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Modulation of brain delivery and copulation by intranasal apomorphine hydrochloride

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Abstract

Clinical studies showed apomorphine (APO) nasal spray was well tolerated, with lower dose and less side effect in treatment of erectile dysfunction compared with its sublingual formulation. The aim of this paper was to find out whether there exists any direct drug transport from nasal cavity to brain following intranasal administration to rats. Pharmacokinetic results illustrated there were no significant differences of $AUC_{0\rightarrow120}$ values in most brain regions and cerebrospinal fluid (CSF) through intranasal delivery route compared with subcutaneous injection, while its plasma $AUC_{0\rightarrow120}$ was only one-half. APO brain and CSF profiles after intranasal administration displayed faster onset compared with subcutaneous delivery. About 35–50% of APO content at 2 h, by calculating brain drug direct transport percentage, were transported to different brain regions via the olfactory pathway. In addition, the similar brain drug concentration–time profiles through intranasal delivery compared with subcutaneous route had good correlation with its equivalent sexual stimulant activity on copulatory behaviour in rats. Therefore, we could conclude a nose-to-brain pathway for APO intranasal delivery, which significantly increased brain accumulation of APO. Current experiments also explained the reason why the intranasal application of APO could be an effective alternative to subcutaneous and oral formulations.

Keywords: Apomorphine; Intranasal delivery; Brain distribution; Copulatory behaviour

1. Introduction

Erectile dysfunction (ED) is a common medical condition that affects the sexual life of millions of men worldwide. Compared with those phosphodiesterase type 5 inhibitors such as sildenafil, tadalafil and vardenafil, sublingual apomorphine (Uprima™) is a centrally acting agent licensed for the treatment of ED in most European and South American countries (Kalsi and Kell, 2004).

Apomorphine (APO) is a dopamine D_1 and D_2 receptor agonist, which has been used in the treatment of a variety of medical conditions, including Parkinson's disease. The dopaminergic properties of APO were first recognized in the 1960s, when it was successfully used to suppress refractory motor oscillations in Parkinson's disease (Cotzias et al., 1970). Furthermore, through activation of dopamine D_1 and D_2 postsynaptic receptors which are mainly located in the paraventricular nucleus of the hypothalamus, APO has been shown to be effective in eliciting penile erection in both rat and human models (Lal, 1988; Elabbady et al., 1995; Mas et al., 1995). The involvement of dopaminergic mechanisms in the regulation of masculine sexual behaviour, as well as other motivational processes, has long been suggested by a wealth of pharmacological studies (Melis et al., 1989; Mas et al., 1995).

Because of severe first-pass metabolism, oral delivery of APO appears to be virtually ineffective, with only 1–2% of the activity seen after intravenous or subcutaneous administration being observed (Montorsi et al., 2003; Briganti et al., 2006). The bioavailability of sublingual APO is estimated to be 16–18%, as it is rapidly absorbed and can avoid the first-pass metabolism (Argiolas and Hedlund, 2001). However, as a marked increase in the prevalence of nausea (14.1% of incidence) was evident in the group treated with sublingual APO 4 mg per patient, the patient should always start at the 2 or 3 mg dose per patient followed by

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a dose-optimization schedule (Dula et al., 2001; Heaton et al., 2002).

In recent years, systemic drug delivery through nasal route has received a lot of attention, because it offers some advantages including rapid absorption, avoidance of hepatic first-pass metabolism, and the preferential drug delivery to brain via the olfactory region (Behl et al., 1998; Illum, 2000). Therefore, the nasal delivery of centrally acting APO may provide a better alternative to oral and subcutaneous administration (Ikechukwu Ugwoke et al., 1999, 2000; Ugwoke et al., 1999).

Recently, results of a randomized, double-blind, placebo-controlled study of 122 men with ED demonstrated that intranasal APO 0.5 mg administered 15–20 min before sexual intercourse was associated with successful sexual intercourse 80% of the time. Furthermore, even the dose up to 1.75 mg of nasal spray APO were well tolerated, without any reported cardiovascular or syncope event or nausea (Kendirci and Hellstrom, 2004). Because of its intranasal delivery, which avoids first-pass metabolism, intranasal APO was supposed unlikely to interact with food or any other drug (Kendirci and Hellstrom, 2004). However, no detailed data were reported to explain the brain delivery mechanisms of intranasal APO.

The aim of this paper was to investigate the concentration profiles of APO in plasma, cerebrospinal fluid (CSF) and different brain tissue after intranasal (i.n.) delivery compared with subcutaneous (s.c.) administration in rats to elucidate the degree of drug targeting to brain following i.n. route. Consequently, we could find out whether there exists a direct nose–brain pathway into brain and CSF. Finally, a modulatory activity of i.n. APO on copulatory behaviour was tested to confirm this mechanism in rats.

2. Materials and methods

2.1. Materials

Apomorphine hydrochloride (APO·HCl) was purchased from Qinghai Pharmaceutical Ltd. (Xining, China). Pipemidic acid was obtained from Shanghai FDA (China). Double distilled water was purified using a Millipore Simplicity System (Millipore, Bedford, MA, USA). HPLC grade acetonitrile was purchased from Shanghai Chemical Reagents Research Institute (China). All other chemicals were of commercially analytical grade.

