CENTRAL MECHANISMS REGULATING PENILE ERECTION IN CONSCIOUS RATS: THE DOPAMINERGIC SYSTEMS RELATED TO THE PRO-ERECTILE EFFECT OF APOMORPHINE


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Abbreviation: ED, erection dysfunction; CNS, central nervous system; i.c.v., intracerebroventricularly; MPOA, medial preoptic area, PVN, praventricular nucleus

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ABSTRACT

Apomorphine has been used as a pharmacological probe of dopaminergic receptors in a variety of CNS 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 µmol/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 agonist), but not by SKF38393 and SKF81297 (D_1 receptor agonists), or a D_2 receptor agonist PNU-95666E. The role of D_4 receptors in penile erection was demonstrated using selective D_4 receptor agonists, PD168077 and 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 pro-erectile activity remains to be elucidated by use of selective subtype agonists. These results suggest that the pro-erectile 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 paraventricular nucleus (PVN) (McKenna, 2000). The MPOA and PVN nuclei play a critical role in sexual behavior and related sexual responsiveness as 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 non-selective 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, though there is evidence that apomorphine can act via spinal sites in rats as determined by the rises of 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 pro-erectile 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 Chemical Co. (St. Louis, MO).

Animals

Wistar rats (male, ~ 300 g body weight, obtained from Charles River, Portage, MI) were used for all experiments. All animals were housed in AAALAC-approved facilities at Abbott Laboratories in a temperature-regulated environment under a controlled 12-h light-dark cycle, with lights on at 6:00 a.m. 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 1.5-2.0 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 µl) 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 µl) 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 PE10 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 PE5 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 (towards the tail) through the skin to keep the catheter in place and out of reach of the animal (the external catheter portion points towards 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.

Following 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 prior to behavioral testing. For compound intrathecal injection, a Hamilton syringe (50 µl) was connected to the external portion (4 cm) of the catheter and 10 µl 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 previously reported (Hsieh et al., 2003). All experiments were carried out between 9:00 a.m. and 3:00 p.m. On the day of testing, animals were allowed to adapt to a diffusely illuminated testing room with
red light for one hour prior to the start of the experiment. Rats were placed individually into a transparent Plexiglas cage (20x30x30 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 which the rat proceeded to groom. Apomorphine or other compounds were freshly prepared and administered to rats via subcutaneous injection into the back neck area (1ml/kg injection volume), i.c.v. infusion (5 µl), or intrathecal injection (10 µl).

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 µmol/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 intraperitoneally (i.p.) or intracerebrally (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 µmol/kg) injection.

The penile erection episodes were recorded by direct observation for a period of 60 min following the compound dosing, and erection incidence (%) was defined as the % of animals exhibiting one or more erections during the observation period. Data were expressed as incidence (%) ± S.E. calculated by using Wald equation. Statistical evaluation of the results was performed by Chi-
Square test. A $p < 0.05$ was considered significant. The number of penile erections was also counted and the data, expressed as mean ± 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) prior to 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. Following evaporation of the supernatant, the plasma samples were constituted with mobile phase. The components of interest were separated from co-extracted contaminants on a 50 x 3 mm, 5 µm, C$_{18}$ column (Keystone Scientific, Inc., Bellfonte, PA) with an acetonitrile: 1% formic acid 40:60 (by volume) mobile phase at a flow rate of 0.4 ml/minute, with quantitation by HPLC-MS/MS (API Sciex) in the MRM mode.

In another series of experiments, apomorphine was given 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 resulted 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 D₄ dopamine receptors (D₄.2, D₄.4, and D₄.7 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 Pharmacia Biotech Inc., Piscataway, NJ) and were incubated at room temperature for 2 hr with gentle shaking. The final concentration of [³H]-spiperone was 0.2 nM. Non-specific 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 MgCl₂, 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 (IBMX) in the binding buffer. The reaction was terminated by rapid filtration through Unifilter-96 GF/B filter using a Filtermate Harvester (Packard Instruments, Meriden, CT). Filter was washed 3x with 700 µl ice-cold 50 mM Tris-HCl wash buffer (pH 7.4). Radioactivity was counted by a TopCount Microplate Scillation Counter after adding 45 ul scintillation cocktail (Microscint-20, Packard). Protein concentrations were determined by BCA Protein Assay Kit (Pierce, Rockford, IL) using BSA as a standard. All assays were performed in triplicated and IC₅₀ values converted to Kᵢ values (Cheng and Prusoff, 1973).
**Agonist-stimulated GTP\textsubscript{γ}S binding assay.**

