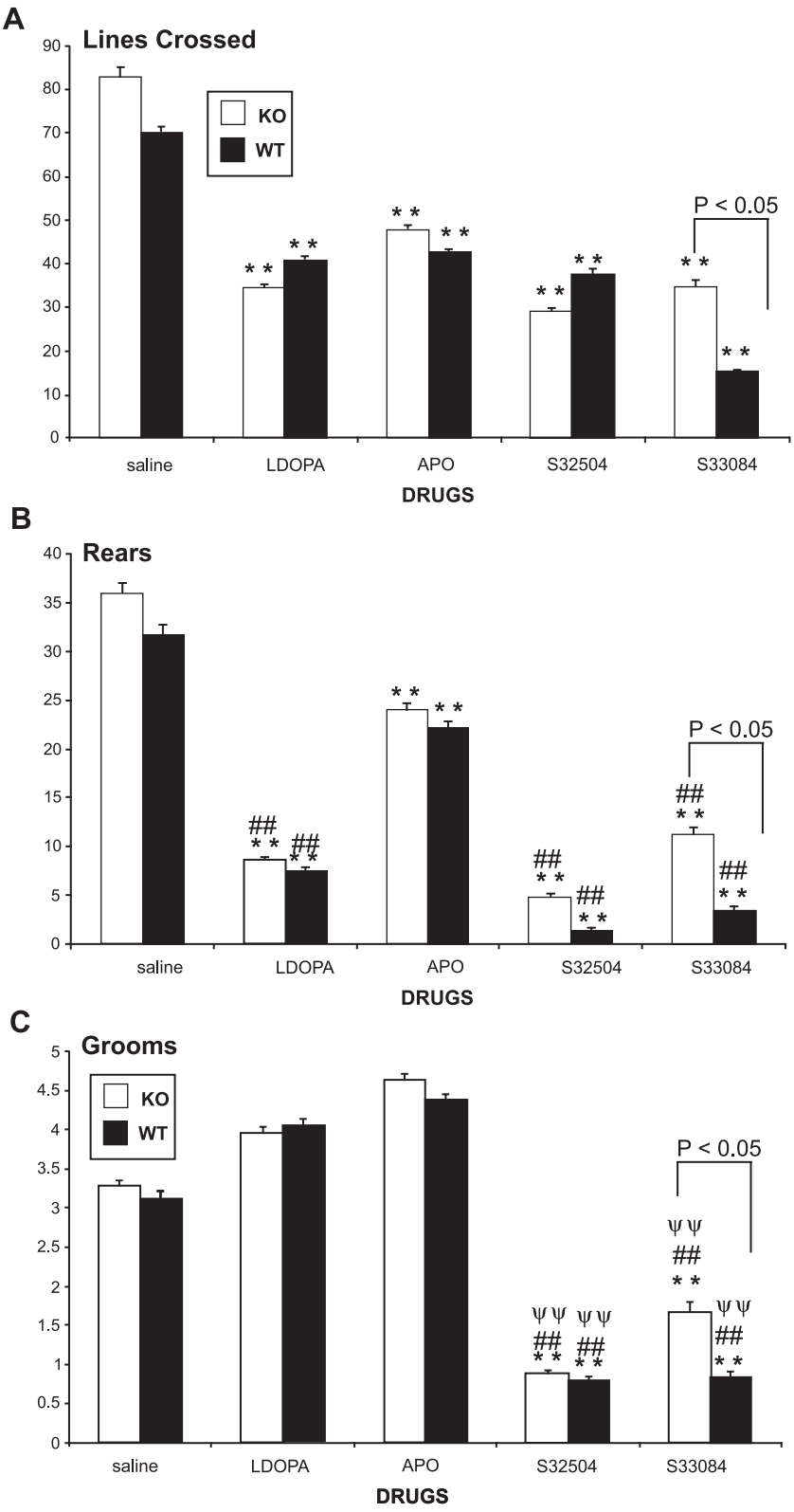
2. Method

2.1. Subjects and experimental design

We established D_3R −/− (D_3 KO) mice from a breeding pair obtained from Jackson Laboratories (B6,129-$Drd3^{tm1Dac}$) and backcrossed to an inbred genetic background (C56/6J) for more than 10 generations and produced D_3 KO animals and WT littermates with defined hybrid genetic backgrounds. Primers for genotyping D_3 KO and WT mice were #215 (5′ to 3′) GCA GTG GTC ATG CCA GTT CAC TAT CAG; #216: CCT GTT GTC TTG AAA CCA AAG AGG AGA GG; #217: TGG ATG TGG AAT GTG TGC GAG; and #218: GAA ACC AAA GAG GAG AGG GCA GGA C. Both 215/216 and 217/218 primers were used at a stock concentration of 50 μM and were combined at a 1:6 ratio, respectively, to form a two-plex mix. A true deletion of the 200-bp band indicated the +/+ (WT) and a true deletion of the 137-bp band indicated the −/− (KO). Forty-eight male and female C57BL/6 mice ranging from 8 to 10 months of age were used for the experiments and were handled for 1 week prior to drug treatment and group housed four per cage. The room was

Fig. 1. Locomotor activity (lines crossed), frequency of rears, and frequency of grooming bouts in WT (D_3 +/+, $n=24$) and KO (D_3 −/−, $n=24$) mice administered different DA agonists. Mice were administered saline (1 ml/kg ip), L-dopa/carbidopa (20/2 mg/kg ip), apomorphine (0.05 mg/kg sc), S32504 (1.0 mg/kg ip), or S33084 (2 mg/kg ip) in a counter balanced design. The presence or absence of four independent behaviors was measured for 1 min of every 5-min block of the 60-min test per the procedures in the Method section. The mean ± S.E.M. is shown for (A) the total number of lines crossed of each 1-min observation block for the last 55 min of the test session; (B) for the number of rears the average number for each 1-min observation block for the entire 60 min; and (C) for the grooming bouts the average number for each 1-min observation block for the entire 60 min of the test session. As there were no significant effects of genotype, all comparisons were made for drug within genotype (WT or KO). Significant effects shown with ** represents $P<.001$ versus saline; ψψ represents $P<.01$ versus L-dopa; and ## represents $P<.01$ versus apomorphine. Abbreviations: WT, D_3 +/+; KO, D_3 −/−; APO, apomorphine.

