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 $\mu$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 significantly induced by quinpirole ($D_2-D_3-D_4$ receptor agonist), but not by $R(+)1$-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine-7,8-diol (SKF83939) and $R(+)1$-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine (SKF81297) ($D_1$ receptor agonists), or a $D_2$ receptor agonist $R$-5,6-dihydro-N,N-dimethyl-4H-imidazo[4,5,1-ij]quinolin-5-amine (PNU-95666). The role of $D_3$ receptors in penile erection was demonstrated using selective $D_3$ receptor agonists [4-(phenylpiperazinyl) methyl]benzamide (PD168077) and 5-fluoro-2-[(4-(2-pyridinyl)-1-piperazinyl)methyl]-1H-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

ABBREVIATIONS: ED, erectile dysfunction; CNS, central nervous system; MPOA, medial preoptic area; PVN, paraventricular nucleus; PE, polyethylene; CHO, Chinese hamster ovary; GTPyS, guanosine 5′-O-(3-thio)triphosphate; 5HT, 5-hydroxytryptamine; SKF83939, $R(+)1$-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine-7,8-diol; SKF81297, $R(+)1$-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine; PNU-95666, $R$-5,6-dihydro-N,N-dimethyl-4H-imidazo[4,5,1-ij]quinolin-5-amine; PD168077, [4-(phenylpiperazinyl)methyl]benzamide; CP226269, 5-fluoro-2-[(4-(2-pyridinyl)-1-piperazinyl)methyl]-1H-indole; BP897, 1-4-2-naphthoylaminobutyl-4-2-methoxyphenyl-1A-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; Anderson 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 erekctogenic 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.e.v.) Injection.** Anesthetized rats (pentobarbital sodium 50 mg/kg i.p.) were placed into stereotactic 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 (stereotactic 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 stereotactic 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 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 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 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 × 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.e.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 i.p. or i.e.v. with various dopamine receptor antagonists such as haloperidol (D₂-like), clozapine (D₂ 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 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) ± S.E. calculated by using Wald equation. Statistical evaluation of the results was performed by χ² 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) 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 μm, 2.1 × 150 mm) (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/MD Sciex, Foster City, CA) in the multiple reaction monitoring 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 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/g and 0.4 ng/g on the plasma and brain tissue samples, respectively.

**Radioligand Binding Assay.** Membranes containing recombinant human D₃ dopamine receptors (D₃.2, D₃.4, and D₃.7 variants) transfected into CHO cells were obtained from Receptor Biology (Beltsville, MD). Membranes suspended in 10 mM Tris-Cl, 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]apomorphine (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]apomorphine was 0.2 nM. Nonspecific binding was determined in the presence of 10 μM haloperidol. The incubation buffer consisted of 5 mM Tris-Cl, 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-methoxanthine 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). Filters were washed three times with 700 μL ice-cold 50 mM Tris-Cl 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 IC₅₀ values converted to Kᵢ values (Cheng and Prusoff, 1973).

**Agonist-Stimulated GTP-γS Binding Assay.** Agonist activity was measured by GTP-binding assay using a DELFIA GTP-binding kit (PerkinElmer Life Sciences). Membranes containing recombinant human D₂ dopamine receptors (D₂.3 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 MgCl₂, 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 min. The nonspecific binding was defined by the addition of 100 μM GTPγS. 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 × the difference between the counts of agonist and 10 μM of dopamine-stimulated binding. All assays were performed in triplicate, and EC₅₀ values were calculated by nonlinear regression using curve-fitting Prism program (GraphPad Software Inc., San Diego, CA).

### Results

Apomorphine exhibited potent binding to D₁-like (D₁ and D₃) and D₂-like (D₂, D₃, and D₄) receptor subtypes when examined in more than 70 neurotransmitter receptors/uptake/enzymes/ion channels (Table 1). The affinity (Kᵢ) 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 (Kᵢ) to α₂, 5HT₁A, 5HT₂C, and 5HT₇, and with >1000 nM affinity to other receptors such as α₁, β₁, β₂, H₁, κ, µ, 5HT₁B, 5HT₂A, 5HT₃A, and 5HT₆ (Table 1).

Subcutaneous injections of apomorphine (0.003–1 μmol/kg) dose dependently induced penile erections in conscious rats (Fig. 1). The maximum effect of 91 ± 5% (p < 0.001 versus vehicle control) was seen in rats injected with apomorphine at 0.1 μmol/kg s.c. (Fig. 1, top). A significant proerectic effect (56% incidence versus 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 (Fig. 1, bottom). 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 t1/2 (0.2 h) after i.v. administration with a high total plasma clearance (10.4 l/h · 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 min after systemic s.c. dosing (Fig. 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 proerectile effects of apomorphine (Tables 2 and 3). Haloperidol is a dopaminergic receptor antagonist (Kᵩ = 80, 1.2, 7, 2.3, and 100 nM for D₁, D₂, D₃, D₄, and D₅ 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. apomorphine. Clozapine is a preferential D₄ receptor antagonist (Kᵩ = 170, 230, 170, 21, and 330 nM for D₁, D₂, D₃, D₄, and D₅ subtype, respectively) (Seeman and Van Tol, 1994). The preadministration of clozapine at the doses of 3 to 10 µmol/kg i.p. significantly blocked the penile activity induced by apomorphine. Domperidone is a D₂-like receptor antagonist (Kᵩ = 0.4, 9.5, and 30.4 nM for D₂, D₃, and D₄ 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 (0.1 µmol/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 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 D₁ receptor agonist) nor SKF81297 (a full D₁ receptor agonist) (Seeman and Van Tol, 1994; Vallone et al., 2000) exhibited any proerectile effects. These data suggest that D₁-like receptors participate in apomorphine-induced penile erection.