Agonist activity was measured by GTP-binding assay using a DELFIA GTP-binding kit (Perkin Elmer Life Science, Boston, MA). Membranes containing recombinant human D\textsubscript{4} dopamine receptors (D\textsubscript{4,2} variant) transfected into CHO cells were obtained from Receptor Biology (Beltsville, MD). In this assay, cell membrane homogenates (8 µg protein) were incubated with agonists or without antagonists in a GTP binding buffer consisting of 50 mM HEPES, pH7.4, 10 mM MgCl\textsubscript{2}, 25 mM NaCl, 100 µg/ml Saponin, 1 µM GDP and 10 nM GTP in a 96-well Filter plate at room temperature for 40 minutes. The non-specific binding was defined by the addition of 100 µM GTP\textsubscript{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\textsuperscript{TM} Multilabel Counter (Perkin Elmer, Gaithersburg, MD). Since 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 x the difference between the counts of agonist and 10 µM of dopamine–stimulated binding. All assays were performed in triplicates and EC\textsubscript{50} was calculated by nonlinear regression using curve-fitting Prism program (GraphPad Software, San Diego, CA).
RESULTS

Apomorphine exhibited potent binding to D1-like (D1 and D5) and D2-like (D2, D3, and D4) 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 D1, D2, D3, D4, and D5, respectively. The binding studies also demonstrate that apomorphine binds with < 1000 nM affinity (K_i) to α2, 5HT1A, 5HT2C, and 5HT7, and with >1000 nM affinity to other receptors such as α1, β1, β2, H1, κ, μ, 5HT1B, 5HT2A, 5HT3, 5HT5A, 5HT6 (Table 1).

Subcutaneous injections of apomorphine (0.003 – 1 µmol/kg) dose-dependently induced penile erections in conscious rats (Figure 1). The maximum effect of 91 ± 5 % (p<0.001 vs. vehicle control) was seen in rats injected with apomorphine at 0.1 µmol/kg s.c. (Figure 1 upper graph). A significant pro-erectile effect (56 % incidence vs. 22% for vehicle control, p<0.01) was observed at the 0.01 µmol/kg dose. Apomorphine also elicited a dose-dependent enhancement in the number of penile erections. At the most efficacious dose of 0.1 µmol/kg apomorphine produced a mean of 1.5 ± 0.2 erections during the observation period (Figure 1 lower graph). Additional groups of rats received s.c. injections of apomorphine 0.1 µmol/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/hr.kg), in spite of a large apparent volume of distribution (3.5 l/kg). More importantly, pharmacokinetics studies revealed that apomorphine readily crossed the blood brain barrier and that maximal brain levels were reached within 15 minutes after systemic s.c. dosing (Figure 2). At the maximally efficacious dose (0.1 µmol/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 on the pro-erectile effects of apomorphine (Table 2 and Table 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 µmol/kg i.p. significantly inhibited the penile erection facilitated by 0.1 µmol/kg s.c. of 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 pre-administration of clozapine at the doses of 3-10 µmol/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 µmol/kg i.p.) did not block the penile erection facilitated by apomorphine in rats. The penile erection facilitated by apomorphine (0.1µmol/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 nmoles) (Table 3). These results reveal that the pro-erectile 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 not exhibit significant changes in penile activity as compared with spontaneous erection in the vehicle-treated animals.

To determine whether the pro-erectile 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% following i.c.v. injections of 3 nmoles apomorphine (Figure 3, upper graph). In contrast, only 50% maximal incidence was elicited following intrathecal injection of 30 nmol apomorphine (Figure 3, lower graph), 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 non-selective 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 D1 receptor agonist) nor SKF81297 (a full D1 receptor agonist) (Seeman and Van Tol, 1994; Vallone et al., 2000) exhibited any pro-erectile effects. These data suggest that D2–like receptors participate in apomorphine-induced penile erection.

