

Central Mechanisms Regulating Penile Erection in Conscious Rats: The Dopaminergic Systems Related to the Proerectile Effect of Apomorphine

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ABSTRACT

Apomorphine has been used as a pharmacological probe of dopaminergic receptors in a variety of central nervous system disorders. The utility of apomorphine as an agent for the treatment of erectile dysfunction has also been demonstrated clinically. Apomorphine is a nonselective dopaminergic receptor agonist with potent binding affinity (K_i) of 101, 32, 26, 2.6, and 10 nM for D_1 , D_2 , D_3 , D_4 , and D_5 , respectively. When administered either subcutaneously (s.c.) or intracerebroventricularly (i.c.v.), apomorphine fully evoked penile erections in conscious rats with maximum effect at 0.1 μ mol/kg s.c. and 3 nmol/rat i.c.v., respectively. Apomorphine was less efficacious when injected intrathecally (i.t.) to L4-L6 spinal levels (50% at 30–100 nmol/rat i.t.). Penile erection facilitated by apomorphine was blocked by haloperidol and clozapine (i.p. and i.c.v.) but not by domperidone (a peripherally acting dopaminergic receptor antagonist). In this model using conscious rats, penile erection was significantly induced by quinpirole (D_2 - D_3 - D_4 receptor ag-

onist), but not by $R(+)$ -1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine-7,8-diol (SKF38393) and $R(+)$ -6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzapine (SKF81297) (D_1 receptor agonists), or a D_2 receptor agonist R -5,6-dihydro- N,N -dimethyl-4*H*-imidazo[4,5,1-*ij*]quinolin-5-amine (PNU-95666E). The role of D_4 receptors in penile erection was demonstrated using selective D_4 receptor agonists [(4-phenylpiperazinyl)-methyl]benzamide (PD168077) and 5-fluoro-2-[[4-(2-pyridinyl)-1-piperazinyl]methyl]-1*H*-indole (CP226269), whether administered systemically (s.c.) or locally in the brain (i.c.v.). The ability of apomorphine to activate D_3 receptors in relation to its proerectile activity remains to be elucidated by use of selective subtype agonists. These results suggest that the proerectile action of apomorphine in rats is mediated at supraspinal levels and that this effect is not mimicked by a D_2 receptor agonist but associated with activation of D_4 receptors.

Penile erection is one component of a complex series of integrated physiological processes and biochemical events coordinated at the level of the peripheral and central nervous system (Moreland et al., 2001). Different brain regions and neuroanatomical connections have been proposed to regulate penile erection (deGroat and Booth, 1993; McKenna, 2000; Steers, 2000), and perturbation of the neural pathways can lead to erectile dysfunction (ED).

The central nervous system (CNS) is an attractive target for discovery of novel therapeutic approaches for the treatment of ED, and many potential sites for CNS-acting drugs have been hypothesized (Andersson and Hedlund, 2002). Pharmacological experiments have focused on the role of neurotransmitters and neuropeptides involved in the central control of erection, via spinal and supraspinal pathways (Andersson, 2001). Substantial evidence indicates that the central dopaminergic pathway plays an important role in penile erection (Heaton, 2000), mainly the central dopaminergic neurons that comprise the incerto-hypothalamic system with projections to the medial preoptic area (MPOA) and

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ABBREVIATIONS: ED, erectile dysfunction; CNS, central nervous system; MPOA, medial preoptic area; PVN, paraventricular nucleus; PE, polyethylene; CHO, Chinese hamster ovary; GTP γ S, guanosine 5'-O-(3-thio)triphosphate; 5HT, 5-hydroxytryptamine; SKF38393, $R(+)$ -1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine-7,8-diol; SKF81297, $R(+)$ -6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzapine; PNU-95666E, R -5,6-dihydro- N,N -dimethyl-4*H*-imidazo[4,5,1-*ij*]quinolin-5-amine; PD168077, (4-phenylpiperazinyl)-methyl]benzamide; CP226269, 5-fluoro-2-[[4-(2-pyridinyl)-1-piperazinyl]methyl]-1*H*-indole; BP897, 1-4-2-naphthoylaminobutyl-4-2-methoxyphenyl-1*A*-piperazine.

paraventricular nucleus (PVN) (McKenna, 2000). The MPOA and PVN nuclei play a critical role in sexual behavior and related sexual responsiveness because lesions of these areas abolish male sexual behavior (Argiolas et al., 1987; Melis et al., 1987; Hull et al., 1995). Dopaminergic neurons have also been identified traveling from the caudal hypothalamus within the diencephalo-spinal dopamine pathway to innervate the lumbosacral spinal cord (Ridet et al., 1992; Andersson and Wagner, 1995; Holstege et al., 1996).

Apomorphine is a nonselective dopaminergic receptor agonist that activates D₁-like (D₁ and D₅) and D₂-like (D₂, D₃, and D₄) receptors (Seeman and Van Tol, 1994; Vallone et al., 2000; Moreland et al., 2003). Systemic administration of apomorphine facilitates penile erection in rats, rabbits, and monkeys, and the effect can be blocked by haloperidol, a dopamine receptor antagonist (Bitran and Hull, 1987; Pomerantz, 1990; Heaton et al., 1991). More recently, apomorphine has been shown to be effective for restoring penile erection in patients with erectile dysfunction (Dula et al., 2001). The mechanisms underlying the erectogenic effects induced by apomorphine have not been fully elucidated, although there is evidence that apomorphine can act via spinal sites in rats as determined by rises in intracavernous pressure (Giuliano et al., 2001; Ishizuka et al., 2002). In the present study, we have conducted a series of *in vivo* studies in a conscious rat model to determine the site of action in mediating penile erection evoked by apomorphine (spinal, supraspinal, or peripheral) and to determine which dopamine receptor subtype(s) may be responsible for its proerectile effect.

Materials and Methods

Chemicals and Reagents. Apomorphine was obtained from Aldrich Chemical Co. (Milwaukee, WI). PD168077, CP226269, and PNU-95666E were synthesized at Abbott Laboratories (Abbott Park, IL). All other chemicals or reagents, unless indicated otherwise, were obtained from Sigma-Aldrich (St. Louis, MO).