maintained at a temperature of 28 °C and the cages at a temperature of 36 °C with the use of thermal barrier pads (Large Thermal Barrier, Harvard Apparatus, Holliston, MA). Mice had free access to food and water and were maintained in a 12:12-h light/dark cycle. All mice were tested for the behavioral response to DA agonists and antagonists prior to their segregation to treatment groups (Experiment 1). Subsequently, one half of the WT and KO



mice were assigned to one of the two treatment groups. One group received only vehicle, the second received MPTP (Sigma, St Louis, MO), and mice were given injections of either MPTP (20 mg/kg sc) or vehicle (0.9% NaCl) twice daily at 8-h intervals for 2 days. Thus, all mice were assigned to one of four groups ($n = 12$ per group): WT administered vehicle (WT-CO), WT administered MPTP (WT-MPTP), KO administered vehicle (KO-CO), and KO administered MPTP (KO-MPTP). At the end of a 14-day recovery period following the last injection of MPTP or vehicle, mice were administered all drugs in a counterbalanced design (Experiment 2). At the conclusion of Experiment 2, the mice were anesthetized with 1.0 mg/kg ip pentobarbital, brains quickly removed, frozen in isopentane, and stored at −70 °C prior to sectioning. All animals were treated in accordance with a protocol approved by the Sun Health Research Institute Animal Care and Use Committee.

2.2. Drugs

L-Dopa/carbidopa (ratio 10:1; Sigma) was dissolved in 0.9 % NaCl and injected intraperitoneally at a volume of 1 ml/kg, and carbidopa was administered 20 min (change to min throughout document) before L-dopa. The D1/D2/D3 agonist apomorphine (Sigma) was dissolved in 0.9% NaCl and injected subcutaneously at a volume of 0.5 ml/kg. The D_3-preferring agonist S32504 (Institut de Servier, Croissy, France) was dissolved in a small amount of ethanol, brought to pH 7.0 with PBS, and administered intraperitoneally at a volume of 1 ml/kg. The D_3 antagonist S33084 (Institut de Servier) was dissolved in Lactic acid (80% to saline) brought to pH 7.0 with NaOH (10 N) and administered intraperitoneally at a volume of 1 ml/kg.

2.3. Experiment 1

All mice were administered saline (1 ml/kg ip), L-dopa/carbidopa (20/2 mg/kg ip), apomorphine (0.05 mg/kg sc), S32504 (1.0 mg/kg ip), or S33084 (2 mg/kg ip) in a counterbalanced design. Doses were chosen based on the literature so as to be D_3 receptor preferring. Each drug/dose was administered one time at 3-day intervals, except for saline, which was administered on the first and last behavioral test days. All testing occurred during the light phase of the light/dark cycle. After injection, mice were placed into a circular arena 23 cm in diameter with a 15-cm high wall and a covered Plexiglas top. The floor of the chamber was made of wire mesh supported on a Plexiglas platform with lines at 7.3 cm apart dividing the floor into six areas of approximately equal size. The presence or absence of four independent behaviors was measured for 1 min of every 5-min block of the 60-min test per our standard procedures (Neal-Beliveau and Joyce, 1998; Neal and Joyce, 1991). The behaviors were (1) lines crossed, (2) number of rears, (3) number of grooms, and (4) gnawing. Rears were scored when front paws reaching upward while standing on hind limbs occurred (Wong et al., 2003); groom was scored when snout remained in contact with any surface of the body for more than 5 sec and was terminated with return to another activity (Wong et al., 2003); gnawing was recorded when the incisors were placed over the wire mesh and accompanied by the typical jaw movements of biting (Geter-Douglass et al., 1997).

2.4. Experiment 2

At 14 days following MPTP/vehicle treatment, the mice were administered saline (1 ml/kg ip), L-dopa/carbidopa (20/2 mg/kg ip), S32504 (1 mg/kg ip), apomorphine (0.05, 1.0, and 5.0 mg/kg sc), S33084 (0.1 and 2 mg/kg ip), and apomorphine (5 mg/kg sc) 5 min after administration of S33084 (0.1 and 2 mg/kg ip) in a counterbalanced design. Doses were chosen based on the literature so as to be D_3 receptor preferring or exhibit pronounced D1 and D2 receptor effects (apomorphine). Each drug/dose was administered one time at 3-day intervals, except for saline, which was administered on the first and last behavioral test days. Behavioral testing was completed as in Experiment 1.

2.5. Quantitative autoradiography

Dopamine transporter (DAT) sites were labeled with [^{125}I]RTI-55 (3β-(4-iodophenyl)tropan-2 β-carboxylic acid methyl ester) (Dupont, New England Nuclear, Boston, MA) according to our previously published conditions (Joyce et al., 2000; Joyce, 2001a). D_3 receptor binding was conducted with [^{125}I]*trans* 7-OH-PIPAT (Dupont) according to our previously published conditions (Joyce et al., 2000; Joyce, 2001a; Ryoo et al., 1998). Sections were apposed to ^{3}H-Hyperfilm for 18 h for DAT and 24 h for [^{125}I]*trans* 7-OH-PIPAT. Autoradiographs were analyzed using a computer-based image analysis system (AIS, Imaging Research, ON, Canada) that converts transmitted optical density to the amount of radioligand bound in femtomole per milligram of protein.