Quinpirole (a nonselective D2-D3-D4 receptor agonist) (Moreland et al., 2003) significantly produced a dose-dependent facilitation of penile erection in rats with maximal incidence of 75% at 0.3 µmol/kg (Table 4). The effect of the selective D2 receptor agonist PNU-95666E (Heier et al., 1997) was investigated in rats at the doses of 0.1, 0.3, 1, and 3 µmol/kg. As shown in Figure 4, the s.c. administration of PNU-95666E did not facilitate penile erection. PNU-95666E has been reported as a centrally acting D2 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 D4 receptor activation in relation to the pro-erectile activity of apomorphine, two D4 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 (Kᵢ) with little difference across the three D4 alleles (Table 5). Apomorphine also showed no difference in binding to the D4 alleles, having potencies in the nM range. The agonist activities of PD168077 and CP226269, determined by measuring GTPᵢS binding activity followed by D4 receptor activation on the cloned human D4.2 cell membranes, are shown in Figure 5. Dopamine induced concentration-dependent increases in GTPᵢS binding (EC₅₀ 7.9 nM). The agonist effect was mimicked by PD168077 (EC₅₀
5.5nM, 88% efficacy), CP226269 (EC\textsubscript{50} 10nM, 69% efficacy), and apomorphine (88% agonist activity with an EC\textsubscript{50} 1.2nM). GTP\textsubscript{γS} binding activity reflects the activation of G-protein-couple receptors, an early event in the cascade of signal transduction following the receptor ligation and has been successfully applied to the stimulation of human dopamine D\textsubscript{4} receptors (Chabert et al., 1994; Newman-Tancredi et al., 1997; Patel et al., 1997).

Administration of PD168077 (0.03 - 1.0 \(\mu\)mol/kg s.c.) or CP226269 (0.03 - 3.0 \(\mu\)mol/kg s.c.) induced dose-dependent penile erections in conscious Wistar rats. The maximum effect of 79 ± 11 % (p< 0.01 vs. 22% for vehicle control) was displayed in rats injected with PD168077 at 0.3 \(\mu\)mol/kg s.c. during a 60 minute observation period (Figure 6, upper graph). A significant pro-erectile effect was also elicited by CP226269 with the maximum effect of 83 ± 10 % (p< 0.001 vs. 17% for vehicle control) at the 1 \(\mu\)mol/kg dose (Figure 6 lower graph). At the most efficacious dose of 0.3 \(\mu\)mol/kg PD168077 and 1.0 \(\mu\)mol/kg CP226269 produced a mean of 1.2 ± 0.3 and 1.0 ± 0.2, respectively, in the number of penile erections during the observation period. The pro-erectile 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\textsubscript{4} receptor agonists directly injected into brain at a dose of 10 nmoles 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, potently facilitates penile erection in conscious rats. The pro-erectile 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 5 dopamine receptor subtypes (Table 1). Functional studies have indicated that apomorphine elicits agonist activity with 80-90% 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 5-HT receptors in penile erection (Andersson, 2001), their potential role in the pro-erectile effect of apomorphine cannot be ruled out at the present time. However, since plasma level of apomorphine at the maximally efficacious dose (0.1 µmol/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 non-dopaminergic 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 pro-erectile effect of
apomorphine is biphasic with low doses facilitating and high doses inhibiting erection after either s.c. or i.c.v. injections (Figures 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 D₂-like receptor blocker that does not penetrate the blood-brain barrier (Barone, 1999). The lack of effect of domperidone further demonstrates that the pro-erectile 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 following 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 (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 pro-erectile 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 (Figure 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 D₁ receptor agonists SKF38393 and SKF81297 nor a D₂ receptor agonist PNU-95666E has any significant pro-erectile effects, whereas quinpirole (a D₂-D₃-D₄ receptor agonist) produces a dose-dependent response in facilitating penile erection. On the other hand, the pretreatment with haloperidol (a D₂-like receptor antagonist) or clozapine (a preferential D₄ receptor antagonist) completely inhibits the penile erection facilitated by 0.1 µmol/kg s.c. of 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.) induce 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 appears to involve particularly the D$_2$-like receptor subtypes, and this seems to be the case also in man (Heaton, 2000; Andersson and Hedlund, 2002). PNU-95666E is a centrally acting agonist that reportedly activates the D$_2$ 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 (Figure 4), elicits full D$_2$ agonism (90%) but is totally devoid of D$_4$ functional activity (Moreland et al., 2003). Quinpirole is a potent agonist that elicits full functional activity at D$_2$, D$_3$, and D$_4$ receptor subtypes with efficacy of 90%, 90%, and 94%, respectively (Moreland et al., 2003). Taken together, the present results suggest that D$_4$ and/or D$_3$ receptors may be responsible for the pro-erectile effects of apomorphine.