Animals. Wistar rats (males, ~300 g body weight, obtained from Charles River, Portage, MI) were used for all experiments. All animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care approved facilities at Abbott Laboratories in a temperature-regulated environment under a controlled 12-h light/dark cycle, with lights on at 6:00 AM. Food and water were available *ad libitum* at all times except during testing. All testing was done following procedures outlined in protocols approved by Abbott's Institutional Animal Care and Use Committee.

Intracerebroventricular (i.c.v.) Injection. Anesthetized rats (pentobarbital sodium 50 mg/kg *i.p.*) were placed into stereotaxic apparatus and a midline incision of approximately 1.5 to 2.0 cm in length was made longitudinally. A stainless steel guide cannula (22-gauge) was stereotaxically aimed at the left lateral ventricle (stereotaxic coordinates: 1.0 mm posterior to bregma, 1.6 mm left lateral to midline, and 4.5 mm vertical from surface of the skull). After the skull was cleaned and dry, a small amount of dental acrylic cement was pasted on the surface of the skull so that it covered the skull screws and secured the implantation cannula in place. After the cement was completely dry and hardened, a stainless steel stylet was used to occlude the guide cannula during recovery and between drug injections. The incision was closed using wound clips. The rat was then removed from the stereotaxic apparatus, and placed into a 37°C warming plate to allow them to recover from anesthesia after surgery. Animals were individually housed and allowed to recover for 7 days before any experimental treatment.

Compounds of interest were infused intracerebrally alone (5 μ l) or in conjunction with other systemically administered agents. After

the experiment was completed, cannula placement was confirmed by the infusion of 0.5% fast-green dye in saline solution (5 μ l) and subsequent dissection.

Intrathecal (i.t.) Injection. Rats were placed under halothane anesthesia and mounted onto an intrathecal stereotaxic instrument by placing the animal into blunt ear bars, which held the animal's head firmly. An incision was made vertically from the dorsal surface of the occipital bone to the base of the skull (2 cm). Tissue was then displaced using a blunt probe so that the alanto-occipital membrane at the base of the skull was clearly seen. Custom-made intrathecal catheters, constructed of 8.5 cm of PE-5 tubing glued to 4 cm of PE-10 tubing separated by a small notch between them (PSS Select, Jacksonville, FL), were used to implant into the rat spinal subarachnoid space with the caudal tip of the PE-5 catheter at the L4-L6 spinal level. The PE-5 tubing was slowly and gently inserted from the incision point to the lumbar enlargement (L4-L6) (LoPachin et al., 1981). Using the smaller PE-5 internal tubing facilitated the surgical manipulation and significantly decreased the chances of motor impairment. Once the notch rested on the alanto-occipital membrane (indicating the tip is in the lumbar enlargement), an 18-gauge needle tip was slid through the posterior (to the initial incision) skin surface. With the needle remaining in the skin, the external portion of the catheter was threaded into the needle. Both the needle and the external catheter were pulled (toward the tail) through the skin to keep the catheter in place and out of reach of the animal (the external catheter portion points toward the animal's tail). The incision was closed with surgical wound clips. The catheter was filled with sterile physiological saline and the ends of the catheter were heat-sealed.

After the recovery from surgery, animals were individually housed. If motor impairment was noticed, the animals were immediately euthanized. Animals with catheters were allowed at least 1 week of recovery from surgery before behavioral testing. For compound intrathecal injection, a Hamilton syringe (50 μ l) was connected to the external portion (4 cm) of the catheter and 10 μ l of drug solution was slowly injected into the catheter. The tip of the catheter was then cauterized. Using the fast-green dyed saline solution demonstrated that, under this condition, the diffusion of the injection solution was restricted to the spinal areas of the injection site.

In Vivo Rat Penile Erection. Male adult Wistar rats, weighing ~300 g, were used as an animal model to study penile erection *in vivo* as reported previously (Hsieh et al., 2003). All experiments were carried out between 9:00 AM and 3:00 PM. On the day of testing, animals were allowed to adapt to a diffusely illuminated testing room with red light for 1 h before the start of the experiment. Rats were placed individually into a transparent Plexiglas cage (20 \times 30 \times 30 cm) immediately after the drug injection. A mirror was placed behind and under the observation cages to facilitate observation of the animals. Each rat was used only once. A penile erection was considered to occur when the following behaviors were presented: repeated pelvic thrusts immediately followed by an upright position, and an emerging, engorged penis that the rat proceeded to groom. Apomorphine or other compounds were freshly prepared and administered to rats via subcutaneous injection into the back neck area (1 ml/kg injection volume), *i.c.v.* infusion (5 μ l), or intrathecal injection (10 μ l).

In another series of *in vivo* studies, repeated dosing experiments were also conducted in the rat conscious penile erection model. For these studies, rats were dosed with either vehicle or apomorphine (0.1 μ mol/kg *s.c.*) once daily for 5 days. Animals were tested in the morning on days 1 and 5, respectively, immediately after drug injection.

In the pharmacological blockade experiments, rats were injected either *i.p.* or *i.c.v.* with various dopamine receptor antagonists such as haloperidol (D₂-like), clozapine (D₄ preferential), and domperidone (D₂-like) before *s.c.* apomorphine (0.1 μ mol/kg) injection.

The penile erection episodes were recorded by direct observation for a period of 60 min after the compound dosing, and erection

incidence (percentage) was defined as the percentage of animals exhibiting one or more erections during the observation period. Data were expressed as incidence (percentage) \pm S.E. calculated by using Wald equation. Statistical evaluation of the results was performed by χ^2 test. A $p < 0.05$ was considered significant. The number of penile erections was also counted and the data, expressed as mean \pm S.E.M. of erection over the observation period, were analyzed by the Mann-Whitney nonparametric test. A $p < 0.05$ was considered significant.

Apomorphine Pharmacokinetics. Apomorphine was freshly prepared in 20% ethanol, 30% propylene glycol, and 50% dextrose (5%) in water (v/v) before i.v., s.c., and p.o. administration in rats. Blood samples were obtained at 0.1 (i.v. and s.c. only), 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, and 8 h after dosing. Plasma was separated by centrifugation and frozen (-20°C) until analysis. Apomorphine and an internal standard were separated from the plasma using a single protein precipitation with 1% formic acid acetonitrile. After evaporation of the supernatant, the plasma samples were constituted with mobile phase. The components of interest were separated from co-extracted contaminants on a C18 column (50×3 mm, $5 \mu\text{m}$) (Thermo Hypersil, Keystone Scientific Operations, Bellfonte, PA) with an acetonitrile: 1% formic acid 40:60 (by volume) mobile phase at a flow rate of 0.4 ml/min, with quantitation by high-performance liquid chromatography-tandem mass spectrometry (Applied Biosystems/MDS Sciex, Foster City, CA) in the multiple reaction monitoring mode.

