Title Page:

Pro-erectile Effects of Dopamine D₂-like Agonists are Mediated by the D₃ Receptor in Rats and Mice


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Abstract:

Dopamine D_2-like agonists induce penile erection (PE) and yawning in a variety of species, effects that have recently been suggested to be specifically mediated by the D_4 and D_3 receptors, respectively. The current studies were aimed at characterizing a series of D_2, D_3, and D_4 agonists with respect to their capacity to induce PE and yawning in the rat, as well as the pro-erectile effects of apomorphine in wild-type and D_4 receptor (R) knock-out (KO) mice. All D_3 agonists induced dose-dependent increases in PE and yawning over a similar range of doses, whereas significant increases in PE or yawning were not observed with any of the D_4 agonists. Likewise, D_2, D_3, and D_4 antagonists were assessed for their capacity to alter apomorphine- and pramipexole-induced PE and yawning. The D_3 antagonist, PG01037, inhibited the induction of PE and yawning, whereas the D_2 antagonist, L-741,626, reversed the inhibition of PE and yawning observed at higher doses. The D_4 antagonist, L-745,870, did not alter apomorphine- or pramipexole-induced PE or yawning. A role for the D_3 receptor was further supported as apomorphine was equipotent at inducing PE in wild-type and D_4R KO mice, effects that were inhibited by the D_3 antagonist, PG01037, in both wild-type and D_4R KO mice. Together, these studies provide strong support that D_2-like agonist-induced PE and yawning are differentially mediated by the D_3 (induction) and D_2 receptors (inhibition). These studies fail to support a role for the D_4 receptor in the regulation of PE or yawning by D_2-like agonists.
Introduction:

The involvement of dopamine in the regulation of penile erection (PE) has been a long-studied phenomenon (Hyppa et al., 1970). Systemic administration of the non-selective dopamine agonist, apomorphine, is known to induce PE and yawning in a variety of species including rats (Benassi-Benelli et al., 1979), mice (Rampin et al., 2003), monkeys (Gisolfi et al., 1980), and man (Lal et al., 1987), suggesting that the receptor regulation of these effects may be similar across species. Several D₃-preferring agonists, including 7-OH-DPAT, pramipexole, and quinpirole have been shown to induce PE over low doses with inhibition of PE occurring at higher doses (Melis et al., 1987; Ferrari et al., 1993; Ferrari and Giuliani, 1995), as has previously been demonstrated for yawning (e.g., Collins et al., 2005; Collins et al., 2007). D₂-like agonist-induced PE and yawning are thought to be centrally mediated as they are inhibited by relatively non-selective, centrally active, D₂-like antagonists such as haloperidol, sulpiride, and clozapine, but not the peripheral D₂-like antagonist domperidone (Benassi-Benelli et al., 1979; Gower et al., 1984; Doherty and Wisler, 1994; Hsieh et al., 2004). Moreover, a significant body of literature supports a common role for the paraventricular nucleus (PVN) in the induction of PE and yawning by both physiologic and pharmacologic means (e.g., Argiolas and Melis, 1998; Melis and Argiolas, 1999; Melis and Argiolas, 2003; Argiolas and Melis, 2005), however, the specific receptor(s) mediating the pro-erectile effects of D₂-like agonists are yet to be elucidated.

Recently, a specific role for the D₄ receptor in the induction of PE by D₂-like agonists has been suggested. Dose-dependent increases in the percent incidence of PE were reported following systemic administration of D₄-selective agonists (Hsieh et al., 2004). Similar dose-dependent inductions of PE following systemic (Brioni et al., 2004;
Enguehard-Gueiffier et al., 2006; Melis et al., 2006) or intra-PVN (Melis et al., 2005; Melis et al., 2006) administration of a variety of D₄-selective agonists (e.g., ABT-724, CP226269, PD-168,077 and PIP3EA), with the D₄-selective antagonist, L745,870, reported to block PD-168,077- and PIP3EA-induced PE (Melis et al., 2005; Enguehard-Gueiffier et al., 2006; Melis et al., 2006). Although these findings support a role for the D₄ receptor in the mediation of PE, it should be noted that D₄-selective agonists have generally been reported to induce fewer erections compared to less selective D₂-like agonists such as apomorphine, and L-745,870 has been shown to be ineffective at altering the induction of PE by apomorphine (Melis et al., 2006), suggesting that other receptor(s) are also involved in the mediation of D₂-like agonist-induced PE. Interestingly, a variety of D₃-preferring agonists (e.g., (+)-3-PPP, 7-OH-DPAT, pramipexole, quinelorane, and quinpirole) have also been reported to increase PE (Melis et al., 1987; Ferrari et al., 1993; Doherty and Wisler, 1994; Ferrari and Giuliani, 1995) suggesting that D₃ receptors may be involved in the induction of PE by D₂-like agonists.