Results from in vivo experiments in conscious rats have clearly indicated that penile erection is fully facilitated by two D$_4$ receptor agonists, PD168077 and CP226269, via either systemic administration or locally i.c.v. microinjection (Figure 6 and Figure 7). Functionally in vitro, both agents potently activate D$_4$ receptor with agonist activity on the stimulation of GTP$_{\gamma}$S binding of D$_4$ receptor activation on D$_4$ expressing cell membranes (Figure 5), but have no D$_2$ or D$_3$ activity as determined by the increases in intracellular calcium levels in the cells transfected with cloned human D$_2$ and D$_3$ (Moreland et al., 2003). To our knowledge, this presents the first in vivo evidence of the activation of D$_4$ 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 (Hsieh et al., unpublished data), and all possess potent agonist activity at D₄ (Moreland et al., 2003). While 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 pro-erectile 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 pro-erectile 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. While the ability of apomorphine to activate D₃ receptors in relation to its pro-erectile 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.
REFERENCES


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 percent inhibition (IC50) of an enzymatic reaction. *Biochemical Pharmacology* **22**:3099-3108.


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FIGURE LEGENDS

Figure 1. Pro-erectile 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 minutes post-dosing period. Erection incidence (%) is defined as the % of animals exhibiting one or more erections during the observation period. Data are expressed as incidence (%) ± S.E. or mean of number of penile erection ± S.E.M. (n=30). * p<0.05, **p<0.01, ***p<0.001 vs. vehicle control.

Figure 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 µmol/kg in rats. The concentrations of apomorphine are expressed as ng/ml and ng/g on plasma and brain tissue samples (mean ± S.E.M., n=3), respectively.

Figure 3. Pro-erectile effects of intracerebravalventricular (i.c.v., n=8, upper graph) or intrathecal (i.t., n=12, lower graph) 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 minutes post-dosing period. Data are expressed as erection incidence (%) ± S.E. * p<0.05, **p<0.01, vs. vehicle control.
Figure 4. The selective D₂ agonist PNU-95666E is not pro-erectile in rats. Penile erection was assessed during a 60 minutes post-dosing period in individual observation cage. Data are expressed as erection incidence (%) ± S.E. (n=8).

Figure 5. Agonist–stimulation of GTPγS binding activity by dopamine, apomorphine, PD168077, and CP226269 in recombinant human D₄.2 dopamine receptor. Data for each concentration point were normalized to the maximal effect of dopamine (10 µM) and presented as the mean (± S.E.M.) of the triplicate assays (n=4).

Figure 6. Pro-erectile effects of D₄ 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 minutes post-dosing period. Data are expressed as erection incidence (%) ± S.E. * p<0.05, **p<0.01, ***p<0.001 vs. vehicle control.

Figure 7. Pro-erectile effects of intracerebroventricular (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 minutes post-dosing period. Data are expressed as erection incidence (%) ± S.E. * p<0.05, **p<0.01, vs. vehicle control.
Table 1. Receptor selectivity of apomorphine (radioligand binding studies at CEREP)