2.6. Statistical analysis

For each animal and drug administration, (1) the total number of lines crossed of each 1-min observation block for the last 55 min of the test session was calculated; and (2) for the number of rears, grooming bouts, and gnawing bouts, the average number for each 1-min observation block for the entire 60 min of the test session was calculated. For Experiments 1 and 2, a two-way ANOVA, with Sex × Genotype (Experiment 1) and Sex × Group (Experiment 2) as main factors, was initially used to determine any significant sex, Sex × Genotype, or Sex × Group interactions. No main effects of sex or interactions were found, and all subsequent analyses excluded sex as a factor. For Experiment 1, a two-way ANOVA, with drug and genotype as main factors, and pairwise comparisons performed using post hoc Dunnett

with Bonferroni correction. For Experiment 2, the animals were assigned to treatment groups and their behavioral responses to drugs analyzed with two-way ANOVA, with drug and group as main factors, and pairwise comparisons performed using post hoc Dunnett with Bonferroni correction. To test for the possible effect of prior dosing of mice in Experiment 1 on their response in Experiment 2, the behavioral responses to saline, apomorphine, S33084, and S32504 in nonlesioned WT and KO mice were analyzed by ANOVA for between-test differences. For determinations of quantitative autoradiography, the striatum was divided into the caudate putamen (CPu), nucleus accumbens (NAC), islands of Calleja (IC), globus pallidus (GP), and ventral pallidum (VP) according to the atlas of the C57/BL6 mouse brain (Hof et al., 2000). Group means and variances were produced. Statistical analysis of group differences was assessed by two-way ANOVA, with regions and treatment group as main factors, and pairwise comparisons performed using post hoc Dunnett with Bonferroni correction.

3. Results

3.1. Experiment 1

The behavioral responses (lines crossed, average number of rears, and average number of grooms) did not differ between the first and last tests with saline, indicating no shifting baseline. Analysis by two-way ANOVA, with drug and genotype as factors, determined that there were significant drug effects for lines crossed ($P<.0001$; $F=18.87$), average number of rears ($P<.0001$; $F=48.47$), and average number of grooms ($P<.0001$; $F=30.44$). Only for the average number of rears was there significant genotype effects ($P=.0246$; $F=5.121$), and for none of the behavioral measures were there significant Drug × Genotype interaction effects. Since none of the drugs at the doses tested produced gnawing, this behavioral measure was not analyzed. Since there was no Drug × Genotype interaction, data were collapsed across genotype and significant effects tested by one-way ANOVA to examine the source of the drug effect. For lines crossed (Fig. 1A) and average number of rears (Fig. 1B), all drugs tested (L-dopa, apomorphine, S32504, and S33084) produced a reduction in numbers as compared with saline (control) in both WT and D_3 KO mice.

For average number of rears, treatment with L-dopa, S32504, and S33084 reduced rearing more than apomorphine in both WT and D_3 KO mice. For average number of grooms, S32504 and S33084 produced greater reductions than saline, L-dopa, and apomorphine in both WT and D_3 KO mice (Fig. 1C). The only drug that exhibited apparent genotype differences was the D_3 antagonist S33084, which showed greater suppression of lines crossed ($P=.0339$), average number of rears ($P=.0129$), and average number of grooms ($P=.0456$) in WT than D_3 KO mice when analyzed for significance by post hoc *t* test.

Analysis of the effects of the drugs on lines crossed, average number of rears, and average number of grooms was also made based on their assignment to group (WT-CO, WT-MPTP, KO-CO, and KO-MPTP) but prior to the MPTP/vehicle injections. Two-way ANOVA with drug and group showed no significant effect of drug and group or interaction of drug with group as factors.