In another series of experiments, apomorphine was give subcutaneously and the rats were decapitated at different time points. Brains were immediately removed, placed on an ice-cold dissection plate, and rapidly freed from blood vessels as much as possible. The resulting brain tissues were immediately frozen at -20°C followed by weighing and homogenization with aliquot taken and stored at -20°C until analysis as described above. The limit for detection for apomorphine was 0.05 ng/ml and 0.4 ng/g on the plasma and brain tissue samples, respectively.

Radioligand Binding Assay. Membranes containing recombinant human D_4 dopamine receptors ($D_{4.2}$, $D_{4.4}$, and $D_{4.7}$ variants) transfected into CHO cells were obtained from Receptor Biology (Beltsville, MD). Membranes suspended in 10 mM Tris-HCl, pH 7.2, with 2 mM EDTA were stored at -80°C until use. Binding assays for human receptors were initiated by adding 250 μl of the prepared membrane suspension to 200 μl of [^3H]spiperone (107 Ci/mmol; Amersham Biosciences Inc., Piscataway, NJ) and were incubated at room temperature for 2 h with gentle shaking. The final concentration of [^3H]spiperone was 0.2 nM. Nonspecific binding was determined in the presence of 10 μM haloperidol. The incubation buffer consisted of 5 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, 5 mM MgCl_2 , and 1 mM EDTA. In competition binding studies, 50 μl of drug solution prepared in 0.1% ascorbic acid with 0.5 mM 3-isobutyl-1-methylxanthine in the binding buffer. The reaction was terminated by rapid filtration through Unifilter-96 GF/B filter using a Filtermate Harvester (PerkinElmer Life Sciences, Boston, MA). Fil-

ter was washed three times with 700 μl of ice-cold 50 mM Tris-HCl wash buffer, pH 7.4. Radioactivity was counted by a TopCount microplate scintillation counter after adding 45 μl of scintillation cocktail (Microscint-20; PerkinElmer Life Sciences). Protein concentrations were determined by bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL) using bovine serum albumin as a standard. All assays were performed in triplicate, and IC_{50} values converted to K_i values (Cheng and Prusoff, 1973).

Agonist-Stimulated GTP γ S Binding Assay. Agonist activity was measured by GTP-binding assay using a DELFIA GTP-binding kit (PerkinElmer Life Sciences). Membranes containing recombinant human D_4 dopamine receptors ($D_{4.2}$ variant) transfected into CHO cells were obtained from Receptor Biology. In this assay, cell membrane homogenates (8 μg of protein) were incubated with agonists or without antagonists in a GTP binding buffer consisting of 50 mM HEPES, pH 7.4, 10 mM MgCl_2 , 25 mM NaCl, 100 $\mu\text{g}/\text{ml}$ saponin, 1 μM GDP, and 10 nM GTP in a 96-well Filter plate at room temperature for 40 min. The nonspecific binding was defined by the addition of 100 μM GTP γ S. The assay reaction was terminated by rapid filtration and the filter was washed three times with 225 μl of ice-cold washing solution in a vacuum manifold. The plate was read using time-resolved fluorometer, 1420 VICTOR multilabel counter (PerkinElmer Life Sciences). Because GDP-GTP exchange takes place continuously in the absence of G protein-coupled receptor activation, basal signal caused by automatic GTP binding was also determined in the absence of agonists. Percentage of stimulation was calculated as $100 \times$ the difference between the counts of agonist and 10 μM of dopamine-stimulated binding. All assays were performed in triplicate, and EC_{50} values were calculated by nonlinear regression using curve-fitting Prism program (GraphPad Software Inc., San Diego, CA).

Results

Apomorphine exhibited potent binding to D_1 -like (D_1 and D_5) and D_2 -like (D_2 , D_3 , and D_4) receptor subtypes when examined in more than 70 neurotransmitter receptors/uptake binding assays/ion channels/enzymes (Table 1). The affinity (K_i) was 101, 32, 26, 2.6, and 10 nM for D_1 , D_2 , D_3 , D_4 , and D_5 , respectively. The binding studies also demonstrate that apomorphine binds with <1000 nM affinity (K_i) to α_2 , 5HT $_{1A}$, 5HT $_{2C}$, and 5HT $_7$, and with >1000 nM affinity to other receptors such as α_1 , β_1 , β_2 , H_1 , κ , μ , 5HT $_{1B}$, 5HT $_{2A}$, 5HT $_3$, 5HT $_{5A}$, and 5HT $_6$ (Table 1).

Subcutaneous injections of apomorphine (0.003–1 $\mu\text{mol}/\text{kg}$) dose dependently induced penile erections in conscious rats (Fig. 1). The maximum effect of $91 \pm 5\%$ ($p < 0.001$ versus vehicle control) was seen in rats injected with apomorphine at 0.1 $\mu\text{mol}/\text{kg}$ s.c. (Fig. 1, top). A significant proerectile effect (56% incidence versus 22% for vehicle control; $p < 0.01$) was

TABLE 1

Receptor selectivity of apomorphine (radioligand binding studies at Cerep, Redmond, WA)

Apomorphine exhibited low affinities ($K_i > 10,000$ nM) for more than 50 other neurotransmitters/uptake/enzymes/ion channels as determined by Cerep screening.