2.2. Animals

Adult male and female Sprague–Dawley rats (Shanghai Slac Laboratory Animal Co. Ltd., Shanghai, China) weighing 250–300 g were housed in groups of four, of the same sex, with food and water *ad libitum* and on a reversed 12 h light cycle, from 7 a.m. to 7 p.m., for at least 2 weeks prior to the start of the experiments. For evaluation of copulatory behaviour, the experiments were performed between 9 p.m. and 1 a.m. in a sound-attenuated, air-conditioned room (under red light), where the animals were monitored by trained observers unaware of the experimental design, the controls being handled in the same way as the treated animals. Animals were treated according to protocols approved by the ethical committees of Fudan University and Shanghai Jiaotong University.

2.3. Formulation

APO·HCl nasal spray (10 mg/mL) was prepared by the following procedures: 100 mg APO·HCl raw drug was dissolved in excipient solution containing 0.2% sodium pyrosulfite (reductant), 0.01% EDTA-2Na (chelating reagent), 0.01% chlorhexidine acetate (antiseptic) and 1.2% Na_2HPO_4. The solution was adjusted to pH 4.5 by adding 2 mol/L H_3PO_4 followed by adjusting the final volume of 10 mL. APO·HCl subcutaneous injection was formulated by dissolving the drug into 0.9% NaCl immediately before the administration and sterilized by passing through 0.22 μm filter (Millipore, Bedford, MA, USA).

2.4. Nasal ciliotoxicity

Nasal ciliotoxicity studies were carried out using *in situ* toad palate model (Jiang et al., 1995). In brief, the upper palate of toads (30–40 g, Experimental Animal Center of Fudan University, China) were randomly divided into four groups ($n = 5$) and exposed with the following samples for 1 h: (a) APO·HCl nasospray solution (10 mg/mL), (b) APO·HCl nasospray solution without the drug (excipient), (c) saline (negative control) and (d) 1% (w/v) sodium deoxycholate solution (a severe nasal mucociliary toxicity agent, positive control). The volume of sample solution was ensured much enough to cover the application site during the whole experiment. Afterwards, the palate was rinsed with saline and dissected. The mucocilia was examined with an optical microscope (Nikon Fx-35A, Japan), and *in vitro* lasting time (LT) of ciliary movement was measured. The relative percentage of ciliary movement was calculated by dividing the LT of ciliary movement of different sample by that of saline group. The higher value the percentage LT is, the lower ciliotoxicity the sample is regarded as (Jiang et al., 1995). For *in vivo* evaluation, SD rats were divided into four groups ($n = 5$), which were intranasally administered to left nostril with the same testing solutions as those in toad palate model experiment at a volume of 40 μL/day for 7 successive days (Hsieh, 1994; Zhang et al., 2001). The rats were sacrificed 24 h after the last dosing, with the nasal mucosa peeled off. The mucocilia samples were prepared routinely and examined under a scanning electronic microscope (S-520, Hitachi, Japan).

2.5. Pharmacokinetic study of plasma and CSF

The experimental procedure of serial CSF sampling with cistern puncture was performed according to the literature with some adaptations (Van den Berg et al., 2002; Shi et al., 2005). Briefly, rats were anesthetized with an intraperitoneal dose of 1% (w/v) sodium pentobarbital (45 mg/kg) and fixed onto a stereotaxic apparatus (Jiangwan I-C, Shanghai, China). The skin overlying the occipital bone was incised and then the underlying muscle and tissue were bluntly dissected so that the atlanto-occipital (a-o) membrane could be exposed. A 25-gauge needle

connected with a 5 cm PE-10 tube was punctured into the bottom part of a-o membrane, which CSF could flow into the PE tube. The mucilage was used to fasten the needle. Afterwards, the rat was placed in the supine-90° position in order to let CSF drip out of the tube by gravity. Before drug administration, a clamp was used to close the tube.

After tying off the esophagus, a PE-10 tube connected with a microsyringe was inserted into the right nostril of rats to administer the nasal formulation at a dose of 5 mg/kg. Another group of rats were received subcutaneous injection of the same dose of drug solution. Each group contained five rats. CSF and blood sample were collected at the predetermined time points 5, 10, 20, 30, 45, 60, 80, 100 and 120 min. The blood sample taken from tail vein was placed into a heparinized PE conical tube, then centrifuged for 10 min at 3000 rpm for at least 100 μL plasma. The CSF sample of 30 μL was collected in the PE tube without heparin and stored at −20 °C.

2.6. Brain drug distribution

The animals were received either i.n. or s.c. administration of 5 mg/kg APO formulations and decapitated at each time points described above. The skull was cut open and the olfactory bulb (OB), olfactory tract (OT), corpus striatum (CS), hippocampus (HC), cerebellum (CL) and cerebrum (CR) were carefully excised. Each brain tissue was quickly rinsed with saline and blotted up with filter paper to get rid of blood-taint and visible blood vessels as much as possible, storing in a deep freezer (−70 °C). Five rats were repeated for each time point data collected.

2.7. Analytical procedure

The analytical procedure of APO was modified according to previous report (Priston and Sewell, 1996). After weighing, the brain tissue samples were homogenized with saline solution with the identical weight. Twenty microliters of pipemidic acid (1 μg/mL in methanol, internal standard) was added into 0.1 mg of brain homogenate or 100 μL of plasma sample, and extracted with 1 mL extraction solvent, dichlormethane, vortexing for 1.5 min. The dichlormethane was transferred to a conical tube, followed by mixing with 0.2 mL of HCl (0.1 mol/L). After vortexing for 1 min, the supernatant was transferred for HPLC analysis. The standard was prepared by adding known amount of APO·HCl into blank sample, run at the same time and treated in a similar way as samples. CSF samples were centrifuged at 14,000 rpm for 5 min and 20 μL supernatant was injected into the HPLC system directly.