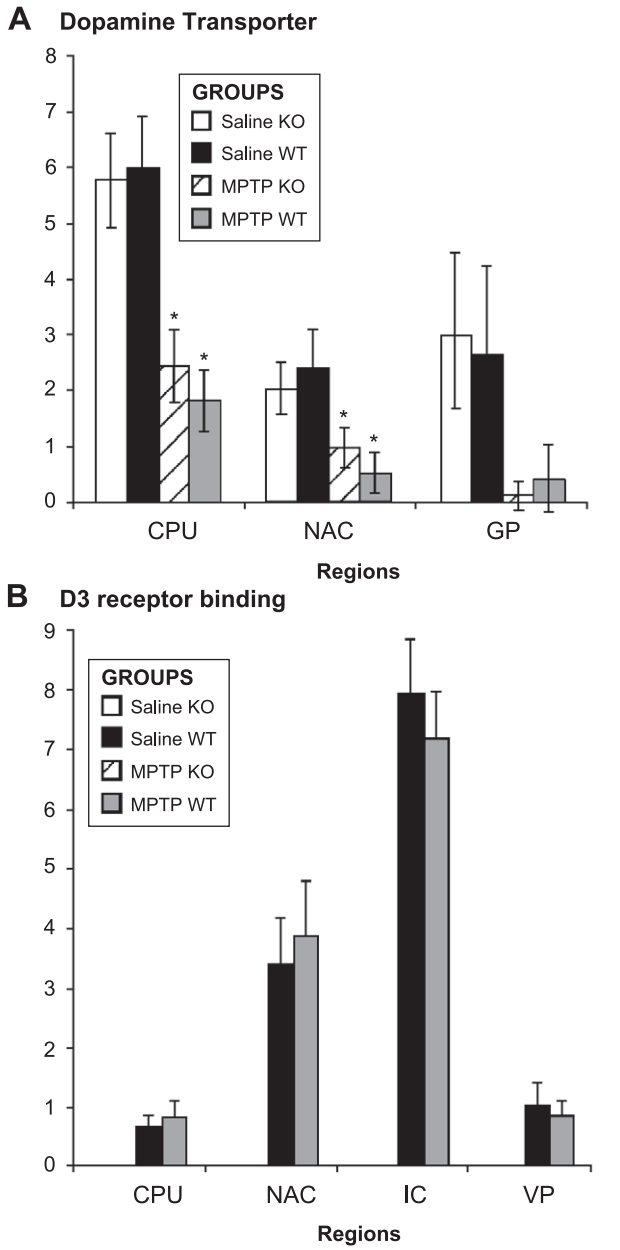


Fig. 2. Neurochemical effects of the administration of MPTP in WT and KO mice. (A) The mean ± S.D. for DAT sites labeled with [^{125}I]RTI-55. (B) The mean ± S.D. for D_3 receptor binding sites labeled with [^{125}I]*trans* 7-OH-PIPAT. Note the significant reduction in DAT sites produced by MPTP (* represents $P<.05$ vs. saline) in KO and WT mice for the CPu and NAC. D_3 receptor binding sites were absent in KO mice and not different in WT mice treated with MPTP as compared to the vehicle saline. Abbreviations: CPU, caudate putamen; GP, globus pallidus; IC, islands of Calleja; NAC, nucleus accumbens; VP, ventral pallidum.

3.2. Neurochemical results

[^{125}I]RTI-55 binding to DAT is considered to be a measure of the integrity of DA fibers as they are localized to DA terminals (Joyce et al., 1986; Murray et al., 1995). The density of sites was not different between WT-CO and KO-CO groups, with the highest density of binding sites in the CPu and less in the NAC and GP (Fig. 1A). MPTP had a significant effect in both WT and D_3 KO mice, with significant differences between WT-CO and WT-MPTP groups ($P<.05$) and between KO-CO and KO-MPTP groups ($P<.05$). The reduction of [^{125}I]RTI-55 binding was slightly but not significantly less in the WT-MPTP than the KO-MPTP groups in the CPu (-70% vs. -59%, respectively) and NAC (-79% vs. -60%). [^{125}I]*trans* 7-OH-PIPAT binding to D_3 receptors was absent in D_3 KO mice (Fig. 1B) and similar in WT-CO and WT-MPTP groups. The concentration of D_3 receptors was highest in the IC, less in the NAC, and very low in the CPu and VP (Fig. 2).

3.2.1. Experiment 2: Behavioral effects of dopamine agonists

The behavioral responses (lines crossed, average number of rears, and average number of grooms) did not differ between the first and last tests with saline, indicating no shifting baseline. Analysis of behavioral responses to the agonists (L-dopa, apomorphine at 0.05, 1.0, and 5.0 mg/kg, and S32504) by two-way ANOVA, with drug and group as factors, determined that there were significant drug effects (Fig. 3) for lines crossed ($P<.0001$; $F=36.51$), average number of rears ($P<.0001$; $F=55.35$), and average number of grooms ($P<.0001$; $F=24.26$) but not Group or Group-× Drug interactions. For lines crossed (Fig. 3A), L-dopa and S32504 produced a reduction in numbers as compared with saline (control) for all groups ($P<.05$). Apomorphine at doses of 0.05 and 1.0 mg/kg did not differ from saline. S32504 not only reduced lines crossed as compared to saline but also as compared to apomorphine at the 0.05 mg/kg dose ($P<.01$). At the highest dose tested, apomorphine (5.0 mg/kg) increased numbers of lines crossed as compared to saline in ($P<.05$), to L-dopa ($P<.001$), and S32504 ($P<.01$) in all groups.

For the analysis of average number of rears (Fig. 3B), L-dopa, apomorphine at 1.0 and 5.0 mg/kg, and S32504 significantly suppressed numbers as compared to either saline ($P<.05$) or apomorphine at a dose of 0.05 mg/kg ($P<.01$) in all groups. For the analysis of average number of grooms (Fig. 3C), apomorphine at a dose of 5.0 mg/kg and S32504 suppressed numbers as compared to saline ($P<.05$) and apomorphine at a dose of 0.05 mg/kg ($P<.01$) in all groups.