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>Kᵢ, nM</th>
<th>Receptor</th>
<th>Ligand</th>
<th>Kᵢ, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>D₁</td>
<td>[³H]-SCH23390</td>
<td>101</td>
<td>κ</td>
<td>[³H]-U69593</td>
<td>5000</td>
</tr>
<tr>
<td>D₂</td>
<td>[³H]-Spiperone</td>
<td>32</td>
<td>μ</td>
<td>[³H]-DAMGO</td>
<td>2000</td>
</tr>
<tr>
<td>D₃</td>
<td>[³H]-Spiperone</td>
<td>26</td>
<td>5HT₁ₐ</td>
<td>[³H]-8-OH-DPAT</td>
<td>121</td>
</tr>
<tr>
<td>D₄</td>
<td>[³H]-Spiperone</td>
<td>2.6</td>
<td>5HT₁₈</td>
<td>[³H]-Cyanopindolol</td>
<td>6000</td>
</tr>
<tr>
<td>D₅</td>
<td>[³H]-SCH23390</td>
<td>10</td>
<td>5HT₂ₐ</td>
<td>[³H]-Ketanserin</td>
<td>1000</td>
</tr>
<tr>
<td>α₁</td>
<td>[³H]-Prazosin</td>
<td>3000</td>
<td>5HT₂₈</td>
<td>[³H]-Mesulergine</td>
<td>200</td>
</tr>
<tr>
<td>α₂</td>
<td>[³H]-RX821002</td>
<td>100</td>
<td>5HT₃</td>
<td>[³H]-BRL43694</td>
<td>5000</td>
</tr>
<tr>
<td>β₁</td>
<td>[³H]-CGP12177</td>
<td>&gt;10000</td>
<td>5HT₅₈</td>
<td>[³H]-LSD</td>
<td>5000</td>
</tr>
<tr>
<td>β₂</td>
<td>[³H]-CGP12177</td>
<td>&gt;10000</td>
<td>5HT₆</td>
<td>[³H]-LSD</td>
<td>2000</td>
</tr>
<tr>
<td>H₁</td>
<td>[³H]-Pyrilamine</td>
<td>&gt;10000</td>
<td>5HT₇</td>
<td>[³H]-LSD</td>
<td>200</td>
</tr>
</tbody>
</table>

* Apomorphine exhibited low affinities (Kᵢ >10000 nM) for more than 50 other neurotransmitters/uptake/enzymes/ion channels as determined by CEREP screening.
Table 2. Effects of dopaminergic antagonists on penile erection facilitated by subcutaneous (s.c.) injection of 0.1 µmol/kg apomorphine in conscious Wistar rats. Penile erection data are expressed as the mean of incidence (%) ± S.E.

<table>
<thead>
<tr>
<th>Treatment†</th>
<th>µmol/kg i.p.</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apomorphine</td>
<td>88 ± 12</td>
<td></td>
</tr>
<tr>
<td>+ haloperidol 0.1</td>
<td>63 ± 17</td>
<td></td>
</tr>
<tr>
<td>+ haloperidol 0.3</td>
<td>13 ± 12**</td>
<td></td>
</tr>
<tr>
<td>Apomorphine</td>
<td>88 ± 12</td>
<td></td>
</tr>
<tr>
<td>+ clozapine 1</td>
<td>50 ± 18</td>
<td></td>
</tr>
<tr>
<td>+ clozapine 3</td>
<td>38 ± 17*</td>
<td></td>
</tr>
<tr>
<td>+ clozapine 10</td>
<td>0 ± 0**</td>
<td></td>
</tr>
<tr>
<td>Apomorphine</td>
<td>100 ± 0</td>
<td></td>
</tr>
<tr>
<td>+ domperidone 3</td>
<td>88 ± 12</td>
<td></td>
</tr>
<tr>
<td>+ domperidone 10</td>
<td>100 ± 0</td>
<td></td>
</tr>
</tbody>
</table>

†Dopaminergic antagonists were administered i.p. 60 minutes before s.c. apomorphine injection (0.1 µmol/kg).

*p<0.05, **p<0.01, as compared to apomorphine control
Table 3. The inhibitory effects of intracerebroventricular (i.c.v) microinjection of haloperidol or clozapine on penile erection facilitated by apomorphine (0.1 µmol/kg s.c.) in conscious Wistar rats. Penile erection was assessed during a 60 minutes post-dosing period. Data are expressed as erection incidence (%) ± S.E. (n=8).