Chromatographic separation was achieved on a 4.6 mm × 200 mm, C_{18} analytical column (5 μm, Diamonsil™, Dikma) attached to a guard column (Nova-Pak, 10 μm, C_{18} 15220, Waters) at 30 °C. The HPLC system (Shimadzu Scientific Instrument Inc., Japan) consisted of a pump (LC-10ATVP) and a fluorescence detector (RF-10AXL, Ex 270 nm/Em 450 nm). The mobile phase was 15% acetonitrile/85% 20 mmol/L NH_4Ac (pH 3.2) containing 1‰ triethylamine (v/v) and 1 mmol/L EDTA-2Na at flow rate of 1.5 mL/min for plasma and brain tissue extractions and 0.8 mL/min for CSF samples. The retention time was 6.1 min for APO and 3.0 min for the internal standard.

Results obtained from the HPLC analyses were plotted as drug concentration vs. time curves in brain tissues, CSF or plasma. The AUC values for each curve were calculated from the time zero to the last data point using the stand trapezoidal method without extrapolation to infinity. Statistical differences between i.n. and s.c. administration were concluded using the unpaired Student's *t*-test and a value of $P < 0.05$ was considered statistically significant. Results are presented as mean values ± S.D.

To evaluate the brain targeting after nasal dosing, two indexes were adopted:

(1) According to Hunt et al. (1986) and Wang et al. (2003), the degree of APO targeting to different brain tissues or CSF after intranasal administration can be evaluated by the drug targeting index (DTI), which was described as the ratio of the value of AUC_{brain}/AUC_{plasma} or AUC_{CSF}/AUC_{plasma} following intranasal administration to that following subcutaneous injection. The higher the DTI is, the further degree of APO targeting to brain or CSF can be expected after intranasal administration.

(2) It is believed that drug uptake into brain from the nasal mucosa via two different pathways. One is systemic pathway that some of the drug is absorbed into the systemic circulation and subsequently reaches the brain by crossing blood–brain barrier (BBB). The other is olfactory pathway that partial drug can travel from the olfactory region in the nasal cavity directly into CSF and brain tissue (Illum, 2000). We supposed that the amount of drug in different brain regions after nasal application attributes to these two parts. The term of "brain drug direct transport percentage (DTP)" was used to clarify nose–brain direct transport (Zhang et al., 2004) in our lab, which was based on the following equations:

$$\text{Hypothesis}: \frac{B_{\text{s.c.}}}{P_{\text{s.c.}}} = \frac{B_x}{P_{\text{i.n.}}} \quad (1)$$

$$\text{DTP}\% = \frac{B_{\text{i.n.}} - B_x}{B_{\text{i.n.}}} \times 100\% \quad (2)$$

where $P_{\text{s.c.}}$, $B_{\text{s.c.}}$, $P_{\text{i.n.}}$, $B_{\text{i.n.}}$, denote the AUC of APO in plasma and different brain regions that obtained after s.c. and i.n. administration, respectively. B_x represents the brain AUC fraction contributed by systemic pathway after nasal dosing. Since a linear pharmacokinetics in APO was reported (Aymard et al., 2003), B_x was proportional to plasma AUC. The value of B_x divided by plasma AUC from nasal route is equal to that of $B_{\text{s.c.}}$ divided by $P_{\text{s.c.}}$ via s.c. route as (see Eq. (1)). Therefore, the value of $B_{\text{i.n.}}$ subtracted by B_x represents the brain AUC fraction contributed by olfactory pathway after nasal dosing. DTP in different brain regions represents the drug amount directly transported to that brain region via olfactory pathway accounts for the percentage of the total amount of drug uptake in that region from the nasal mucosa (both olfactory and systemic pathway).

2.8. Copulatory behaviour in rats

2.8.1. Establishment oestrus female model of rat

All female rats were brought into oestrus with estradiol benzoate (30 mg/rat, s.c.), followed 48 h later by progesterone (0.5 mg/rat, s.c.), and used 4–5 h thereafter. Before the copulatory tests, they were screened with sexually experienced males; only those exhibiting good receptivity (solicitation behaviour and lordosis in response to mounting) and no rejection were used according to literature (Ottani et al., 2002).

2.8.2. Evaluation of first copulatory behaviour of normal male rats

Fifty male rats were transferred singly to an observation cage (55 cm × 35 cm × 20 cm). The rats were divided into five groups ($n = 10$), after a 5-min adaptation period, followed by treated as follows: (a) saline (0.2 mL/kg, i.n.), (b) APO·HCl solution (200 μg/kg, s.c.), (c) APO·HCl nasospray (low dose, 100 μg/kg, i.n.), (d) APO·HCl nasospray (medium dose, 200 μg/kg, i.n.), or (e) APO·HCl nasospray (high dose, 400 μg/kg, i.n.). Fifteen minutes later, a receptive female was introduced (totally 50). Male copulatory behaviour was evaluated as in previous works (Dewsbury, 1972; Giuliani and Ferrari, 1996). The parameters considered were mount latencies (ML: the time from the introduction of the female until the first mount); ejaculation latency (EL: the interval between the first intromission and ejaculation); mount and intromission frequencies (MF and IF: the number of mounts and intromissions preceding the first ejaculation, respectively). Those that never mounted or intromitted during these tests were discarded. Those with intromission latencies (IL: the time from the introduction of the female until the first intromission) or post-ejaculatory interval (PEI: the time between the first ejaculation and the next mount or intromission) were >15 min or EL was >30 min were discarded (Ottani et al., 2002).