Receptor	Ligand	K_i	Receptor	Ligand	K_i
		nM			nM
D_1	[^3H]SCH23390	101	κ	[^3H]U69593	5000
D_2	[^3H]Spiperone	32	μ	[^3H]DAMGO	2000
D_3	[^3H]Spiperone	26	5HT $_{1A}$	[^3H]8-OH-DPAT	121
D_4	[^3H]Spiperone	2.6	5HT $_{1B}$	[^3H]Cyanopindolol	6000
D_5	[^3H]SCH23390	10	5HT $_{2A}$	[^3H]Ketanserin	1000
α_1	[^3H]Prazosin	3000	5HT $_{2C}$	[^3H]Mesulergine	200
α_2	[^3H]RX821002	100	5HT $_3$	[^3H]BRL43694	5000
β_1	[^3H]CGP12177	$>10,000$	5HT $_{5A}$	[^3H]LSD	5000
β_2	[^3H]CGP12177	$>10,000$	5HT $_6$	[^3H]LSD	2000
H_1	[^3H]Pyrilamine	$>10,000$	5HT $_7$	[^3H]LSD	200

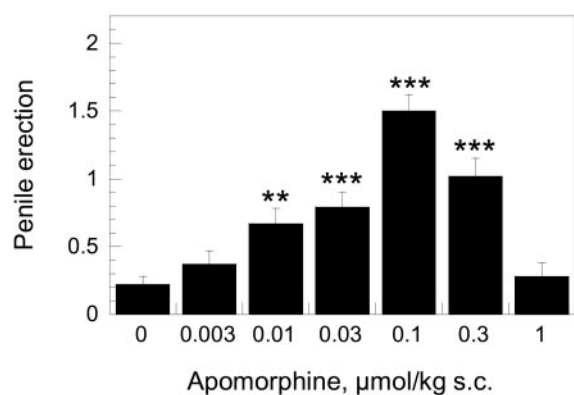
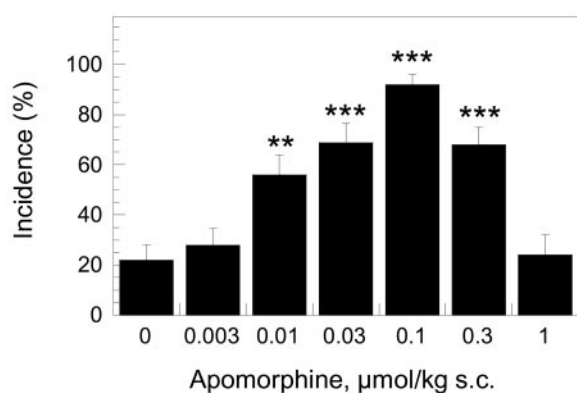


Fig. 1. Proerectile effects of apomorphine in conscious rats. Rats were injected subcutaneously with apomorphine immediately before introduction in individual observation cage. Penile erection was assessed during a 60-min postdosing period. Erection incidence (percentage) is defined as the percentage of animals exhibiting one or more erections during the observation period. Data are expressed as incidence (percentage) \pm S.E. or mean of number of penile erection \pm S.E.M. ($n = 30$). **, $p < 0.01$; ***, $p < 0.001$ versus vehicle control.

observed at the 0.01- $\mu\text{mol/kg}$ dose. Apomorphine also elicited a dose-dependent enhancement in the number of penile erections. At the most efficacious dose of 0.1 $\mu\text{mol/kg}$ apomorphine produced a mean of 1.5 ± 0.2 erections during the observation period (Fig. 1, bottom). Additional groups of rats received s.c. injections of apomorphine 0.1 $\mu\text{mol/kg}$ once daily for 5 days. The occurrence of penile erection was determined on day 1 and on day 5 immediately after dosing. The effect of apomorphine on day 5 (87%) was similar to the effects on day 1 (83%), indicating that no tolerance to the erectogenic effect of apomorphine was developed in rats under this dosing regimen.

In the present study, the systemic s.c. injection was selected as a route of apomorphine administration in the in vivo efficacy testing due to the pharmacokinetic studies, demonstrating apomorphine is not orally bioavailable in rats. Apomorphine was rapidly absorbed after s.c. injection with 93.3% bioavailability. Apomorphine exhibited a short terminal $t_{1/2}$ (0.2 h) after i.v. administration with a high total plasma clearance (10.4 l/h \cdot kg), in spite of a large apparent volume of distribution (3.5 l/kg). More importantly, pharmacokinetics studies revealed that apomorphine readily crossed

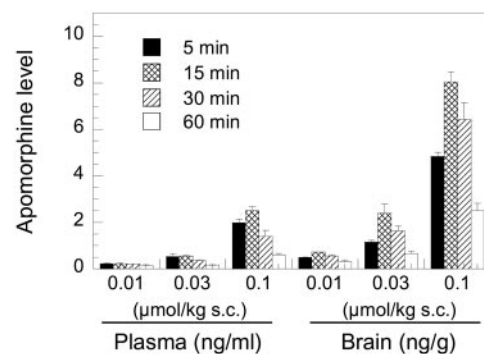


Fig. 2. Plasma and brain concentrations of apomorphine after a single subcutaneous (s.c.) administration at the doses of 0.01, 0.03, 0.1, and 0.3 $\mu\text{mol/kg}$ in rats. The concentrations of apomorphine are expressed as nanograms per milliliter and nanograms per gram on plasma and brain tissue samples (mean \pm S.E.M., $n = 3$), respectively.

the blood-brain barrier and that maximal brain levels were reached within 15 min after systemic s.c. dosing (Fig. 2). At the maximally efficacious dose (0.1 $\mu\text{mol/kg}$ s.c.) plasma levels reached 2 ng/ml, whereas the apomorphine brain concentrations reached 8 ng/g tissue.

Several experiments were carried out to examine the differential effects of various dopaminergic receptor antagonists

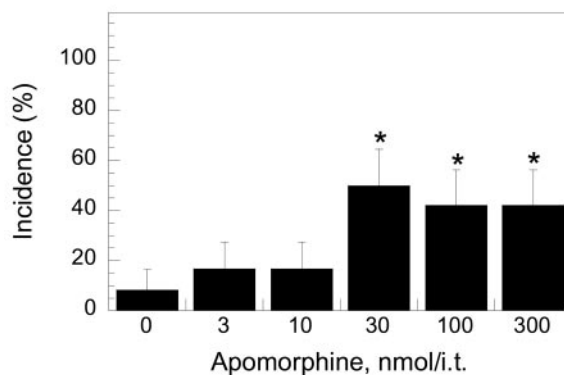
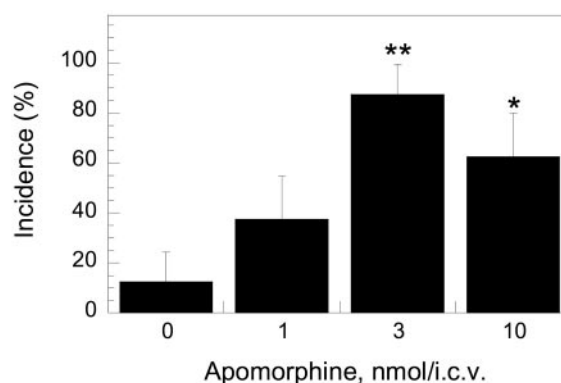


Fig. 3. Proerectile effects of i.c.v. ($n = 8$, top) or i.t. ($n = 12$, bottom) microinjections of apomorphine in conscious rats. Rats were injected with apomorphine immediately before introduction in individual observation cage. Penile erection was assessed during a 60-min post-dosing period. Data are expressed as erection incidence (percentage) \pm S.E. *, $p < 0.05$; **, $p < 0.01$, versus vehicle control.