Quinpirole (a nonselective D₂-D₃-D₄ receptor agonist) (Moreland et al., 2003) significantly produced a dose-depen-

### TABLE 3

Inhibitory effects of 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-min post-dosing period. Data are expressed as erection incidence (%) ± S.E. (n = 8).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haloperidol</td>
<td>0.001</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>Clozapine</td>
<td>0.03</td>
<td>29 ± 17**</td>
</tr>
<tr>
<td>Apomorphine</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>0.003</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>+ 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>0.01</td>
<td>14 ± 13**</td>
</tr>
</tbody>
</table>

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

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

### 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 (%) ± S.E.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKF81297</td>
<td>Vehicle</td>
<td>13 ± 12</td>
</tr>
<tr>
<td>SKF83393</td>
<td>Vehicle</td>
<td>14 ± 13</td>
</tr>
<tr>
<td>Quinpirole</td>
<td>Vehicle</td>
<td>14 ± 13</td>
</tr>
<tr>
<td>SKF81297</td>
<td>0.3</td>
<td>14 ± 13</td>
</tr>
<tr>
<td>SKF83393</td>
<td>0.3</td>
<td>14 ± 13</td>
</tr>
<tr>
<td>Quinpirole</td>
<td>0.3</td>
<td>14 ± 13</td>
</tr>
<tr>
<td>SKF81297</td>
<td>0.1</td>
<td>38 ± 17</td>
</tr>
<tr>
<td>SKF83393</td>
<td>0.1</td>
<td>38 ± 17</td>
</tr>
<tr>
<td>Quinpirole</td>
<td>0.3</td>
<td>75 ± 15*</td>
</tr>
<tr>
<td>SKF81297</td>
<td>1</td>
<td>25 ± 15</td>
</tr>
</tbody>
</table>

*p < 0.05, compared with vehicle control.
dent facilitation of penile erection in rats with maximal incidence of 75% at 0.3 \( \mu \text{mol/kg} \) (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 \( \mu \text{mol/kg} \). 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 \(^{[3]H}\)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\(_S\) binding activity followed by D_4 receptor activation on the cloned human D_{1.4} cell membranes, are shown in Fig. 5. Dopamine induced concentration-dependent increases in GTP\(_S\) 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\(_S\) 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 \( \mu \text{mol/kg s.c.} \)) or CP226269 (0.03–3.0 \( \mu \text{mol/kg s.c.} \)) 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 \( \mu \text{mol/kg s.c.} \) during a 60-min observation period (Data are expressed as erection incidence (percentage) \( \pm \) S.E. * \( p < 0.05; ** \), \( p < 0.01; *** \), \( p < 0.001 \) versus vehicle control.

**Discussion**

The present study demonstrates that apomorphine, a non-selective dopaminergic receptor agonist, potently facilitates...
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 D₄ receptors, but it is not mimicked by the injections of a selective D₂ 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 D₂, D₃, and D₄ receptors (Moreland et al., 2003). Affinity of apomorphine for several other selected receptors such as α₉, 5HT₁A, and 5HT₂C is comparably weaker (Kᵢ > 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 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 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 D₂-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 D₁ receptor agonists SKF38393 and SKF81297 nor a D₂ receptor agonist PNU-95666E has any significant proerectile effects, whereas quinpirole (a D₂-like 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. apomorphine.
The lack of effect of D₃ 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 D₃-like receptor antagonist SCH-23390 (D’Aquila et al., 2003). However, our findings are in agreement with previous data that D₃ 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 Heddlund, 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 (Durham et al., 1997; Heier et al., 1997; Sethy et al., 1997). PNU-95666E, which does not induce penile erection (Fig. 4), elicits full D₃ agonism (90%) but is totally devoid of D₂ 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 D₃ and/or D₄ 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 D₃ receptor agonists, PD168077 and CP226269, via either systemic administration or locally i.c.v. microinjection (Figs. 6 and 7). Functionally, in vitro, both agents potentely activate D₃ receptor with agonist activity on the stimulation of GTPγS binding of D₃ receptor activation on D₃-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 D₃ 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 D₃ (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 D₃ 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 D₃ receptor agonist PNU-95666E, which does not significantly induce penile erection, also exhibits 79% functional efficacy at D₃ but is totally devoid of D₂ 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 D₃ 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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