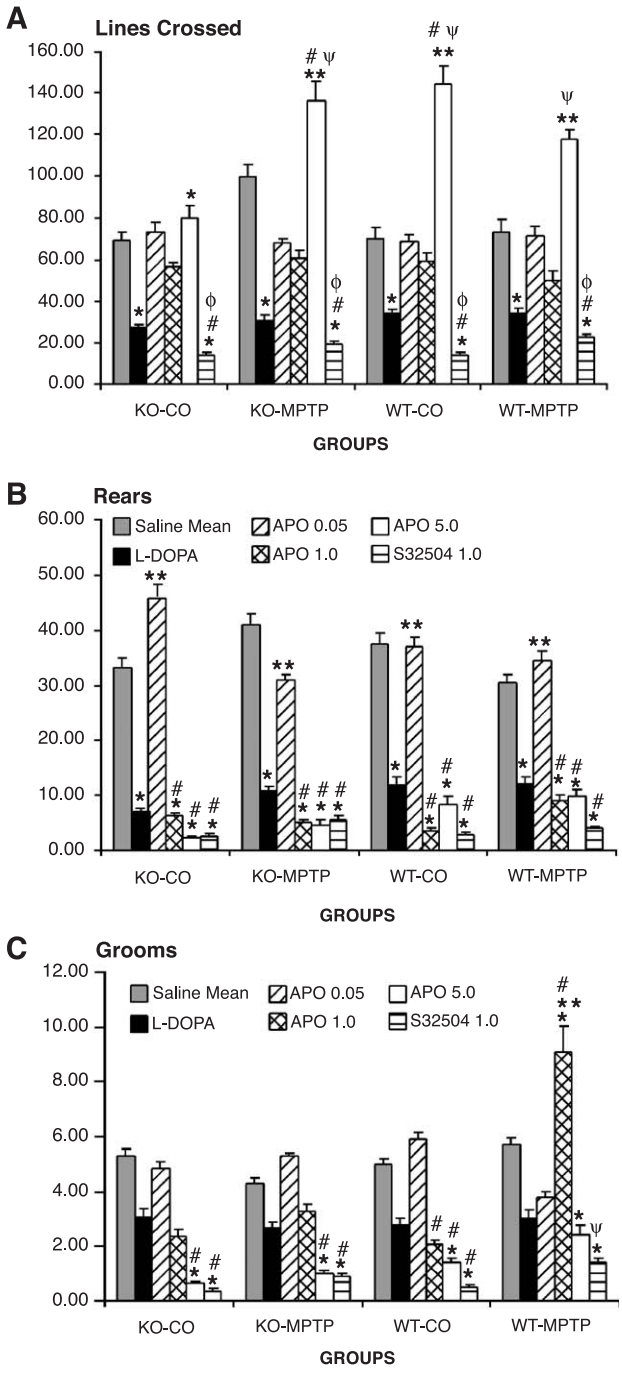


Fig. 3. The mean ± S.E.M. for (A) locomotor activity (lines crossed), (B) frequency of rears, and (C) frequency of grooming bouts in response to DA agonists in different experimental treatment groups. At 14 days following MPTP/vehicle treatment, the mice were administered saline (1 ml/kg ip), L-dopa/carbidopa (20/2 mg/kg ip), S32504 (1 mg/kg ip), and apomorphine (0.05,1.0, and 5.0 mg/kg sc) in a counter-balanced design. Behavioral observations were made as in Fig. 1. As there were no significant effects of group, all comparisons were made for drug within group (KO-CO, KO-MPTP, WT-CO, and WT-MPTP). Significant effects: * represents $P<.05$ versus saline; ** represents $P<.001$ versus saline; # represents $P<.01$ versus apomorphine (0.05 mg/kg dose); ψ represents $P<.01$ versus apomorphine (1.0 mg/kg dose); and Φ represents $P<.01$ versus apomorphine (5.0 mg/kg dose). Abbreviations: WT-CO, D_3 +/+ administered vehicle ($n=12$); WT-MPTP, D_3 +/+ administered MPTP ($n=12$); KO-CO, D_3 −/− administered vehicle ($n=12$); KO-MPTP, D_3 −/− administered MPTP ($n=12$).

Only in the WT-MPTP group were there significant effects of apomorphine at a dose of 1.0 mg/kg, with significant increases as compared to saline ($P<.05$), L-dopa ($P<.01$), apomorphine 0.05 ($P<.01$) and 5.0 mg/kg ($P<.01$), and S32504 ($P<.01$).

3.2.2. Experiment 2: Behavioral effects of S33084 with and without apomorphine

Analysis of behavioral responses to the S33084 (0.1 and 2.0 mg/kg) alone or with apomorphine (5.0 mg/kg) in comparison with saline or apomorphine by two-way ANOVA, with drug and group as factors, determined that for lines crossed (Fig. 4A) there were significant Drug ($P<.0001$; $F=18.46$) and Group effects ($P=.0252$; $F=3.166$) but not Group × Drug interactions. For the analysis of lines crossed, S33084 at 0.1 and 2.0 mg/kg increased numbers as compared to saline, this was significant ($P<.05$) for KO-CO, KO-MPTP, and WT-CO for the 0.1 mg/kg dose and significant ($P<.05$) for all groups at the 2.0 mg/kg dose. Apomorphine at a dose of 5.0 did not increase lines crossed but when combined with S33084 at 0.1 and 2.0 mg/kg increased numbers as compared to saline ($P<.05$). Apomorphine with S33084 at 0.1 mg/kg dose produced significantly ($P<.05$) higher activity than S33084 (0.1 mg/kg dose) for KO-CO, KO-MPTP, and WT-CO. Apomorphine with S33084 at 2.0 mg/kg dose produced significantly ($P<.01$) higher activity than S33084 (2.0 mg/kg dose) for all groups. Apomorphine with S33084 at 2.0 mg/kg dose produced significantly ($P<.05$) higher activity than apomorphine alone for KO-MPTP and WT-MPTP groups.

For the analysis of average number of rears (Fig. 4B), there were significant Drug ($P<.0001$; $F=33.80$), Group ($P=.0052$; $F=4.363$), and Group × Drug interactions ($P=.0006$; $F=2.753$). Apomorphine alone or in combination with S33084 (0.1 and 2.0 mg/kg doses) significantly ($P<.05$) decreased the average number of rears as compared to saline in all groups. S33084 at a dose of 0.1 mg/kg was ineffective but S33084 at a dose of 2.0 mg/kg significantly ($P<.05$) reduced the rearing frequency in KO-CO and WT-CO mice but not MPTP-treated groups. Apomorphine alone or in combination with S33084 at a dose of 2.0 mg/kg produced significantly less rearing than S33084 at the dose of 2.0 mg/kg in KO-CO and WT-CO mice but not MPTP-treated groups. Similar results were obtained for the analysis of average number of grooms (Fig. 4C); there were significant Drug ($P<.0001$; $F=31.14$), Group ($P=.0282$; $F=3.081$), and Group × Drug interactions ($P=.0004$; $F=2.832$). Apomorphine alone or in combination with S33084 (0.1 and 2.0 mg/kg doses) significantly ($P<.05$) decreased the average number of grooms as compared to saline in all groups. S33084 at a dose of 0.1 mg/kg was ineffective but S33084 at a dose of 2.0 mg/kg significantly ($P<.05$) reduced the grooming frequency in KO-CO and WT-CO mice but not MPTP-treated groups. Apomorphine alone or in combination with S33084 at a dose of 2.0 mg/kg