<table>
<thead>
<tr>
<th>Treatment †</th>
<th>µmol/kg i.c.v.</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apomorphine</td>
<td>100±0</td>
<td>100±0</td>
</tr>
<tr>
<td>+ haloperidol</td>
<td>0.001</td>
<td>100±0</td>
</tr>
<tr>
<td>+ haloperidol</td>
<td>0.003</td>
<td>71±17</td>
</tr>
<tr>
<td>+ haloperidol</td>
<td>0.01</td>
<td>43±19**</td>
</tr>
<tr>
<td>+ haloperidol</td>
<td>0.03</td>
<td>29±17**</td>
</tr>
<tr>
<td>+ haloperidol</td>
<td>0.1</td>
<td>14±13***</td>
</tr>
<tr>
<td>Apomorphine</td>
<td>75±15</td>
<td></td>
</tr>
<tr>
<td>+ clozapine</td>
<td>0.01</td>
<td>57±19</td>
</tr>
<tr>
<td>+ clozapine</td>
<td>0.03</td>
<td>57±19</td>
</tr>
<tr>
<td>+ clozapine</td>
<td>0.1</td>
<td>14±13**</td>
</tr>
</tbody>
</table>

†Dopaminergic antagonists were administered i.p. 15 minutes before s.c. apomorphine injection (0.1 µmol/kg).

*p<0.05, **p<0.01, ***p<0.001, as compared to apomorphine control.
Table 4. Pro-erectile effects of dopaminergic agonists in conscious Wistar rats after a single subcutaneous (s.c.) injection. Penile erection was assessed during a 60 minutes post-dosing period. Data are expressed as the mean incidence (%) ± S.E.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>µmol/kg s.c.</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKF81297</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vehicle</td>
<td></td>
<td>13 ± 12</td>
</tr>
<tr>
<td>0.3</td>
<td></td>
<td>14 ± 13</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>25 ± 15</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>SKF38393</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vehicle</td>
<td></td>
<td>14 ± 13</td>
</tr>
<tr>
<td>0.3</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>12 ± 12</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>38 ± 17</td>
</tr>
<tr>
<td>Quinpirole</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vehicle</td>
<td></td>
<td>14 ± 13</td>
</tr>
<tr>
<td>0.1</td>
<td></td>
<td>38 ± 17</td>
</tr>
<tr>
<td>0.3</td>
<td></td>
<td>75 ± 15*</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>25 ± 15</td>
</tr>
</tbody>
</table>

*p<0.05, as compared to vehicle control
Table 5. Radioligand binding affinity (Ki, nM) for PD168077, CP226269, and apomorphine on recombinant human D₄ variants hD₄.2, hD₄.4, and hD₄.7.

<table>
<thead>
<tr>
<th>Compound</th>
<th>hD₄.2</th>
<th>hD₄.4</th>
<th>hD₄.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apomorphine</td>
<td>8.1 ± 0.4</td>
<td>9.6 ± 0.6</td>
<td>9.3 ± 0.7</td>
</tr>
<tr>
<td>PD168077</td>
<td>6.0 ± 2.8</td>
<td>22.3 ± 0.1</td>
<td>29.0 ± 1.3</td>
</tr>
<tr>
<td>CP226269</td>
<td>2.4 ± 0.7</td>
<td>3.6 ± 0.1</td>
<td>5.6 ± 0.9</td>
</tr>
</tbody>
</table>

Competition binding with [³H] spiperone for dopaminergic receptor agonists.

Data are presented as mean ± S.E.M. (n=4)
Hsieh et al., Figure 1

Incidence (%)

Penile erection

Apomorphine, µmol/kg s.c.

Apomorphine, µmol/kg s.c.
Hsieh et al., Figure 2
Hsieh et al., Figure 3
Hsieh et al., Figure 4
Hsieh et al., Figure 5
Hsieh et al., Figure 6

**Figure Caption:**

The figure shows the incidence (%) of a particular trait for two different compounds, PD168077 and CP226269, at various concentrations given s.c. (subcutaneously). The x-axis represents the concentration of each compound (in µmol/kg), while the y-axis represents the incidence percentage. Doses of 0, 0.03, 0.1, 0.3, 1, and 3 µmol/kg were tested for each compound. The bars are accompanied by statistical symbols indicating significance: ***(p < 0.001)***, ***(p < 0.01)***, ***(p < 0.05)***. The error bars indicate the standard error of the mean (SEM).
Hsieh et al., Figure 7