The current studies were aimed at characterizing the roles of the D₂, D₃, and D₄ receptors in the regulation of D₂-like agonist-induced PE. Thus, in vitro binding affinities for a series of D₂-like agonists and antagonists with varying degrees of selectivity for the D₂, D₃, and D₄ receptors were first determined to compare receptor selectivity. Agonists were then assessed for their capacity to induce PE and yawning, and antagonists were assessed for their capacity to alter the induction of PE and yawning by apomorphine or pramipexole in rats. Similarly, the pro-erectile effects of apomorphine were evaluated in D₄R WT and KO mice alone, and in combination with the D₃ antagonist, PG01037. Convergent evidence from the characterization of the pro-erectile effects of D₂-like agonists, as well as the agonist-antagonist interactions in rats and D₄R WT and KO mice, supports the notion that the induction of PE and yawning by D₂-like agonists used
herein are similarly mediated by the $D_3$ receptor, whereas the inhibition of PE and yawning observed at higher doses results from a concomitant activation of the $D_2$ receptor.
Methods:

Subjects. Male Wistar rats (250-275 g) were obtained from Harlan (Indianapolis, IN), whereas wild-type (WT) and D₄R KO mice (30-35 g) were derived from the mating of D₄R heterozygote mice (129/Ola C57Bl/6J) for more than 20 generations (Rubinstein et al., 1997). Rats were housed three to a cage, and mice were singly housed in temperature and humidity controlled rooms on a 12-h dark/light cycle with lights on at 7:00 AM. Food and water were freely available; however, no food or water was available during observations. All studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the National Institutes of Health, and all procedures were approved by the University of Michigan Committee on the Use and Care of Animals, and National Institutes of Health Guidelines under Institutional Animal Care and Use Committee-approved protocols.

Behavioral observations. On the day of testing rats were transferred from their home cage to a test chamber (48cm x 23cm x 20cm, clear rodent cage; cob bedding present in rat studies and absent in mouse studies), and allowed to habituate for a period of 30 min prior to vehicle or antagonist pretreatment. Following a 30 min pretreatment, one dose of agonist was administered and the total number of yawns and PEs were recorded for a period of 45 min (rats) or 30 min (mice) thereafter; yawning was not observed in mice. Yawning was defined as a prolonged (~1s), wide opening of the mouth followed by a rapid closure, whereas PE was defined as an emerging, engorged penis usually followed by an upright posture, repeated pelvic thrusts, and genital grooming. All observations of drug-induced behavioral effects were separated by at least 48 hr to allow for drug washout.
D₂-like agonist-induced yawning and penile erection in rats. The following D₂-like agonists were assessed for their capacity to induce PE and yawning: apomorphine (0.01 - 0.32 mg/kg), pramipexole (0.01 - 1.0 mg/kg), PD-128,907 (0.01 - 0.32 mg/kg), quinpirole (0.0032 - 0.32 mg/kg), sumanirole, (0.1 - 3.2 mg/kg), ABT-724 (0.001 - 0.32 mg/kg), PD-168,077 (0.0032 - 0.32 mg/kg), PD-168,077 (0.0032 - 0.32 mg/kg), and PIP3EA (0.0032 - 0.32 mg/kg). All agonists were investigated in separate groups of 8 rats, with each rat receiving each dose of one agonist presented in random order.

Effects of D₂-, D₃-, and D₄-selective antagonists on apomorphine- and pramipexole-induced yawning and penile erection in rats. The following D₂-like antagonists were assessed for their capacity to alter the induction of PE and yawning by apomorphine (0.01 - 0.32 mg/kg) and pramipexole (0.01 - 1.0 mg/kg): PG01037 (32.0 mg/kg), L-741,626 (1.0 mg/kg), and L-745,870 (1.0 mg/kg). PG01037 and L-741,626 was administered as 30 min pretreatments, whereas L-745,870 was administered 15 min prior to agonist injection. Each antagonist X agonist combination was assessed in separate groups of 8 rats, with each rat receiving all dose combinations in random order.

Effects of D₂-like antagonists on pramipexole-induced yawning and penile erection in rats. The following series of D₂-like antagonists were assessed for their capacity to alter the induction of PE and yawning by pramipexole (0.1 mg/kg): PG01037 (1.0 - 32.0 mg/kg), SB-277011A (1.0 - 32.0 mg/kg), raclopride (0.0032 - 0.1 mg/kg), haloperidol (0.0032 - 0.1 mg/kg), L-741,626 (0.32 - 10.0 mg/kg), Ro-61-6270 (1.0 - 32.0 mg/kg) and L-745,870 (0.32 - 10.0 mg/kg). Each antagonist was assessed in separate groups of 8 rats with each rat receiving all dose combinations, presented in random order.
Apomorphine-induced penile erection in wild-type and D₄ receptor knock-out mice. The capacity of apomorphine (0.0003 – 0.032 mg/kg) to induce PE was assessed in WT and D₄R KO mice. Each group of mice was comprised of 6 littermates (one group each of WT and KO mice), with saline injections administered 30 min prior to apomorphine doses. All mice were exposed to each dose of apomorphine presented in random order.

Effects of PG01037 on apomorphine-induced penile erection in wild-type and D₄ receptor knock-out mice. The capacity of the D₃-selective antagonist, PG01037 (10.0 and 30.0 mg/kg) to alter apomorphine-induced (0.0003 – 0.032 mg/kg) PE was assessed in both WT and D₄R KO mice. PG01037 was administered 30 min prior to doses of apomorphine or saline injections, with each mouse receiving each combination of doses presented in random order. One WT mouse was euthanized due to health problems after 5 of the 19 treatments, and was therefore not included in the analysis.