2.8.3. Evaluation of male sexual behaviour of different copulatory patterns

Another 80 male rats and 70 females were used for the following experiments. After having verified the consistency of the rats' copulatory pattern in the sixth and seventh tests, the animals were categorized as sluggish ejaculators (SE) or normal ejaculators (NE), on the basis of the following calculated indexes: copulatory efficacy (CE) as IF/(MF + IF), and interintromission interval (III) as EL/IF (Clark et al., 1987; Ottani et al., 2002). Arbitrarily, rats were considered SE when CE was <0.5 and III >35. About 90% of the males fulfilled both indexes, which can be admitted to the experiments.

SE ($n = 35$) and NE ($n = 35$) rats were divided into five subgroups ($n = 7$), which were treated with saline, APO·HCl solution (s.c.) or APO·HCl nasospray (low, medium or high doses, i.n.) same doses as those in experiment of first copulatory behaviour evaluation, 15 min before the eighth test. The following parameters were recorded: ML, IL, MF, IF, EL and PEI to calculate CE and III. Data for the parameters of sexual behaviour are the means ± S.D. of the values recorded in the animals in the eighth test, after treatment. Significant difference was calculated by ANOVA followed by Student–Newman–Keul's test.

3. Result

3.1. Nasal mucociliary toxicity evaluation

The optical microscopic results showed that there were a great number of cilia at a fast beating rate on the edge of the mucosa treated with APO·HCl nasospray or excipient vehicle for 1 h, indicating that both the drug and excipient showed no obvious effect on the cilia movement. The cilia beating of saline group lasted for 1119 ± 63 min after the palate was dissected, while those treated with excipient vehicle and APO·HCl nasospray were 921 ± 111 (82.38 ± 9.92% vs. control) and 802 ± 121 min (71.75 ± 10.82% vs. control), respectively. The positive control was 76 ± 14 min (6.82 ± 1.22% vs. control). According to our previous report which the formulation generally exhibits mild mucociliary toxicity with criteria score more than 60% (Jiang et al., 1995), the APO·HCl nasospray showed no severe toxicity.

By *in vivo* evaluation, the scanning electron microscopic results displayed that the cilia were bushy and intact but unorderly on the surface of the mucosa after the successive administration of APO·HCl nasospray or excipient vehicle for 7 days compared with saline control (Fig. 1), indicating that they also presented no marked effect on the length and density of the cilia. However, the positive control showed that nearly no cilia on the mucosa were observed but a few exfoliated ones. Therefore, both APO·HCl nasospray and excipient vehicle possess no severe nasal ciliotoxicity and irritability.

3.2. Validation of plasma, CSF and brain tissues APO assay

Selectivity and specificity were found for the determination of drugs in plasma, CSF and brain tissue samples (Fig. 2). No endogenous sources of interference were observed at the retention time of the analyte and internal standard. The detection limit of APO in those three kinds of samples were all 0.05 ng (S/N = 3). The linearity was observed over the concentration range 5–1000 ng/mL plasma ($r = 0.9997$), 5–1000 ng/mL brain tissues ($r = 0.9999$) and 5–100 ng/mL CSF ($r = 0.9999$). The method recovery of APO fell into 96.45–100.88% (plasma), 97.08–100.38% (brain tissues) and 97.09–103.74% (CSF) when low, medium and high APO within the linearity was added. The extraction recovery of APO ranged from 75.46 to 82.36% in plasma and brain tissue samples at APO concentration within the linearity. The Relative standard deviation (R.S.D.) of APO ranged from 4.67 to 5.01% (plasma), from 3.95 to 5.92% (brain tissue) and from 1.31 to 2.91% (CSF) for inter-day precision, respectively. The R.S.D. of APO ranged from 2.39 to 4.58% (plasma), from 3.25 to 4.16% (brain tissue) and from 2.35 to 4.20% (CSF) for intra-day precision, respectively.

3.3. Pharmacokinetics of APO in plasma and CSF

APO concentration profiles in plasma and CSF following s.c. or i.n. administration are shown in Fig. 3. It was found that the APO levels in plasma of nasal route were significantly lower than those obtained after s.c. injection, which was nearly one-half (337.79 ± 19.33 ng/mL vs. 659.64 ± 25.86 ng/mL). However,