TABLE 2

Effects of dopaminergic antagonists on penile erection facilitated by s.c. injection of 0.1 $\mu\text{mol/kg}$ apomorphine in conscious Wistar rats
Penile erection data are expressed as erection incidence (%) \pm S.E.

Treatment ^a	Dose	Incidence (%)
	$\mu\text{mol/kg i.p.}$	
Apomorphine		88 \pm 12
+ haloperidol	0.1	63 \pm 17
+ haloperidol	0.3	13 \pm 12**
Apomorphine		88 \pm 12
+ clozapine	1	50 \pm 18
+ clozapine	3	38 \pm 17*
+ clozapine	10	0 \pm 0**
Apomorphine		100 \pm 0
+ domperidone	3	88 \pm 12
+ domperidone	10	100 \pm 0

^a Dopaminergic antagonists were administered i.p. 60 min before s.c. apomorphine injection (0.1 $\mu\text{mol/kg}$).

* $p < 0.05$, ** $p < 0.01$, compared with apomorphine control.

TABLE 3

Inhibitory effects of i.c.v. microinjection of haloperidol or clozapine on penile erection facilitated by apomorphine (0.1 $\mu\text{mol/kg}$ s.c.) in conscious Wistar rats

Penile erection was assessed during a 60-min post-dosing period. Data are expressed as erection incidence (%) \pm S.E. ($n = 8$).

Treatment ^a	Dose	Incidence (%)
	$\mu\text{mol/kg i.c.v.}$	
Apomorphine		100 \pm 0
+ haloperidol	0.001	100 \pm 0
+ haloperidol	0.003	71 \pm 17
+ haloperidol	0.01	43 \pm 19**
+ haloperidol	0.03	29 \pm 17**
+ haloperidol	0.1	14 \pm 13***
Apomorphine		75 \pm 15
+ clozapine	0.01	57 \pm 19
+ clozapine	0.03	57 \pm 19
+ clozapine	0.1	14 \pm 13**

^a Dopaminergic antagonists were administered i.p. 15 min before s.c. apomorphine injection (0.1 $\mu\text{mol/kg}$).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with apomorphine control.

on the proerectile effects of apomorphine (Tables 2 and 3). Haloperidol is a dopaminergic receptor antagonist ($K_i = 80, 1.2, 7, 2.3,$ and 100 nM for $D_1, D_2, D_3, D_4,$ and D_5 subtype, respectively) (Seeman and Van Tol, 1994). Pretreatment with haloperidol 0.3 $\mu\text{mol/kg}$ i.p. significantly inhibited the penile erection facilitated by 0.1 $\mu\text{mol/kg}$ s.c. apomorphine. Clozapine is a preferential D_4 receptor antagonist ($K_i = 170, 230, 170, 21,$ and 330 nM for $D_1, D_2, D_3, D_4,$ and D_5 subtype, respectively) (Seeman and Van Tol, 1994). The preadministration of clozapine at the doses of 3 to 10 $\mu\text{mol/kg}$ i.p. significantly blocked the penile activity induced by apomorphine. Domperidone is a D_2 -like receptor antagonist ($K_i = 0.4, 9.5,$ and 30.4 nM for $D_2, D_3,$ and D_4 subtype, respectively) (Vallone et al., 2000) that does not cross the blood-brain barrier. The pretreatment with domperidone (3–10 $\mu\text{mol/kg}$ i.p.) did not block the penile erection facilitated by apomorphine in rats. The penile erection facilitated by apomorphine (0.1 $\mu\text{mol/kg}$ s.c.) was also significantly blocked in conscious rats pretreated via i.c.v. microinjection with either haloperidol (10–100 nmoles) or clozapine (100 nmol) (Table 3). These results reveal that the proerectile action of apomorphine in conscious rats is centrally mediated through dopaminergic pathways. The rats administered with dopaminergic receptor antagonists alone at the doses used in the present studies did

TABLE 4

Proerectile effects of dopaminergic agonists in conscious Wistar rats after a single s.c. injection

Penile erection was assessed during a 60-min post-dosing period. Data are expressed as erection incidence (%) \pm S.E.

Treatment	Dose	Incidence (%)
	$\mu\text{mol/kg s.c.}$	
SKF81297	Vehicle	13 \pm 12
	0.3	14 \pm 13
	1	25 \pm 15
SKF38393	Vehicle	14 \pm 13
	0.3	0
	1	12 \pm 12
	3	38 \pm 17
Quinpirole	Vehicle	14 \pm 13
	0.1	38 \pm 17
	0.3	75 \pm 15*
	1	25 \pm 15

* $p < 0.05$, compared with vehicle control.

not exhibit significant changes in penile activity compared with spontaneous erection in the vehicle-treated animals.

To determine whether the proerectile effects of apomorphine are mediated via supraspinal or spinal sites of action, studies were conducted in conscious rats given various doses of apomorphine via i.c.v. or intrathecal microinjection. Maximal efficacy was observed at 88% after i.c.v. injections of 3 nmol of apomorphine (Fig. 3, top). In contrast, only 50% maximal incidence was elicited after intrathecal injection of 30 nmol of apomorphine (Fig. 3, bottom), i.e., 10-fold higher than the most efficacious i.c.v. dose. These results demonstrate that when the drug is injected systemically, the major site of action of apomorphine to induce penile erection in rats is likely at the supraspinal levels.