Binding Analysis. All Kᵢ values were assessed using membranes prepared from cells recombinantly expressing the hD₂, hD₃ and hD₄ receptors. Ligands were assessed for their capacity to inhibit [³H]PD-128,907 (or [³H]spiperone) binding to the D₃ receptor, or [³H]spiperone binding to the D₂, or D₄ receptor. Membranes for D2, D3, and D4 receptor binding assays were prepared as previously described (Enguehard-Gueiffier et al., 2006) from hD₂-baculovirus-infected insect cells (~2-5 pmol/mg protein), or SH-SY5Y neuroblastoma cells stably expressing either the hD3, or hD₄ receptor (~1-2 pmol/mg protein). Competitions using [³H]PD-128,907 were performed in a buffer containing 25 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1 mM MgSO₄ and 1 mM CaCl₂ with 5 μg of hD₃- SH-SY5Y membranes in the presence of 2 nM [³H]PD-128,907 and varying concentrations of competing ligands (10-11 M to 10-4 M, final), whereas competitions
using [3H]spiperone for D₃ (5 μg membrane), D₂ (5 μg membrane), and D₄ (10 μg membrane) receptors were performed in 25 mM Tris-HCl, pH 8.0, 75 mM NaCl, 0.5 mM EDTA, 1 mM MgSO₄ and 1 mM CaCl₂ with 2 nM (D₃) or 200 pM (D₂ and D₄) [³H]spiperone (final volume of 500 μl) in the presence of varying concentrations of competing ligands (10-11 M to 10-4 M, final). Radioligand binding assays were performed at room temperature in 96-well microtiter plates, and filtered onto GF/B filter plates with radioactivity detected by liquid scintillation counting on a TopCount counter (Perkin-Elmer, Waltham, MA). The IC₅₀ values for inhibition of [³H]spiperone binding to the D₂ and D₄ receptors were calculated using either a single site model (for antagonists) or two-site model (for agonists) using Prism (GraphPad). IC₅₀ values for inhibition of [³H]PD-128,907 binding to the D3 receptor were fit to a single site model. Ki values were derived from the IC₅₀ values according to the Cheng-Prusoff equation (Cheng and Prusoff, 1973) to take into consideration the radioligand concentration and the Kd values for [³H]spiperone on the D2 and D4 receptor and [³H]PD-128-907 on the D3 receptors (not shown). Note that the IC₅₀ values for agonist inhibition of [³H]spiperone binding to the D₂ and D₄ receptor determined from a single-site fit are expressed as a K₀.₅ to reflect the radioligand-corrected value.

**Materials:** ABT-724 (2-[[4-Pyridin-2-yl)piperazin-1-yl]methyl]-1H-benzimidazole) was synthesized by Dr. Kenner Rice (Chemical Biology Research Branch, NIDA, Bethesda, MD). Apomorphine ((R)-(−)-5,6,6a,7-Tetrahydro-6-methyl-4H-dibenzo[de,g]quinoline-10,11-diol hydrochloride), haloperidol (4-[4-(4-Chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butane hydrochloride), PD-128,907 ((S)-(−)-(4aR,10bR)-3,4,4a,10b-Tetrahydro-4-propyl-2H,5H-[1]benzopyrano[4,3-b]1,4-oxazin-9-ol hydrochloride), and quinpirole (trans-(−)-(4aR)-4,4a,5,6,7,8,8a,9-Octahydro-5-propyl-1H-pyrazolo[3,4-g]quinoline hydrochloride) were obtained from Sigma-Aldrich (St. Louis,
L-741,626 (3-[4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl]methyl-1H-indole), L-745,870 (3-[4-(4-Chlorophenyl)piperazin-1-yl]-methyl-1H-pyrrolo[2,3-b]pyridine trihydrochloride), PD-168,077 (N-(Methyl-4-(2-cyanophenyl)piperazinyl-3-methylbenzamide maleate), and raclopride (3,5-Dichloro-N-(1-ethylpyrrolidin-2-ylmethyl)-2-hydroxy-6-methoxybenzamide tartrate salt) were obtained from Tocris (Ellisville, MO).

PG01037 (N-{4-[4-(2,3-Dichlorophenyl)-piperazin-1-yl]-trans-but-2-enyl}-4-pyridine-2-yl-benzamide hydrochloride) was synthesized by Drs. Amy Newman and Peter Grundt (Medicinal Chemistry Section-NIDA, Baltimore, MD). PIP3EA (2-[4-(2-Methoxyphenyl)piperazin-1-ylmethyl]imidazo[1,2-a]pyridine) was synthesized by Drs. Alain Gueiffier and Cécile Enguehard-Gueiffier (Francois-Rabelais Universite, Tours, France). Pramipexole (N'-propyl-4,5,6,7-tetrahydrobenzothiazole-2,6-diamine dihydrochloride) and SB-277011A (trans-N-[4-[2-(6-Cyano-1,2,3,4-tetrahydroisoquinolin-2-yl)ethyl]cyclohexyl]-4-quinolinecarboxamide) were synthesized by Drs. Shaomeng Wang and Jianyong Chen (University of Michigan, Ann Arbor, MI). Ro 61-6270 (2-amino-benzoic acid-1-benzyl-piperidin-4-yl-ester) was provided by Hoffmann-La Roche (Basel, Switzerland).

Sumanirole ([5R]-5,6-dihydro-5-(methylamino) 4H-imidazo[4,5,1-ij]quinolin-2(1H)-one (2Z)-2-butenedioate) was synthesized by Drs. Stephen Husbands and Benjamin Greedy (University of Bath, Bath, U.K.). All drugs were dissolved in sterile water with the exceptions of PG01037 and SB-277,011A which were dissolved in 10% β-cyclodextrin, and haloperidol, L-741,626, PD-168,077, and PIP3EA which were dissolved in 5% ethanol and sterile water. In the rat studies all drugs were administered sub-cutaneously in a volume of 0.1 ml/kg, with the exception of L-745,870 which was administered intraperitoneally. In the mouse studies apomorphine and PG01037 were administered intraperitoneally in a volume of 0.1 ml/kg. The cDNAs for the human dopamine (hD2, hD3, and hD4) receptors were generously provided by Drs. Olivier Civelli.
(University of California at Irvine), Pierre Sokoloff (INSERM, France) and Dr. Hubert VanTol (University of Toronto, Canada).