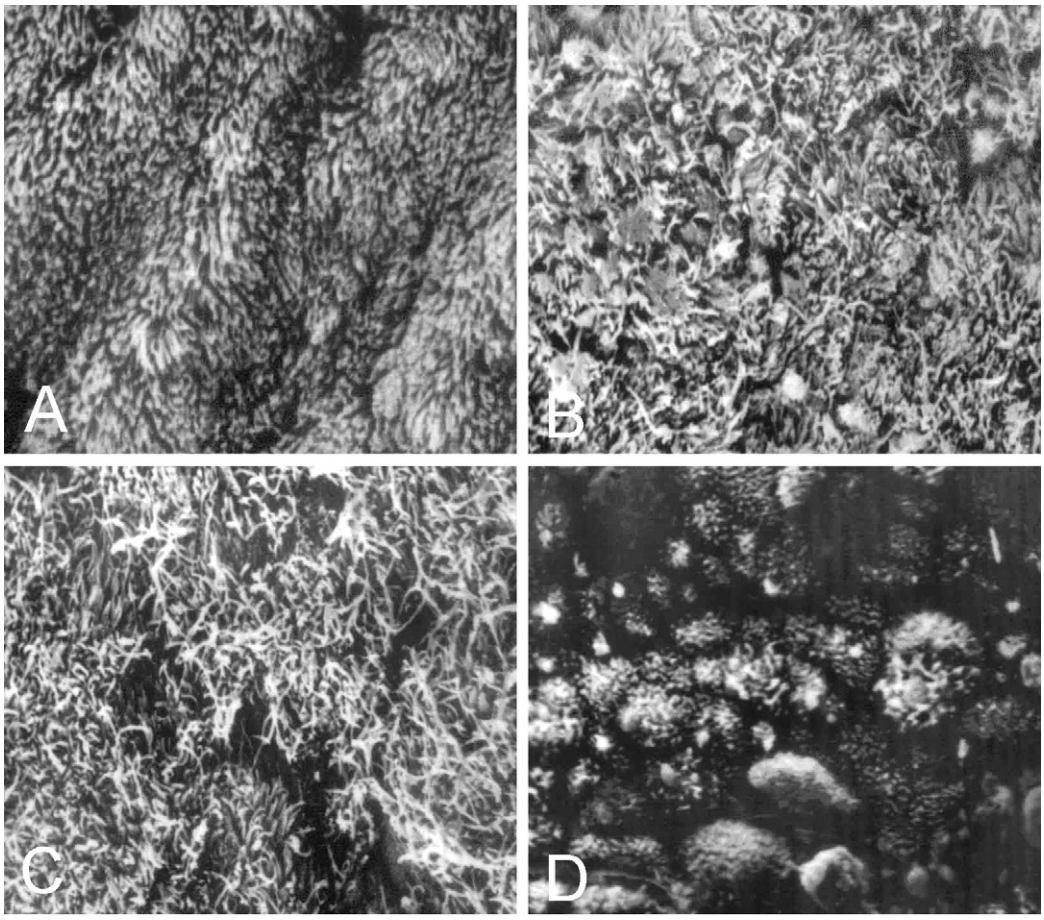

Fig. 1. Scanning electron micrographs of the rat nasal mucosa after nasal administration. (A) Saline; (B) excipient vehicle; (C) APO·HCl nasospray solution (10 mg/mL); (D) 1% sodium deoxycholate solution for 7 consecutive days (×2000).

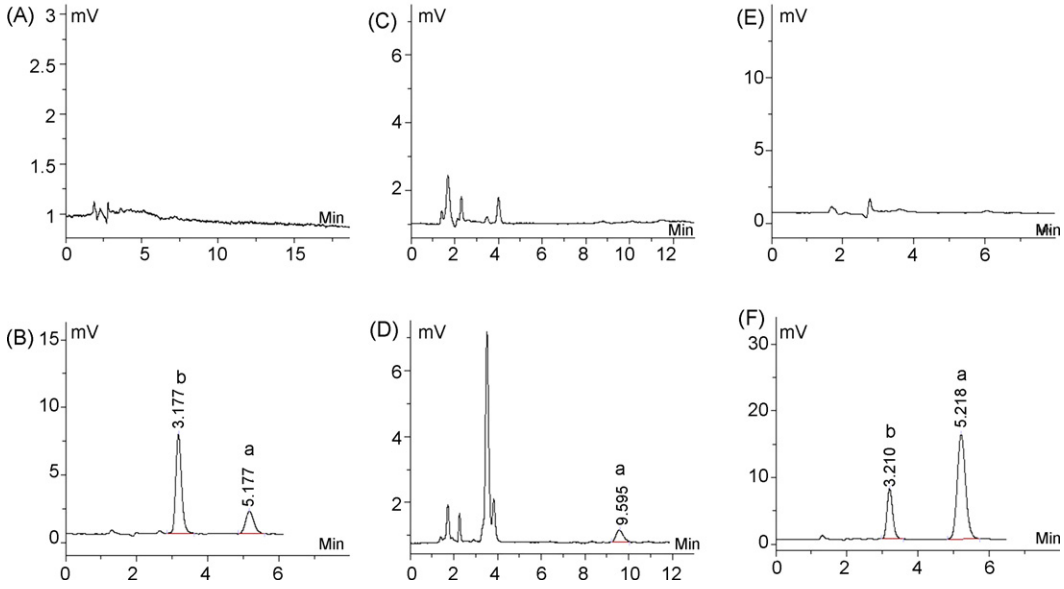

Fig. 2. HPLC chromatograms for the analysis of APO: (A) blank plasma; (B) plasma sample; (C) blank CSF; (D) CSF sample; (E) blank brain tissue; (F) brain tissue samples. Peak (a) for APO and (b) for the internal standard, pipemidic acid.

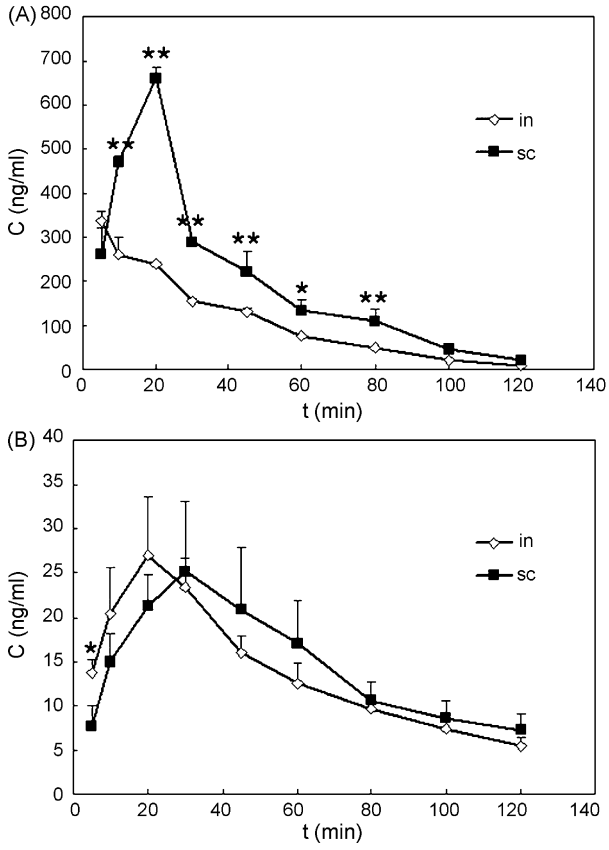

Fig. 3. APO concentrations in plasma (A) and CSF (B) following s.c. or i.n. administration (dose: 5 mg/kg). Data represent the mean ± S.D. ($n = 5$). Any significant difference at each time point between two routes was calculated according to the Student's t-test: $^{*}P < 0.05$; $^{**}P < 0.01$.