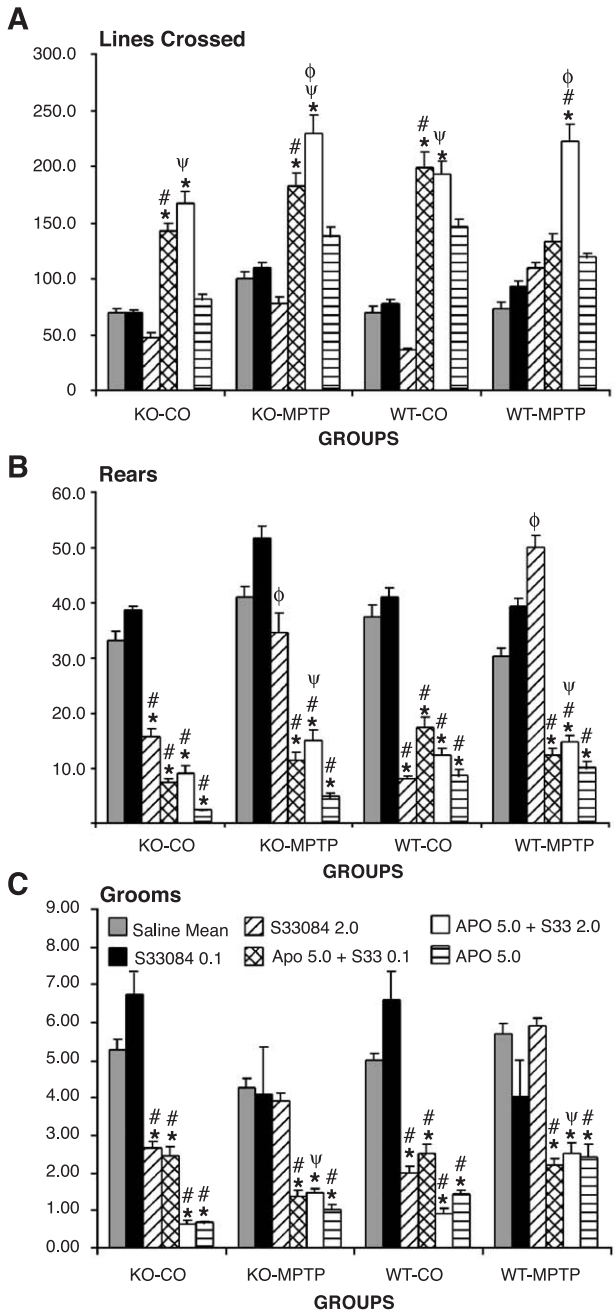


Fig. 4. The mean ± S.E.M. for (A) locomotor activity (lines crossed), (B) frequency of rears, and (C) frequency of grooming bouts in response to D_3 antagonist S33084 and apomorphine with and without S33084 in different experimental treatment groups. Following MPTP/vehicle treatment, the mice were administered saline (1 ml/kg ip), S33084 (0.1 and 2 mg/kg ip), apomorphine (5 mg/kg), and apomorphine (5 mg/kg) 5 min after administration of S33084 (0.1 and 2 mg/kg ip) in a counter-balanced design. Behavioral observations were made as in Fig. 1. As there were no significant effects of group, all comparisons were made for drug within group (KO-CO, KO-MPTP, WT-CO, and WT-MPTP). Significant effects: * represents $P<.05$ versus saline; # represents $P<.01$ versus S33084 (0.1 mg/kg dose); ψ represents $P<.01$ versus S33084 (2.0 mg/kg dose); and Φ represents $P<.05$ versus apomorphine (5.0 mg/kg dose). Abbreviations as shown in Fig. 3.

produced significantly less grooming than S33084 at the dose of 2.0 mg/kg in KO-CO and WT-CO mice but not MPTP-treated groups.

Apomorphine at a dose of 5.0 mg/kg produced stereotyped gnawing accompanied by changes in locomotor activity (Fig. 5), identifiable by snout contact with the floor (Beck et al., 1986; Szechtman et al., 1982). When analyzed for effects on the frequency of gnawing in the presence and absence of S33084 (0.1 and 2.0 mg/kg doses), there were Drug (P=.0072; F=5.143) and Group × Drug interactions (P=.0046; F=3.320) but no significant Group effect. The frequency of stereotyped gnawing was significantly (P<.05) reduced by the low dose of S33084 when combined with apomorphine in all groups, but at the higher dose of S32504 (2.0 mg/kg dose) apomorphine-induced stereotyped gnawing was inhibited in KO-CO and WT-CO but not MPTP-treated mice (Fig. 5A). In fact, in KO-MPTP mice at the higher dose of S32504 apomorphine-induced stereotyped gnawing were elevated as compared to apomorphine alone (P<.05) or in WT-MPTP mice treated with apomorphine with S32504 (P<.05). At the higher dose of S32504 (2.0 mg/kg dose), apomorphine stereotyped locomotor activity was increased as compared with apomorphine alone (Fig. 5B).