**Data analysis.** Radioligand binding data were analyzed using non-linear regression and analyzed for one-or two-site inhibition curves (GraphPad Prism, San Diego, CA). All yawning and PE studies were conducted with 8 rats per group with results expressed as the mean number of yawns or PE observed over 45 min ± standard error of the mean (S.E.M.). Percent incidence represents the number of rats displaying at least one PE during the 45 min observation period. Mouse studies were conducted with 6 littermates per group, and results expressed as the mean number of PE observed over 30 min ± S.E.M.. Due to the fact that the event occurs on less than one occasion per test session, and thus is not normally distributed, the significant effects of agonists on the induction of PE, or antagonists on agonist-induced PE were determined using Mann-Whitney U-Tests (GraphPad Prism). One-way, repeated-measures ANOVA with post-hoc Dunnett’s tests was used to determine significant levels of agonist-induced yawning (GraphPad Prism), whereas significant effects of antagonists on apomorphine-, and pramipexole-induced yawning were determined using two-way ANOVA with post-hoc Bonferroni tests (SPSS, SPSS Inc., Chicago, IL). One-way repeated-measures ANOVA with post-hoc Dunnett’s tests were used to determine significant effects of antagonists on pramipexole-induced yawning. (GraphPad Prism).
Results:

**In vitro binding analysis.** Since a comparison of binding affinities of the ligands used in these studies at the D₂, D₃, and D₄ receptors has not been previously reported in a single study, these data were obtained for each compound against recombinantly expressed human hD₂, hD₃, and hD₄ receptors, and were directly compared using radioligand filter binding assays. The capacity of all of the agonists and antagonists to displace the antagonist, \[^{3}\text{H}]\text{spiperone, was assessed for each receptor subtype, whereas displacement of the D₃-preferring agonist, \[^{3}\text{H}]\text{PD-128,907 was also assessed for the D₃ receptor subtype. Most ligands displaced radioactive probes with a single-phase inhibition, consistent with a one-site model; only agonist binding to D₂ receptors displayed biphasic inhibition curves (composed of a low affinity state and a guanine nucleotide-sensitive high affinity state).** Binding affinities and selectivity ratios for ligands binding to the D₂ and D₃ receptors (D₂/D₃) and D₄ and D₃ receptors (D₄/D₃) are shown in Table 1; note that the more relevant comparisons with the D₂\text{high} state and D₃ receptors (D₂\text{high}/D₃) are also shown. The individual Kᵢ’s and K₀.₅’s obtained in this study are within the range of previously reported values from several studies, using different assay conditions and different radioligand probes. The data presented here, all assayed under similar conditions, provide an appropriate comparison of the receptor subtype selectivity of the D₂-like ligands used in the behavioral studies reported herein. The absence of a strong correlation to *in vivo* potency has been previously described (e.g., Levant, 1997), and is duly noted.

**D₂-like agonist-induced yawning and penile erection in rats.** Dose-dependent increases in PE and yawning were observed for the non-selective D₂-like agonist,
apomorphine, as well as the D₃-preferring agonists, PD-128,907, pramipexole, and quinpirole, with inhibition of both responses occurring at higher doses resulting in inverted U-shaped dose-response curves for PE and yawning (Figure 1). Peak levels of PE and yawning were observed at the same dose for apomorphine (0.1 mg/kg), pramipexole (0.1 mg/kg), and PD-128,907 (0.1 mg/kg), whereas doses of 0.032 and 0.1 mg/kg quinpirole induced peak levels of yawning and PE, respectively. Apomorphine, pramipexole, and PD-128,907 induced at least one PE over the 45 min in 87.5% of rats, whereas the maximal percent incidence of PE for quinpirole was 75%. None of the D₄-selective agonists induced significant levels of PE or yawning (Figure 1). PIP3EA induced at least one PE in 50% of rats at a dose of 0.1 mg/kg, whereas the maximal percent incidence of PE for PD-168,077 and ABT-724 was 25%. Although significant levels of yawning were observed with the D₂-preferring agonist, sumanirole, PE was not induced (Figure 1).

**D₃-, D₂-, and D₄-selective antagonism of apomorphine- and pramipexole-induced yawning and erection in rats.** The effects of the D₃-selective antagonist, PG01037, the D₂-selective antagonist, L-741,626, and the D₄-selective antagonist, L-745,870 on apomorphine- and pramipexole-induced PE and yawning are shown in Figure 2. Significant inhibition of the induction of both PE and yawning by apomorphine and pramipexole was observed following a dose of 32.0 mg/kg PG01037 whereas the inhibition of PE or yawning observed at higher doses was unaffected (Figure 2a-d). PG01037 also reduced the maximal percent incidence of PE for apomorphine from 87.5% to 12.5%, and from 87.5% to 25% for pramipexole (Figure 2e-f). Unlike with PG01037, the D₂-selective antagonist, L-741,626 (1.0 mg/kg) selectively reversed the inhibition of PE and yawning observed at higher doses of apomorphine and pramipexole at a dose that did not affect the induction of yawning or PE at lower doses (Figure 2g-j).
Pretreatment with L-741,626 not only increased the maximal number of PEs and yawns observed, but also shifted the peaks of the PE and yawning dose-response curves for apomorphine and pramipexole ½ log unit to the right. L-741,626 also shifted the descending limb of the dose-response curves for the percent incidence of PE for apomorphine and pramipexole resulting in 100% of rats exhibiting at least one PE at doses of 0.1 and 0.32 mg/kg (Figure 2k and 2l). When given at a behaviorally active dose of 1.0 mg/kg (Enguehard-Gueiffier et al., 2006), L-745,870 failed to modify apomorphine- or pramipexole-induced PE or yawning, and furthermore, did not alter the percent incidence of PE for either apomorphine or pramipexole (Figure 2m-r).