the nasal drug delivery route can reach a rather faster plasma peak time of the drug compared with s.c. administration (5 min vs. 20 min). Following i.n. administration, the APO levels in CSF at the initial time point, i.e. 5 min, was significantly higher than that after s.c. injection. From the time periods of 10–120 min, there were no significant differences of concentrations in CSF between those two routes ($P > 0.05$). Whereas, the CSF peak time of nasal applied APO was 10 min faster than that by s.c. administration. The AUC of drug concentration curves in plasma and CSF were calculated, the results are shown in Table 1. The relative bioavailability of APO obtained following nasal administration was only about 53%, while there was no significant difference of apparent CSF availability between these two routes (Table 1).

3.4. Brain drug distribution

As shown in Fig. 4, there were similar concentration–time profiles in all brain regions following i.n. administration compared with s.c. injection. Except in olfactory tract, there were no significant differences of $AUC_{0\rightarrow120}$ values in all other brain regions between these two delivery routes (Table 1). Except cerebellum, brain APO concentrations reached peak level both at 20 min, followed by a prolonged decline with time. However, the drug concentrations in all brain regions at the initial time point, i.e. 5 min, following nasal delivery were measured significantly higher than those through s.c. injection.

As for the DTI (Table 2), the value achieved ranged from 156 to 194% in different brain tissues or CSF, which were greater than 100%. In addition, DTP results also confirmed that APO content via the olfactory pathway at 2 h constitutes 35–50% of the total drug uptake from both olfactory and system pathway in those regions (Fig. 5).

3.5. Effect of APO·HCl on the first copulatory behaviour of intact sexually active male rats

The occurrence of penile erection, stretching as well as significantly increased activity were observed in male rats 20 min post-treated with APO·HCl s.c. formulation. According to the record in Table 3, a significant effect of APO·HCl s.c. treatment group was detected by ANOVA for decreasing ML and EL ($P < 0.01$) as well as increasing IF and IF/MF ratio ($P < 0.01$) compared with control group. Similar penile erection, stretching and activity increasing were detected in all three different dose groups 15 min after intranasal administration. At a low intranasal dose of APO·HCl (100 μg/kg), there were no significant differences in the parameters of copulatory behaviour compared with control except EL. However, the medium dose of APO·HCl (200 μg/kg) can improve the sexual behaviour in all tested parameters. When increasing the dose to 400 μg/kg, the efficacy slightly decreased, causing no significant differences in IF and IF/MF ratio compared with control. Similar effects were found in medium and high dose of intranasal APO·HCl treated groups in comparison with that of subcutaneous APO·HCl treated group (Table 3).

3.6. Effect of APO·HCl on different copulatory patterns

Table 4 shows that, in the eighth test, saline-treated SE rats behaved differently from those that had received APO·HCl

Table 1
APO AUC in plasma, brain tissue and CSF after i.n. and s.c. administration

Route	AUC							
	Plasma (ng·min/mL)	OB (ng·min/g)	OT (ng·min/g)	CS (ng·min/g)	HC (ng·min/g)	CL (ng·min/g)	CR (ng·min/g)	CSF (ng·min/g)
s.c.	23986 ± 1070	92648 ± 2882	149854 ± 6886	152949 ± 5401	176788 ± 6077	133652 ± 4283	183742 ± 8936	1743 ± 418
i.n.	12709 ± 356**	91351 ± 5605	124388 ± 5585*	145703 ± 6711	168596 ± 8124	137456 ± 6650	175561 ± 9480	1640 ± 193

Values are means ± S.D., $n = 5$. OB: olfactory bulb; OT: olfactory tract; CS: corpus striatum; HC: hippocampus; CL: cerebellum; CR: cerebrum; CSF: cerebrospinal fluid.

* $P < 0.05$ significant difference in comparison with s.c. according to the Student's t-test.