Because apomorphine is a nonselective dopaminergic receptor agonist, the effects of several selective dopaminergic receptor agonists, SKF38393, SKF81297, quinpirole, PNU-95666E, PD168077, and CP226269, on the penile erection were also examined. The results shown in Table 4 demonstrate that neither SKF38393 (a partial D_1 receptor agonist) nor SKF81297 (a full D_1 receptor agonist) (Seeman and Van Tol, 1994; Vallone et al., 2000) exhibited any proerectile effects. These data suggest that D_2 -like receptors participate in apomorphine-induced penile erection.

Quinpirole (a nonselective D_2 - D_3 - D_4 receptor agonist) (Moreland et al., 2003) significantly produced a dose-depen-

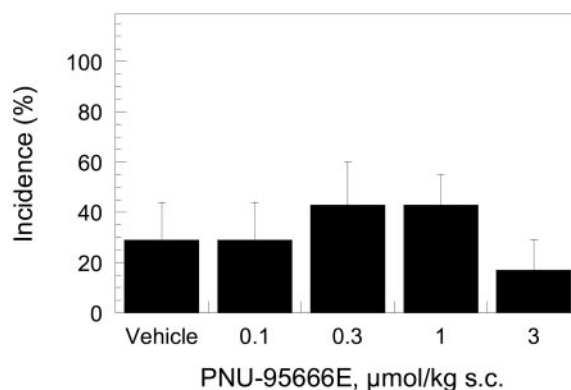


Fig. 4. The selective D_2 agonist PNU-95666E is not proerectile in rats. Penile erection was assessed during a 60-min postdosing period in individual observation cage. Data are expressed as erection incidence (percentage) \pm S.E. ($n = 8$).

TABLE 5

Radioligand binding affinity (K_i , nM) for PD168077, CP226269, and apomorphine on recombinant human D_4 variants hD_{4.2}, hD_{4.4}, and hD_{4.7}. Competition binding with [³H]spiperone for dopaminergic receptor agonists. Data are presented as mean \pm S.E.M. ($n = 4$).

Compound	hD _{4.2}	hD _{4.4}	hD _{4.7}
Apomorphine	8.1 \pm 0.4	9.6 \pm 0.6	9.3 \pm 0.7
PD168077	6.0 \pm 2.8	22.3 \pm 0.1	29.0 \pm 1.3
CP226269	2.4 \pm 0.7	3.6 \pm 0.1	5.6 \pm 0.9

dent facilitation of penile erection in rats with maximal incidence of 75% at 0.3 μ mol/kg (Table 4). The effect of the selective D_2 receptor agonist PNU-95666E (Heier et al., 1997) was investigated in rats at the doses of 0.1, 0.3, 1, and 3 μ mol/kg. As shown in Fig. 4, the s.c. administration of PNU-95666E did not facilitate penile erection. PNU-95666E has been reported as a centrally acting D_2 agent on dopaminergic neurons in rats and mice after systemic administration (Durhama et al., 1997; Heier et al., 1997; Sethy et al., 1997).

To examine the role of D_4 receptor activation in relation to the proerectile activity of apomorphine, two D_4 receptor agonists PD168077 (Glase et al., 1997) and CP226269 (Zorn et al., 1997) were investigated in the present study. In vitro competition binding with [³H]spiperone demonstrated that both PD16807 and CP226269 exhibited potent affinity (K_i) with little difference across the three D_4 alleles (Table 5). Apomorphine also showed no difference in binding to the D_4 alleles, having potencies in the nanomolar range. The agonist activities of PD168077 and CP226269, determined by measuring GTP γ S binding activity followed by D_4 receptor activation on the cloned human $D_{4.2}$ cell membranes, are shown in Fig. 5. Dopamine induced concentration-dependent increases in GTP γ S binding (EC_{50} value of 7.9 nM). The agonist effect was mimicked by PD168077 (EC_{50} value of 5.5 nM, 88% efficacy), CP226269 (EC_{50} value of 10 nM, 69% efficacy), and apomorphine (88% agonist activity with an EC_{50} value of 1.2 nM). GTP γ S binding activity reflects the activation of G protein-coupled receptors, an early event in the cascade of signal transduction after the receptor ligation and has been successfully applied to the stimulation of human dopamine D_4 receptors (Chabert et al., 1994; Newman-Tancredi et al., 1997; Patel et al., 1997).

Administration of PD168077 (0.03–1.0 μ mol/kg s.c.) or

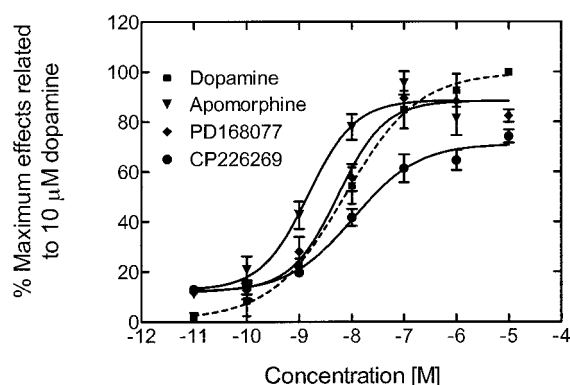


Fig. 5. Agonist-stimulation of GTP γ S binding activity by dopamine, apomorphine, PD168077, and CP226269 in recombinant human $D_{4.2}$ dopamine receptor. Data for each concentration point were normalized to the maximal effect of dopamine (10 μ M) and presented as the mean (\pm S.E.M.) of the triplicate assays ($n = 4$).

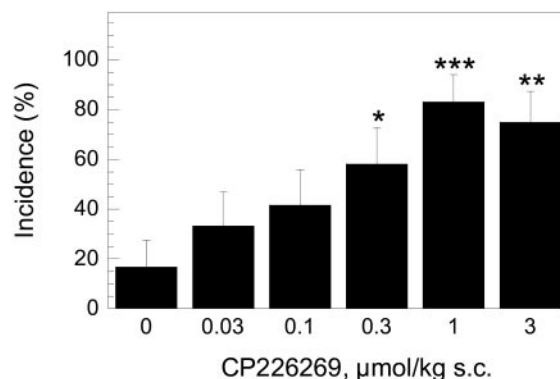
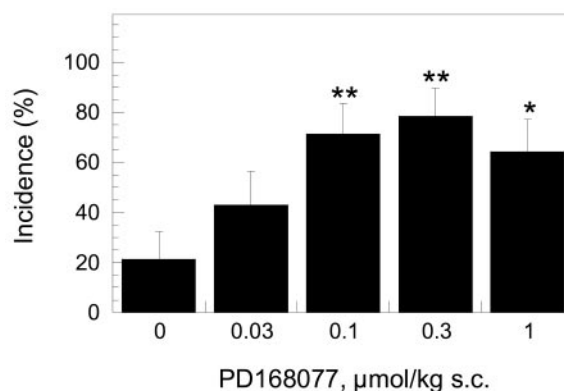


Fig. 6. Proerectile effects of D_4 agonists PD168077 ($n = 14$ rats/group) and CP226269 ($n = 12$ rats/group) in conscious rats. Rats were injected subcutaneously with compound immediately before introduction in the individual observation cage. Penile erection was assessed during a 60-min postdosing period. Data are expressed as erection incidence (percentage) \pm S.E. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus vehicle control.