**D₃, D₂, and D₄ antagonism of pramipexole-induced yawning and penile erection in rats.** The effects of a series of D₂-like antagonists, with varying degrees of selectivity for the D₂, D₃, and D₄ receptors, on PE and yawning induced by the maximally effective dose of pramipexole (0.1 mg/kg) are shown in Figure 3. Dose-dependent inhibition of pramipexole-induced PE and yawning was observed with both of the D₃-selective antagonists, PG01037 and SB-277011A (Figure 3a-b), however, there were slight differences in the relative potencies with PG01037 inhibiting PE at a dose (3.2 mg/kg) ½ log unit lower than that required to inhibit yawning (10.0 mg/kg), whereas SB-277011A was equipotent at inhibiting the induction of yawning and PE (10.0 mg/kg). Similar to SB-277,011A, inhibition of pramipexole-induced yawning and PE was observed at the same dose of the non-selective D₂/D₃ antagonist, raclopride (0.032 mg/kg; Figure 3c), whereas the relatively non-selective D₂-like antagonist, haloperidol, and the D₂-selective antagonist, L-741,626, produced a dose-dependent inhibition of pramipexole-induced PE and yawning with a significant inhibition of yawning observed at a dose ½ log unit lower than was required to inhibit the induction of PE (Figure 3d-e). Unlike all other D₂-like antagonists tested, the D₄-selective antagonists, L-745,870 (Figure 3f) and Ro 61-6270
(Figure 3g), did not alter the induction of either PE or yawning by pramipexole, although a slight, but not significant, reduction of pramipexole-induced PE was observed following a dose of 10.0 mg/kg L-745,870.

**Apomorphine-induced penile erection in wild-type and D₄ receptor knock-out mice.** Similar to the effects of apomorphine in rats, a dose-dependent increase in PE was observed over low doses of apomorphine with inhibition of PE occurring at higher doses resulting in an inverted U-shaped dose-response curve for apomorphine-induced PE in both WT and D₄R KO mice (Figure 4a). No significant differences in the potency or effectiveness of apomorphine to induce PE were observed between the WT and D₄R KO mice, with peak levels of PE observed at a dose of 0.0032 mg/kg apomorphine in both genotypes. Likewise, the effects of the D₃-selective antagonist, PG01037, in WT and D₄R KO mice were similar to the effects observed in rats. Pretreatment with PG01037 resulted in a dose-dependent inhibition of apomorphine-induced PE in both WT and D₄R KO mice, with a dose of 30.0 mg PG01037 producing an almost complete inhibition of apomorphine-induced PE (Figure 4b and 4c).
Discussion:

These studies were aimed at characterizing the receptors involved in the regulation of the pro-erectile effects of D₂-like agonists in rats and mice. Convergent evidence from the pharmacologic evaluation of the effects of a series of D₂-like agonists with varying degrees of selectivity for the D₂, D₃, and D₄ receptors alone, and in combination with D₂-, D₃-, and D₄-selective antagonists suggest that the induction of PE is mediated by an activation of the D₃ receptor, whereas the inhibition of PE observed at higher doses results from the concomitant activation of the D₂ receptor, as has previously been described for D₂-like agonist induced yawning (Collins et al., 2005; Collins et al., 2007). These studies failed to support a role for the D₄ receptor in the mediation of D₂-like agonist-induced PE as D₄-selective agonists failed to induce PE, and D₄-selective antagonists failed to inhibit PE in rats, whereas apomorphine was equally effective at inducing PE in WT and D₄R KO mice.

In agreement with previous reports, apomorphine, pramipexole, and quinpirole induced PE and yawning with inverted U-shaped dose-response curves, and 75 to 87.5% of rats displaying at least one PE at the peak dose, however, these studies are the first to report a similar pro-erectile effect for the D₃-preferring agonist, PD-128,907. The results of these studies suggest that the capacity of these agonists to induce PE is related to their activity at the D₃, but not D₄ receptor as increases in yawning and PE were observed over a similar range of low doses even though large differences exist between their in vitro selectivities for the D₃ compared to D₄ receptor (e.g., apomorphine D₄/D₃ ≈ 0.05 and PD-128,907 D₄/D₃ ≈ 1280; Table 1). In agreement with this notion, but contrary to previous findings (Brioni et al., 2004; Melis et al., 2005; Enguehard-Gueiffier et al., 2006), all of the highly selective D₄ agonists failed to induce PE. It should be noted,
however, that the maximal PE responses for apomorphine, quinpirole, and pramipexole were lower than some previous reports (e.g., Melis et al., 2006), suggesting procedural differences may have affected the PE response. Nevertheless, the percent incidence of PE for apomorphine and the D₃-preferring agonists were similar to previous reports (e.g., Hsieh et al., 2004), suggesting that any procedural differences only affected the maximal number of PEs observed, not the absolute capacity of the agonists to induce PE.