** $P < 0.01$significant difference in comparison with s.c. according to the Student's t-test.

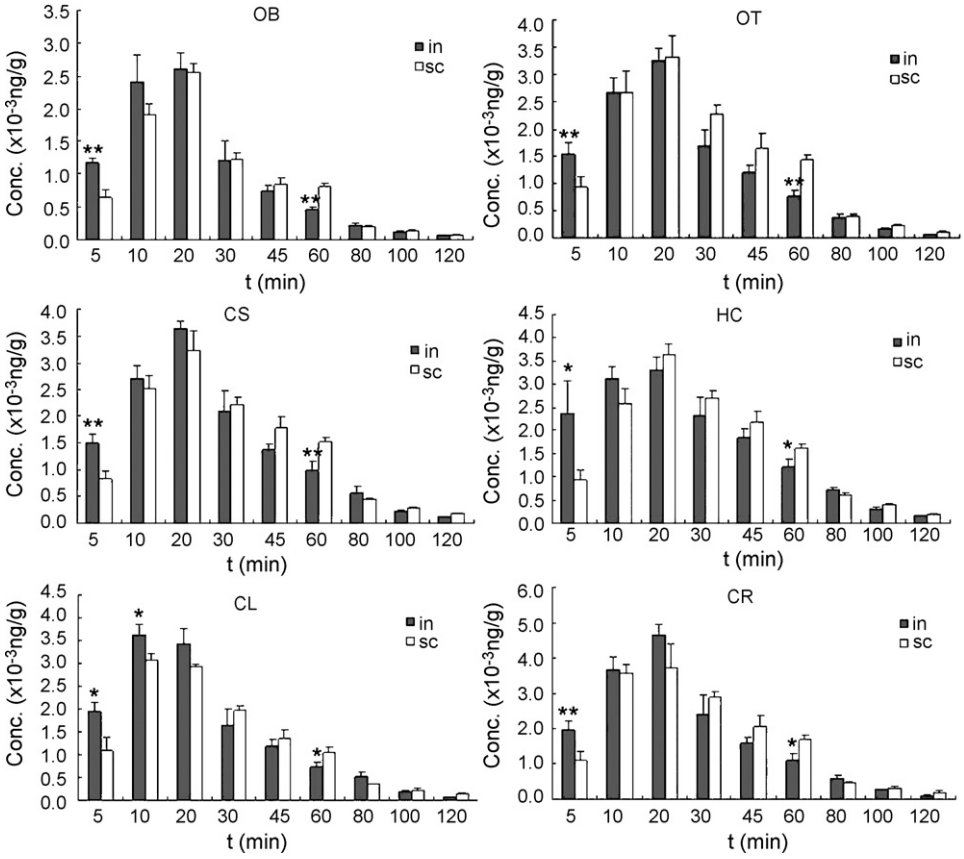

Fig. 4. Average APO concentration in different brain tissue following intranasal (i.n.) and subcutaneous (s.c.) administration. Data represents the mean ± S.D. ($n = 5$). Any significant difference at each time point between two routes was calculated according to the Student's t-test: $^{*}P < 0.05$; $^{**}P < 0.01$. OB: olfactory bulb; OT: olfactory tract; CS: corpus striatum; HC: hippocampus; CL: cerebellum; CR: cerebrum.

Table 2
APO AUC ratio of brain (or CSF)-to-plasma after i.n. and s.c. administration

Route	AUC_{brain}/AUC_{plasma}						
	OB	OT	CS	HC	CL	CR	CSF
s.c.	3.86 ± 0.21	6.25 ± 0.40	6.38 ± 0.36	7.37 ± 0.42	5.57 ± 0.31	7.66 ± 0.51	0.049 ± 0.013
i.n.	7.19 ± 0.48**	9.79 ± 0.52**	11.46 ± 0.62**	13.27 ± 0.74**	10.82 ± 0.60**	13.81 ± 0.84**	0.091 ± 0.014**
DTI[a] (%)	186.09 ± 16.13	156.65 ± 13.02	179.79 ± 14.10	179.98 ± 14.27	194.10 ± 15.22	180.32 ± 16.19	183.76 ± 57.02

Values are means ± S.D., $n = 5$.
[a] The ratios of AUC_{brain}/AUC_{plasma} via i.n. delivery to those through s.c. administration.
** $P < 0.01$, significant difference in comparison with s.c. according to the Student's t-test.

Table 3
Effect of APO·HCl on the first copulatory behaviour of normal male rats

APO·HCl Dose	ML (s)	MF	EL (s)	IF	IF/MF
Control	157 ± 26.0	67 ± 6.0	385 ± 31.7	38 ± 6.3	0.6 ± 0.1
s.c. (200 μg/kg)	87 ± 16.0**	58 ± 7.5	166 ± 36.0**	47 ± 5.3**	0.8 ± 0.1**
i.n. (100 μg/kg)	139 ± 22.7†	67 ± 4.8	290 ± 31.6**,††	38 ± 8.4†	0.6 ± 0.1†
i.n. (200 μg/kg)	74 ± 20.8**	56 ± 9.3*	158 ± 23.1**	49 ± 6.1**	0.9 ± 0.1**
i.n. (400 μg/kg)	134 ± 27.3*,†	64 ± 7.2	161 ± 28.2**	41 ± 6.8	0.7 ± 0.1

ML: mount latencies; MF: mount frequencies; EL: ejaculation latency; IF: intromission frequencies. Values are means ± S.D., $n = 10$.
* $P < 0.05$ significantly different from control (ANOVA followed by Student–Newman–Keul's test).
** $P < 0.01$ significantly different from control (ANOVA followed by Student–Newman–Keul's test).
† $P < 0.05$ significantly different from s.c. (ANOVA followed by Student–Newman–Keul's test).
†† $P < 0.01$ significantly different from s.c. (ANOVA followed by Student–Newman–Keul's test).

Table 4
Effect of APO·HCl on the eighth copulatory behaviour of sluggish ejaculators (SE) male rats

APO·HCl dose	CE	PEI (s)	III	EL (s)
Control	0.37 ± 0.05	396 ± 25.7	43 ± 4.5	648 ± 43.5
s.c. (200 μg/kg)	0.45 ± 0.06**	365 ± 22.4	31 ± 5.7**	528 ± 43.4**
i.n. (100 μg/kg)	0.40 ± 0.04†	384 ± 38.8	36 ± 5.8*	560 ± 36.4**
i.n. (200 μg/kg)	0.48 ± 0.02**	362 ± 22.3*	29 ± 6.1**	522 ± 34.2**
i.n. (400 μg/kg)	0.45 ± 0.03**	369 ± 33.1	33 ± 5.1**	537 ± 50.8**

CE: copulatory efficacy; PEI: post-ejaculatory interval; III: interintromission interval; EL: ejaculatory latency. Values are means ± S.D., $n = 7$.
* $P < 0.05$ significantly different from control (ANOVA followed by Student–Newman–Keul's test).
** $P < 0.01$ significantly different from control (ANOVA followed by Student–Newman–Keul's test).
† $P < 0.05$ significantly different from s.c. (ANOVA followed by Student–Newman–Keul's test).