CP226269 (0.03–3.0 μ mol/kg s.c.) induced dose-dependent penile erections in conscious Wistar rats. The maximum effect of 79 \pm 11% ($p < 0.01$ versus 22% for vehicle control) was displayed in rats injected with PD168077 at 0.3 μ mol/kg s.c. during a 60-min observation period (Fig. 6, top). A significant proerectile effect was also elicited by CP226269 with the maximum effect of 83 \pm 10% ($p < 0.001$ versus 17% for vehicle control) at the 1 μ mol/kg dose (Fig. 6, bottom). At the most efficacious dose of 0.3 μ mol/kg PD168077 and 1.0 μ mol/kg CP226269 produced a mean of 1.2 \pm 0.3 and 1.0 \pm 0.2, respectively, in the number of penile erections during the observation period. The proerectile effects of PD168077 and CP226269 were also examined in conscious rats given the drug via i.c.v. administration. Figure 7 shows that both D_4 receptor agonists directly injected into brain at a dose of 10 nmol fully evoked penile activity as erection was presented in >80% of the animals, an efficacy similar to the maximal effects induced by apomorphine.

Discussion

The present study demonstrates that apomorphine, a non-selective dopaminergic receptor agonist, potentially facilitates

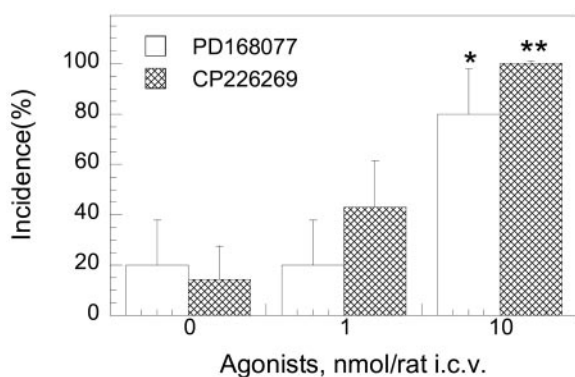


Fig. 7. Proerectile effects of i.c.v. microinjections of PD168077 ($n = 5$ rats/group) and CP226269 ($n = 7$ rats/group) in conscious rats. Rats were injected with drug immediately before introduction in the individual observation cage. Penile erection was assessed during a 60-min postdosing period. Data are expressed as erection incidence (percentage) \pm S.E. *, $p < 0.05$; **, $p < 0.01$, versus vehicle control.

penile erection in conscious rats. The proerectile effect of apomorphine is mediated by central dopaminergic pathway primarily at supraspinal levels and is blocked in animals pretreated with the dopaminergic receptor antagonists haloperidol and clozapine. The facilitatory effect on penile activity is related to the activation of D_4 receptors, but it is not mimicked by the injections of a selective D_2 receptor agonist.

A broad evaluation of receptor binding affinity was carried out for different receptors and apomorphine exhibits high affinity for all five dopamine receptor subtypes (Table 1). Functional studies have indicated that apomorphine elicits agonist activity with 80 to 90% efficacy at the D_2 , D_3 , and D_4 receptors (Moreland et al., 2003). Affinity of apomorphine for several other selected receptors such as α_2 , $5HT_{1A}$, and $5HT_{2C}$ is comparably weaker ($K_i > 100$ nM), and in view of the role of 5HT receptors in penile erection (Andersson, 2001), their potential role in the proerectile effect of apomorphine cannot be ruled out at the present time. However, because plasma level of apomorphine at the maximally efficacious dose ($0.1 \mu\text{mol/kg}$ s.c.) is approximately 2 ng/ml (7.5 nM), it is unlikely that the effect of apomorphine is related to any of these nondopaminergic receptors that would require higher concentrations.

Apomorphine has been demonstrated in animals, primarily rodents, to be a dopaminergic receptor activator of erectile pathways in the brain (Bitran and Hull, 1987; Heaton et al., 1991). In conscious rats, the proerectile effect of apomorphine is biphasic with low doses facilitating and high doses inhibiting erection after either s.c. or i.c.v. injections (Figs. 1 and 3). A biphasic dose-response relationship of apomorphine has been reported in the penile activity of rat models by other investigators (Heaton et al., 1991; Sachs et al., 1994; Matsuoka et al., 1996) as well as in a wide range of pharmacological endpoints, including memory, locomotion, oxytocin release, and prolactin release in rats, mice, dogs, or humans (Calabrese, 2001).

The *in vivo* pharmacological studies show that penile erection is fully evoked by apomorphine when it is injected centrally via i.c.v. and the effect is blocked by pretreatment with haloperidol but not by domperidone, a peripheral D_2 -like receptor blocker that does not penetrate the blood-brain barrier (Barone, 1999). The lack of effect of domperidone further demonstrates that the proerectile activity of apomorphine is

likely mediated through the central dopaminergic system. Additionally, the experiments conducted in the current study also present the first *in vivo* demonstration of the blocking effects of central i.c.v. administration of haloperidol or clozapine on the penile erection facilitated by systemically s.c. apomorphine in a conscious rat model (Table 3). The centrally induced effect of apomorphine has been displayed in anesthetized rats as indicated by the increased intracavernosal pressure after the direct injection of apomorphine into PVN, whereas intracavernosal injection of apomorphine failed to elicit penile erections (Chen et al., 1999). We have also demonstrated that, in the organ bath study, apomorphine does not cause relaxation on corpus cavernosum tissues (our unpublished observations), ruling out a direct peripheral action on the penile tissues.