The effects of D₂-, D₃-, and D₄-selective antagonists on apomorphine- and pramipexole-induced PE and yawning further support specific roles for the D₃ and D₂ receptors in the mediation of D₂-like agonist-induced PE. When given at behaviorally active doses (Collins et al., 2005; Enguehard-Gueiffier et al., 2006; Collins et al., 2007), the D₃-selective antagonist, PG01037, and D₂-selective antagonist, L-741,626, differentially affected apomorphine- and pramipexole-induced PE and yawning, whereas the D₄-selective antagonist, L-745,870, did not alter the induction or inhibition of PE or yawning. Similar to the effects of the D₃ and D₂ antagonists on yawning, PG01037 produced a selective rightward and/or downward shift of the ascending limb, whereas L-741,626 produced a selective rightward shift of the descending limb of the PE dose-response curves for apomorphine and pramipexole with respect to both the absolute number, and percent incidence of PE. Together with previous reports describing specific roles for the D₃ and D₂ receptors in the regulation of D₂-like agonist-induced yawning (Collins et al., 2005; Collins et al., 2007), the differential and selective effects of the D₃ and D₂ antagonists on PE, combined with the fact that both apomorphine and a variety of D₃-preferring agonists were equipotent at inducing PE and yawning suggest that the induction of PE and yawning by D₂-like agonists is mediated by the D₃ receptor, whereas the inhibition of PE and yawning observed at higher doses results from a concomitant activation of the D₂ receptor. It should be noted, however, that unlike pramipexole,
apomorphine also has activity at D₁-like receptors which may also influence the PE response, although the precise role of D₁ receptors in the modulation of PE is currently unclear (Melis et al., 1987; Zarrindast and Jamshidzadeh, 1992; D’Aquila et al., 2003; Hsieh et al., 2004), and may involve peripheral rather than central D₁ receptors (El-Din et al., 2007).

A role for the D₃ receptor in the induction of PE and yawning is further supported by the dose-response analysis of a series of D₂-like antagonists on pramipexole-induced PE and yawning. Dose-dependent inhibition of pramipexole-induced PE was observed following pretreatment with D₃-selective (PG01037 and SB-277011A), non-selective D₂/D₃ (raclopride), non-selective D₂-like (haloperidol), and D₂-selective (L-741,626) antagonists, an effect that was correlated with their capacity to inhibit yawning, but not observed with the D₄-selective antagonists (L-745,870 and Ro 61-6270). Furthermore, all of the D₂-like antagonists inhibited PE and yawning with similar potencies regardless of the fact that large differences exist with respect to their in vitro selectivity for D₃ compared to D₄ receptors (e.g., PG01037 D₄/D₃ = 1.3 x 10⁶⁴, raclopride D₄/D₃ = 64, and haloperidol D₄/D₃ = 0.1; Table 1), whereas antagonists highly selective for D₄ compared to D₃ receptors (e.g., L-745,870 D₄/D₃ = 1.7 x 10⁶⁴ and Ro 61-6270 D₄/D₃ = 9.1 x 10⁶⁵; Table 1) failed to alter pramipexole-induced PE or yawning. Although Ro 61-6270 has not been extensively characterized (Clifford and Waddington, 2000), L-745,870 has been shown to possess favorable pharmacokinetics (0.3 mg/kg; p.o. is thought to be sufficient to occupy ~90% of D4 receptors; Patel et al., 1997), and has been shown to inhibit PD-168,077- and PIP3EA-induced PE at a dose of 1.0 mg/kg (Enguehard-Gueiffier et al., 2006; Melis et al., 2006), suggesting that the doses used in the current studies were sufficient to block D₄ receptors. Together with previous reports that L-745,870 was unable to alter apomorphine-induced PE (Melis et al., 2006), the current
studies suggest that the pro-erectile effects of D$_2$-like agonists (e.g., apomorphine and pramipexole) are mediated by activation of the D$_3$, but not D$_4$ receptor.

Despite the distinct and differential effects of PG01037 and L-741,626 observed in the current studies, the fact that relatively large doses of the D$_3$-selective antagonists (PG01037 and SB-277011A) were required to inhibit pramipexole-induced yawning and PE, whereas similar effects were observed with relatively low doses of non-selective (raclopride and haloperidol) and selective (L-741,626) D$_2$ antagonists, effects that may suggest that the inhibition of PE is mediated by antagonist activity at receptor(s) other than the D$_3$ receptor. These are not, however, the first studies to suggest a disconnect between the in vitro and in vivo potencies of the D$_3$-antagonists, PG01037 and SB-277011A. In fact, a number of previous studies have reported similar in vivo potencies when these antagonists have been evaluated in a variety of operant procedures (3.2-24.0 mg/kg; Andreoli et al., 2003; Xi et al., 2004; Gilbert et al., 2005; Cervo et al., 2007). Moreover, previous studies aimed at characterizing the in vivo selectivity of D$_2$-like agonists and antagonists, suggest that PG01037 and SB-277011A are devoid of significant D$_2$, cholinergic, and serotonergic antagonist activities at doses up to 56.0 mg/kg, whereas L-741,626 displays a much more limited in vivo D$_2$-selectivity with significant D$_3$ antagonist activity observed at doses as low as 3.2 mg/kg (Collins et al., 2005; Collins et al., 2007).

Perhaps the strongest evidence in support of a specific role for the D$_3$ receptor in the induction of PE by D$_2$-like agonists was provided by the evaluation of apomorphine-induced PE in the WT and D$_4$R KO mice. Not only was apomorphine equally effective at inducing PE in the WT and D$_4$R KO mice, but the pro-erectile effect of apomorphine was also dose-dependently inhibited by the D3-selective antagonist, PG01037, in both the
WT and D₄R KO genotypes. Although species differences precluded comparisons of the effects of agonists and antagonists on yawning and PE to be made in mice as D₂-like agonists do not induce yawning in mice (Li et al., submitted), when taken together with the pharmacologic data collected in rats, these data provide strong support for a role for the D₃, but not D₄ receptor in the induction of PE by D₂-like agonists in rodents.