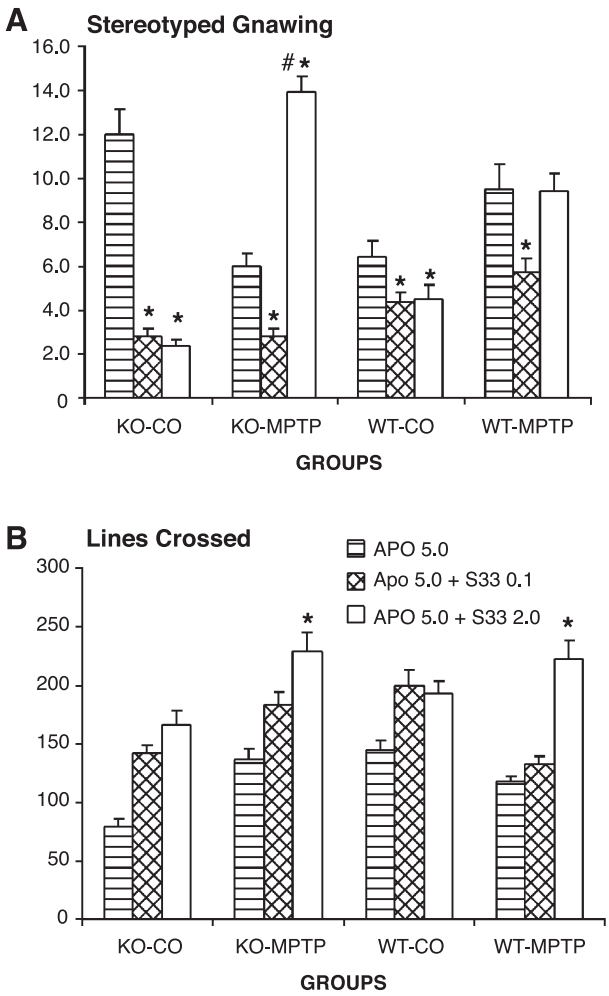


Fig. 5. The mean ± S.E.M. for (A) frequency of gnawing and (B) locomotor activity (lines crossed) in response to apomorphine with and without S33084 in different experimental treatment groups. The presence or absence of gnawing was measured for 1 min of every 5-min block of the 60-min test per the procedures along with three other independent behaviors as in the Method section. Gnawing was only observed with Apomorphine at a dose of 5.0 mg/kg. Significant effects: * represents P<.05 versus apomorphine alone; and # represents P<.05 for KO-MPTP versus WT-MPTP for apomorphine + S33084 (2.0 mg/kg dose).

Due to the possibility that the behavioral sensitization could develop between tests of the behavioral response to the drugs utilized in the study, the behavioral responses to saline, apomorphine, S33084, and S32504 in nonlesioned WT and KO mice were analyzed by ANOVA for between test differences. No significant differences (P=.7217) were observed for the behavioral response in mice between Experiments 1 and 2 for any of the drugs. However, there was a trend for an enhanced response to apomorphine and reduced response to S32504 in both the KO-CO and WT-CO mice in Experiment 2 as compared to Experiment 1.

4. Discussion

We tested the ability of the nonselective agonist apomorphine D_3 receptor preferring agonist S32504 and the selective D_3 antagonist S33084 to alter normal behaviors in WT and D_3KO mice. In addition, we examined their effects following administration of MPTP to significantly diminish DA input in the mice. Finally, we sought to determine if high dose apomorphine-induced stereotypies would be modified in WT and D_3 KO mice or by S33084. This synergistic D1:D2 interaction is thought to be modulated by the D_3 receptor, resulting in the inhibition of the behavioral activation to DA agonists (Damsma et al., 1993; Gilbert and Cooper, 1995; Waters et al., 1994). Consistent with that the D_3-preferring agonist, S32504 inhibited all behaviors measured. However, both apomorphine (at low doses) and S32504 suppressed spontaneous locomotor activity equally in D_3 KO and WT mice. D_3/D_2 agonists are known to decrease locomotor activity in mice (Geter-Douglass et al., 1997) and this effect has previously been reported to be similar in WT and D_3 KO mice (Boulay et al., 1999b; Xu et al., 1999). This has suggested to other investigators that the D_3 receptor does not mediate the inhibitory effects of D_3/D_2 agonists (Depoortere, 1999). Consistent with this, the present findings also suggest that high dose apomorphine-induced gnawing is inhibited by S33084 at low (D_3 selective) as well as the higher dose. As apomorphine-induced gnawing is presumed to involve D1:D2 receptor interaction in the CPu (Costall and Naylor, 1973; Joyce, 1983), a region with very low expression of D_3 receptors, this suggests a non- D_3 receptor-mediated effect. This is further strengthened by the fact that the effect was similar in WT and D_3 KO mice. However, it must be pointed

out that S33084 increased line crossings to high dose apomorphine, which can interfere with gnawing stereotypies resulting in an apparent reduced D1:D2-mediated effect. Again, however, this effect was similar in D_3 KO and WT mice, suggesting that either S32504 is not selective for D_3 receptors or the D_3 receptor does not mediate the inhibitory effects of D_3/D_2 agonists. In fact, S32504 is also active at D_2 receptors and the data support other findings that S32504 may act potently at D_2 receptors in vivo in rats (Dr. Mark Millan, The Institut de Servier, Croissy, France, personal correspondence). S33084 exhibits 100-fold higher affinity at D_3 versus D_2 sites but in vivo shows behavioral responses akin to D_2 selective antagonists (Millan et al., 2000a,b), making it possible that it is interacting with the D_2 receptor in the D_3 KO mice.

An alternative possibility is that the KO strategy does not allow for discrimination of D_3 from D_2 receptor functions in mice. First, there might be a "replacement" of D_3 receptor-mediated functions by the D_2 receptor in D_3 KO mice. That is, that adaptive changes in the D_2 receptor compensate for the loss of D_3 receptors. There is now evidence that D_2 and D_3 receptors can form heterodimers to activate intracellular signaling pathways (Scarselli et al., 2001), and D_2 and D_3 receptors can act through the same G-protein (Zaworski et al., 1999; Watts et al., 1998; Jiang et al., 2001). Thus, in the absence of the D_3 receptor, the D_2 receptor might couple to D_3 receptor-mediated pathways. A number of studies suggest that the occurrence of compensatory changes is the rule rather than the exception in receptor knock out mice, particularly in D_2 receptor KO mice (for a discussion, see (Zapata et al., 2001)). Thus, in the absence of absolute proof that S32504 and S33084 act solely at D_3 receptors in vivo, it remains possible that the KO strategy did not provide an adequate test of D_3 receptor functions.