The spinal cord contains all of the necessary components for achieving penile erection (deGroat and Booth, 1993; Andersson and Wagner, 1995) as well as the intraspinal dopaminergic innervation (Ridet et al., 1992; Holstege et al., 1996; Levant and MaCarson, 2001). To determine the possibility of an additional direct action of apomorphine at the spinal level, apomorphine was delivered into the vicinity of proerectile sacral parasympathetic nucleus by intrathecal injection to spinal L4-L6 levels. Intrathecal injection of apomorphine only produced 50% incidence at a 10-fold higher dose than the maximum efficacious dose of i.c.v. injection (88% at 3 nmol) in conscious rats (Fig. 3), indicating the major site of action of systemically administered apomorphine is likely through supraspinal pathways.

The spinal site of action of apomorphine-induced penile erection has been studied by other investigators. In anesthetized rats, injection of apomorphine intrathecally evokes the intracavernous pressure (Giuliano et al., 2001). This is in contrast to early findings, showing that intrathecal injection of apomorphine into the lumbosacral subarachnoid space is reported to depress the reflexive erections and other sexual behaviors (Pehek et al., 1989). The difference in these results is difficult to explain; however, the presence of dopamine receptors within the spinal cord (Ridet et al., 1992; Holstege et al., 1996; Levant and MaCarson, 2001) raises the possibility of a direct action of apomorphine at the spinal levels in addition to the one in the brain described above. However, due to its relatively smaller magnitude, a direct spinal effect of apomorphine is unlikely to account for the clinical efficacy in humans.

Dopamine neurotransmission plays an important role in the regulation of numerous CNS behaviors, including penile erection and sexual behaviors. Determination of the physiological role of multiple dopamine receptor subtypes identified in recent years has been dependent on the availability of selective agonists. Several pharmacological experiments conducted in the present study in conscious rats, using the selective dopamine agonists or dopamine antagonists, demonstrate that neither D_1 receptor agonists SKF38393 and SKF81297 nor a D_2 receptor agonist PNU-95666E has any significant proerectile effects, whereas quinpirole (a D_2 - D_3 - D_4 receptor agonist) produces a dose-dependent response in facilitating penile erection. On the other hand, the pretreatment with haloperidol (a D_2 -like receptor antagonist) or clozapine (a preferential D_4 receptor antagonist) completely inhibits the penile erection facilitated by $0.1 \mu\text{mol/kg}$ s.c. apomorphine.

The lack of effect of D₁ agonist SKF38393 (0.3–3 μmol/kg s.c.) in the present study (Table 4) is in contrast to the observation recently reported that systemic administration of dopamine D₁-like receptor agonists, including SKF38393 (10–80 μmol/kg s.c.) induces penile erection in rats and that the penile erectile activity is blocked by D₁-like receptor antagonist SCH-23390 (D'Aquila et al., 2003). However, our findings are in agreement with previous data that D₁ agonist SKF38393 does not facilitate penile activity whether administered systemically (s.c.) in conscious rats or injected locally into PVN in anesthetized rats (Melis et al., 1996; Chen et al., 1999). The differences in these results may be explained by the differences in the doses used between the studies. A number of studies have also reported that in rats, dopamine-induced penile erection seems to involve, particularly, the D₂-like receptor subtypes, and this seems to be the case also in humans (Heaton, 2000; Andersson and Hedlund, 2002). PNU-95666E is a centrally acting agonist that reportedly activates the D₂ receptor in central dopamine neurons, increases striatal acetylcholine concentrations, and decreases cerebellar nucleotides in rats and mice after systemic administration (Durhama et al., 1997; Heier et al., 1997; Sethy et al., 1997). PNU-95666E, which does not induce penile erection (Fig. 4), elicits full D₂ agonism (90%) but is totally devoid of D₄ functional activity (Moreland et al., 2003). Quinpirole is a potent agonist that elicits full functional activity at D₂, D₃, and D₄ receptor subtypes with efficacy of 90, 90, and 94%, respectively (Moreland et al., 2003). Together, the present results suggest that D₄ and/or D₃ receptors may be responsible for the proerectile effects of apomorphine.

Results from in vivo experiments in conscious rats have clearly indicated that penile erection is fully facilitated by two D₄ receptor agonists, PD168077 and CP226269, via either systemic administration or locally i.c.v. microinjection (Figs. 6 and 7). Functionally, in vitro, both agents potently activate D₄ receptor with agonist activity on the stimulation of GTPγS binding of D₄ receptor activation on D₄-expressing cell membranes (Fig. 5) but have no D₂ or D₃ activity as determined by the increases in intracellular calcium levels in the cells transfected with cloned human D₂ and D₃ (Moreland et al., 2003). To our knowledge, this presents the first in vivo evidence of the activation of D₄ receptors facilitating penile erections.

D₃ receptor stimulation may induce penile erection in rats based on the preliminary results obtained from the experiments conducted in our laboratories with the reported D₃ receptor agonists such as 7-OH DPAT, BP897, and PD128907, which all been shown to induce penile erections in conscious rats (G. C. Hsieh et al., unpublished data), and all possess potent agonist activity at D₄ (Moreland et al., 2003). Although the role of D₃ receptor in the mediation of this process remains unclear due to the lack of selective agonists, the data seem to indicate that the D₄ receptor mediates penile erection in conscious rats. Therefore, further characterization of the proerectile function mediated via D₃ activation with selective receptor subtype agonists is necessary for dissecting out the role of D₃ activation in apomorphine-induced penile erection. However, it should be noted that the D₂ receptor agonist PNU-95666E, which does not significantly induce penile erection, also exhibits 79% functional efficacy at D₃ but is totally devoid of D₄ functional activity

(Moreland et al., 2003), suggesting D₃ activation may not play an important role in facilitating penile erection.

In conclusion, results from the present in vivo behavioral studies in conscious rats demonstrate that the proerectile action of apomorphine is likely mediated at supraspinal (not spinal) levels, and the effect is related to the activation of D₄ receptors but is not mimicked by a D₂ receptor agonist. Although the ability of apomorphine to activate D₃ receptors in relation to its proerectile activity remains to be elucidated by the use of subtype selective agonists, we have demonstrated that the potent D₄ receptor agonists PD168077 and CP226269 facilitate penile erection in rats through a central dopaminergic pathway. Thus, agonist agents that selectively activate the D₄ receptor subtype may represent a novel approach for studying physiological processes of penile erection.

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