To summarize, a series of D₂-like agonists and antagonists with varying degrees of selectivity for the D₂, D₃, or D₄ receptors were assessed for their capacity to modulate PE and yawning in rats. Similar to the effects of apomorphine, all D₃-preferring agonists induced dose-dependent increases in PE and yawning over a similar range of low doses, with the inhibition of PE and yawning occurring at higher doses; D₄-selective agonists failed to induce PE or yawning. The D₃-selective antagonist, PG01037, and D₂-selective antagonist, L-741,626, had similar effects on PE and yawning, with PG01037 selectively shifting the ascending limbs, and L-741,626 selectively shifting the descending limbs of the dose-response curves for apomorphine- and pramipexole-induced PE and yawning. Additionally, dose-dependent inhibition of pramipexole-induced PE was observed with a series of D₂-like antagonists with a wide range of selectivities for the D₃ and D₂ receptors, an effect that corresponded to their capacity to inhibit pramipexole-induced yawning, but was not observed with D₄-selective antagonists. Furthermore, the pharmacologic evaluation of the pro-erectile effects of D₂-like agonists was validated in D₄R KO mice. Not only was apomorphine was equally effective at inducing PE in both WT, and D₄R KO mice, but the induction of PE by apomorphine was dose-dependently inhibited by the D₃-selective antagonist, PG01037 in both genotypes. In conclusion, although inferences with regard to the receptors mediating the pro-erectile effects of D₄-selective agonists could not be made, these studies provide convergent evidence in support of a role for the D₃ receptor in the
induction of PE by D$_2$-like agonists, with the inhibition of PE observed at higher doses resulting from the concomitant activation of the D$_2$ receptor.
Acknowledgements:

The authors would like to acknowledge the excellent technical work of Davina Barron, Nhu Truong, Dawn French-Evans and Marika B. Cohen throughout the course of these studies. The authors would also like to thank Dr. Pierre Sokoloff (INSERM) and the late Dr. Hubert Van Tol (University of Toronto) for generously providing the cDNAs for the D3 and D4 receptors, respectively.
References:


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099-3108.


Footnotes:

This research was supported by USPHS NIDA grants DA 020669, F013771, GM068603, the NIDA and NIAAA Intramural Research Programs and the University of Michigan Biological Sciences Scholars Program.

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Figure 1. Dose-response curves for D2-like agonist-induced PE and yawning. Characterization of PE and yawning induced by A) apomorphine; B) pramipexole; C) quinpirole; D) PD-128,907; F) ABT-724; G) PD-168,077; H) PIP3EA; and I) sumanirole was conducted in separate groups of rats with data presented as mean (±SEM), n=8, number of PEs and yawns observed in 45 min. E and J) Percent of rats displaying at least one PE over 45 min. *, p<0.05; **, p<0.01. Significant differences in agonist-induced yawning as determined using one-way, repeated-measures ANOVA with post-hoc Dunnett’s tests and, +, p<0.05; ++, p<0.01; agonist-induced PE as determined by Mann-Whitney U-Test compared to vehicle treated animals.

Figure 2. D3-, D2-, and D4-selective antagonists on apomorphine- and pramipexole-induced PE and yawning. Effects of the D3-selective antagonist PG01037 (32.0 mg/kg) on apomorphine- and pramipexole-induced A and B) yawning; C and D) PE; E and F) percent incidence of PE. Effects of the D2-selective antagonist L-741,626 (1.0 mg/kg) on apomorphine- and pramipexole-induced G and H) yawning; I and J) PE; K and L) percent incidence of PE. Effects of the D4-selective antagonist L-745,870 (1.0 mg/kg) on apomorphine- and pramipexole-induced M and N) yawning; O and P) PE; Q and R) percent incidence of PE. Data are presented as mean (±SEM), n=8, number of PEs and yawns observed in 45 min. *, p<0.05; **, p<0.01; ***, p<0.001. Significant effect of antagonist on agonist-induced yawning as determined by a two-way ANOVA with post-hoc Bonferroni tests. +, p<0.05; ++, p<0.01; +++, p<0.001. Significant effect of antagonist on agonist-induced PE as determined by Mann-Whitney U-Test.
Figure 3. Effects of a series of D₂-like antagonists with a range of selectivities for the D₃, D₂, and D₄ receptors on PE and yawning induced by 0.1 mg/kg pramipexole. Effects of the D₃-selective antagonists A) PG01037 (1.0-32.0 mg/kg); and B) SB-277011A (1.0-32.0 mg/kg); the non-selective D₂/D₃ antagonist C) raclopride (0.0032-0.1 mg/kg); the non-selective D₂-like antagonist D) haloperidol (0.0032-0.1 mg/kg); the D₂-selective antagonist E) L-741,626 (0.32-10.0 mg/kg); and the D₄-selective antagonists F) L-745,870 (0.32-10.0 mg/kg); and G) Ro 61-6270 (1.0-32.0 mg/kg). *, p<0.05; **, p<0.01. One-way repeated-measures ANOVAs with post-hoc Dunnett’s tests were used to determine significant effects of antagonists on pramipexole-induced yawning and +, p<0.05; ++, p<0.01; Mann-Whitney U-Tests were used to determine significant effects of antagonists on pramipexole-induced PE.