Second, D_3 and D_2 receptor-mediated inhibition of DA release and resultant hypolocomotor effects (Boulay et al., 1999a) may be so potent that D_3 receptor-mediated functional effects may be difficult to identify even in D_3 KO mice (Jung et al., 1999; Zapata et al., 2001; Zapata and Shippenberg, 2002). Dopaminergic neurons are tonically inhibited by dendritic and terminal autoreceptors operating in interaction with DA transporters and have been proposed to be pharmacologically of the D_2 receptor subtype (Gobert et al., 1995; Koeltzow et al., 1998; L'hirondel et al., 1998; Dickinson et al., 1999). However, it has also been identified that the vast majority of TH-positive neurons of the SN and VTA are double labeled with the D_3 receptor antibody (Lammers et al., 2000). Furthermore, there is evidence that D_3 receptors do modulate DA uptake in mice, which serves as an autoreceptor function (Zapata et al., 2001; Zapata and Shippenberg, 2002). Thus, in the present experiments, D_3 and D_2 receptor-mediated inhibition of DA release could result in potent hypolocomotor effects (Boulay et al., 1999a) overcoming any postsynaptic DA receptor-mediated functional effects of the agonists. Interestingly, the inhibition of apomorphine induced gnawing by S33084 was not apparent in MPTP-lesioned mice, and S33084 elevated apomorphine induced-gnawing in D_3 KO-MPTP-lesioned mice. Hence, another possibility is that the suppressive effect of S32504 is via D_2 and/or D_3 autoreceptor inhibition of DA release, and D_2 and/or D_3 receptor blockade by S33084 leads to release of that inhibition. This may be more apparent in MPTP-lesioned partially DA-denervated mice.

The mixed DA agonist apomorphine produces stereotyped locomotion, climbing, and gnawing at high doses in mice and rats (Arnt, 1985; Geter-Douglass et al., 1997), which do not occur when D1 or D2 agonists are administered alone (Arnt et al., 1987). Apomorphine-induced climbing activity is greatly heightened in mice depleted of DA by MPTP, presumably due to supersensitive DA receptors (Lau and Fung, 1986; Spooren et al., 1998). We hypothesized we would observe differences in apomorphine-induced locomotor activity and gnawing in MPTP-treated mice, which might be absent in D_3 KO mice. Similarly, we predicted that the D_3-preferring agonist S32504 would be stimulatory in MPTP-treated mice and this effect would be absent in D_3 KO mice. However, we did not observe any evidence for behavioral sensitivity to apomorphine in the MPTP-treated mice. S32504 inhibited locomotor activity in unlesioned and MPTP-lesioned mice. Treatment with the DA precursor L-dopa also reduced locomotor activity in unlesioned and MPTP-lesioned mice. DA depletion induced by intracerebral injection of 6-OHDA in rats (Joyce, 1991; LaHoste and Marshall, 1991) or systemic administration of MPTP to monkeys (Joyce et al., 1986; Gagnon et al., 1995; Graham et al., 1990) to produce depletion of striatal DA has consistently demonstrated prolonged elevation in D_2 receptor number in the dorsal–lateral CPu of the rat and striatum of the nonhuman primate. In contrast to that found for the D_2 receptor, D_3 receptor number and mRNA levels are markedly decreased by DA denervation in rats and monkeys (Lévesque et al., 1995; Morissette et al., 1998; Quik et al., 2000). The reduction in D_3 receptor number following DA depletion has recently been confirmed for the mouse following administration of MPTP (Gross et al., 2003). Our MPTP treatment produced 60% to 70% loss of DA fibers in the CPu and NAC, as measured by [^{125}I]RTI-55 binding to DAT, but did not result in any change in D_3 receptor number. This is less than the 90% losses of DA input that appears to be necessary to produce behavioral supersensitivity (Spooren et al., 1998) or losses of D_3 receptor (Gross et al., 2003). Therefore, our MPTP model did not test this aspect of our hypothesis.

Elucidation of the role of D_3 receptors in the compensatory response to DA denervation may have important clinical relevance in the treatment of Parkinson's disease (Bézard et al., 2003; Joyce, 2001b). There is unequivocal evidence for a facilitatory role of striatal and limbic populations of D_2 receptors upon motor function underlying their implication the clinical actions of antiparkinson agents (Joyce, 2001b). On the other hand, it has been suggested

that D_3 receptors, which are primarily localized on different classes of neurons as compared to their D_2 counterparts (Gurevich and Joyce, 1999), exert an opposite, tonic, inhibitory influence upon locomotion (Damsma et al., 1993; Gilbert and Cooper, 1995; Waters et al., 1994). However, this remains disputed and the significance of D_3 receptors to the beneficial (restoration of function) and deleterious (induction of dyskinesia) actions of antiparkinson agents remains controversial (Bézard et al., 2003; Bordet et al., 1997, 2000; Morissette et al., 1998; Joyce et al., 2002). Utilization of D_3 KO mice with comparison to WT mice following DA depletion with MPTP should allow for delineation of D_3 receptor effects of DA agonists. Our results do not support a pronounced role for D_3 receptor effects of DA agonists in motor responses in parkinsonian animals. However, the MPTP regimen utilized in this study (Joyce et al., in press) produces insufficient loss of DA neurons and DA fibers to produce akinesia (loss of movement) and catalepsy (measure of rigidity) associated with experimental Parkinson's in other animal species. In addition, this degree of DA loss appears insufficient to produce a reduction in D_3 receptor number. In the absence of sufficient loss of DA input, the pronounced DA autoreceptor effects of D_3/D_2 agonists may be so profound as to make discrimination of D_3 receptor effects unlikely. Another approach may be to utilize nondopaminergic agents to stimulate behavior in WT and D_3 KO mice, such as that utilized by Sokoloff and associates (Bézard et al., 2003) to demonstrate a D_3 receptor dependency for hyperlocomotion induced by the *N*-methyl-D-aspartate glutamate receptor blocker dizocilpine. This might provide a better model to establish the role of D_3 receptors in the reversal of hypoactivity in DA-depleted mice by D_3/D_2 agonists.

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