Figure 4. Dose-response curves for apomorphine-induced PE in D4R WT and KO mice. A) apomorphine-induced PE in D4R WT and D4R KO mice was conducted in groups of 6 littermates with data presented as mean (±SEM). Effects of PG01037 (10.0 and 30.0 mg/kg) on apomorphine-induced PE in B) D4R WT, and C) D4R KO mice. Significant differences in apomorphine-induced PE in D4R WT (*, p<0.05; **, p<0.01), and D4R KO (+, p<0.05; ++, p<0.01;) as determined by Mann-Whitney U-Test compared to vehicle treated animals. Significant effects of PG01037 (10.0 mg/kg; *, p<0.05, and 30.0 mg/kg; +, p<0.05) on apomorphine-induced PE compared to vehicle treated mice as determined by Mann-Whitney U-Tests.
### Tables:

#### Table 1. *In vitro* binding affinities and selectivity ratios at D₂, D₃, and D₄ receptors for D₂-like agonists and antagonists.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>D₂ [³H]Spip K₀.5</th>
<th>D₂ [³H]Spip Kₕₙₐ₇</th>
<th>D₂ [³H]Spip Kₕₙₒₜ</th>
<th>D₃ [³H]PD-128907 Kᵢ</th>
<th>D₄ [³H]Spip K₀.₅</th>
<th>D₂/D₃ †</th>
<th>D₂ₕᵢₙ₉/D₃ †</th>
<th>D₄/D₃ †</th>
</tr>
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<tbody>
<tr>
<td>pramipexole</td>
<td>&gt;10,000</td>
<td>n.d.⁴</td>
<td>n.d.⁴</td>
<td>0.5</td>
<td>10.2</td>
<td>194</td>
<td>n.a.⁴</td>
<td>388</td>
</tr>
<tr>
<td>PD-128,907</td>
<td>3.5 (29%)</td>
<td>1.9</td>
<td>9.7</td>
<td>2430</td>
<td>490</td>
<td>1.8</td>
<td>1280</td>
<td></td>
</tr>
<tr>
<td>quinpirole</td>
<td>118</td>
<td>6</td>
<td>9.4</td>
<td>109</td>
<td>20</td>
<td>1.7</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>apomorphine</td>
<td>19</td>
<td>3.6 (50%)</td>
<td>570</td>
<td>75</td>
<td>231</td>
<td>3.4</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>ABT-724</td>
<td>&gt;10,000</td>
<td>n.d.⁴</td>
<td>n.d.⁴</td>
<td>947</td>
<td>58</td>
<td>n.a.⁴</td>
<td>n.a.⁴</td>
<td>n.a.⁴</td>
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<tr>
<td>PD-168,077</td>
<td>4250</td>
<td>n.d.⁴</td>
<td>1400</td>
<td>726</td>
<td>23</td>
<td>3.04</td>
<td>n.a.⁴</td>
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<tr>
<td>PIP3EA</td>
<td>32</td>
<td>1.7 (42%)</td>
<td>950</td>
<td>1720</td>
<td>1910</td>
<td>3.7</td>
<td>0.02</td>
<td>9.9x10⁻⁴³</td>
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<tr>
<td>sumanirole</td>
<td>144</td>
<td>0.2 (42%)</td>
<td>256</td>
<td>613</td>
<td>493</td>
<td>&gt;10,000</td>
<td>0.2</td>
<td>3.3x10⁻³⁴</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>PG01037</th>
<th>SB-277011A</th>
<th>raclopride</th>
<th>haloperidol</th>
<th>L-741,626</th>
<th>L-745,870</th>
<th>Ro 61-6270</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agonist</td>
<td>52</td>
<td>527</td>
<td>2.2</td>
<td>3</td>
<td>18.1</td>
<td>3600</td>
<td>1450</td>
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<tr>
<td>SB-277011A</td>
<td>0.06</td>
<td>78</td>
<td>79</td>
<td>16</td>
<td>604</td>
<td>3020</td>
<td>5470</td>
</tr>
<tr>
<td>raclopride</td>
<td>0.03</td>
<td>74</td>
<td>8.8</td>
<td>33</td>
<td>271</td>
<td>872</td>
<td>793</td>
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<tr>
<td>haloperidol</td>
<td>760</td>
<td>3600</td>
<td>5030</td>
<td>21</td>
<td>260</td>
<td>0.5</td>
<td>0.5</td>
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<tr>
<td>L-741,626</td>
<td>867</td>
<td>6.8</td>
<td>0.03</td>
<td>0.2</td>
<td>n.a.⁴</td>
<td>1.2</td>
<td>n.a.⁴</td>
</tr>
<tr>
<td>L-745,870</td>
<td>1.3x10⁴</td>
<td>n.a.⁴</td>
<td>n.a.⁴</td>
<td>n.a.⁴</td>
<td>0.4</td>
<td>1.7 x10⁻⁴³</td>
<td>9.1 x10⁻⁴⁵</td>
</tr>
<tr>
<td>Ro 61-6270</td>
<td>46</td>
<td>0.1</td>
<td>0.4</td>
<td>1.7 x10⁻⁴³</td>
<td>0.4</td>
<td>9.1 x10⁻⁴⁵</td>
<td></td>
</tr>
</tbody>
</table>

† Selectivity ratios were based on radioligand-corrected values (K₀.₅) for D₂ and D₄ using [³H]Spiperone and values for D₃ using [³H]PD128-907. Selectivity ratios for D₂(high) and D₂(low) were calculated based on a two-site model (using Prism) assuming that the Kᵢ for [³H]Spiperone is identical for both sites.

‡ Not determined

a Selectivity ratio could not be calculated
Figure 1.

Filled Symbol - Yawning  Open Symbol - Erection

Apomorphine (A)  ABT-724 (F)

Pramipexole (B)  PD-168,077 (G)

Quinpirole (C)  PIP3EA (H)

PD-128,907 (D)  Sumanirole (I)

PE Incidence (%)  Yawns / 45 min  PE / 45 min

Dose (mg/kg; s.c.)

Veh  0.0032  0.01  0.032  0.1  0.32  1.0  3.2

**  *  +  ***  **  *  +  ***  **

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Figure 4.