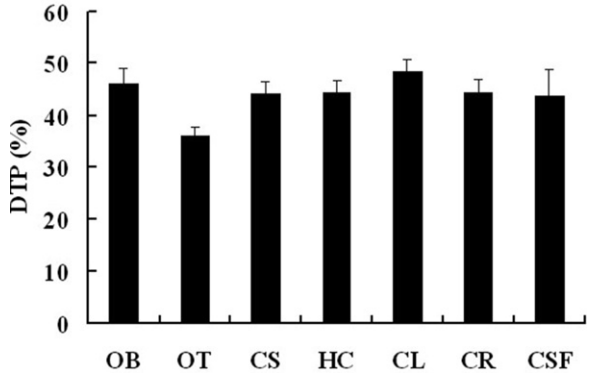

Fig. 5. Brain drug direct transport percentage (DTP) after nasal administration of APO·HCl. Data represent the mean ± S.D. ($n = 5$). OB: olfactory bulb; OT: olfactory tract; CS: corpus striatum; HC: hippocampus; CL: cerebellum; CR: cerebrum; CSF: cerebrospinal fluid.

through subcutaneous or intranasal administration at different doses. APO·HCl subcutaneous formulation significantly increased CE and decreased III and EL compared with control detected by ANOVA. The medium and high intranasal doses treated groups had similar mating effects in comparison with APO·HCl subcutaneous group. The medium dose caused the highest copulatory effect. However, low intranasal dose treated group displayed less improvement in CE than subcutaneous group.

There were no significant differences for CE and PEI, in the eight tests, in NE rats between control and APO·HCl treated groups (Table 5). However, all APO·HCl treated groups significantly decreased III and EL compared with control. APO·HCl medium and high intranasal dose can cause equivalent effects compared to the subcutaneous dose ($P > 0.05$), while low dose failed except for III.

4. Discussion

Literatures reported there could be two direct pathways responsible for the transferring of substances from the nasal cavity into the central nerve system (CNS) (Mathison et al., 1998). The first is the olfactory nerve pathway that involves uptake of the agents into the olfactory nerves and subsequent axoplasmic transport. Viruses, dyes, some inorganic metals, and other large molecular weight substances have been shown to enter the olfactory bulbs via this route after entering the olfactory nerve cells. This pathway was characterized by extremely slow transportation, which usually takes about 24 h to days to reach olfactory bulbs (Mathison et al., 1998). The second is the olfactory epithelial pathway that involves epithelial transferring by passive diffusion across the epithelium or paracellularly through the tight junction of the supporting cells or other means. Low molecular weight drugs ($M_W < 1000$) were supposed to be paracellularly or transcellularly absorbed through this pathway because of their rapid appearances in CSF and brain, such as cephalexin (Sakane et al., 1991), dihydroergotamine (Wang et al., 1998), lidocaine (Chou and Donovan, 1998), cocaine (Chow et al., 1999) and methotrexate (Wang et al., 2003).

In this experiment, APO intranasal administration resulted in a similar AUC value in different brain regions and CSF compared with subcutaneous route, while its plasma AUC was nearly one-half. The results of Pharmacokinetic studies clearly showed that there existed a nose-to-brain pathway for the intranasal delivery of APO. According to DPT results, about one-third to one-half

Table 5
Effect of APO·HCl on the eighth copulatory behaviour of normal ejaculators (NE) male rats

APO·HCl dose	CE	PEI (s)	III	EL (s)
Control	0.46 ± 0.02	340 ± 29.6	27 ± 3.7	348 ± 48.2
s.c. (200 μg/kg)	0.46 ± 0.02	332 ± 20.9	16 ± 2.9**	225 ± 28.4**
i.n. (100 μg/kg)	0.46 ± 0.02	342 ± 34.8	17 ± 3.7*	300 ± 32.2*,††
i.n. (200 μg/kg)	0.46 ± 0.02	323 ± 16.2	13 ± 4.2**	201 ± 39.9**
i.n. (400 μg/kg)	0.46 ± 0.02	347 ± 15.7	16 ± 3.1**	223 ± 32.2**

CE: copulatory efficacy; PEI: post-ejaculatory interval; III: interintromission interval; EL: ejaculatory latency. Values are means ± S.D., $n = 7$.
* $P < 0.05$ significantly different from control (ANOVA followed by Student–Newman–Keul's test).
** $P < 0.01$ significantly different from control (ANOVA followed by Student–Newman–Keul's test).
†† $P < 0.01$ significantly different from s.c. (ANOVA followed by Student–Newman–Keul's test).

of drug content in different brain regions was attributed directly from nose-to-brain transport pathway. Due to quick appearance of APO in brain regions, the nasal transport into the CNS could not occur through the olfactory nerve pathway. Therefore, most APO reached brain through the olfactory epithelial pathway to olfactory bulb, then fast diffused or distributed into other brain region through CSF flow. This was supported by successfully visualizing the olfactory epithelial drug distribution and transferring pathway into the olfactory bulb using the model drug 3 kD fluorescein dextran (FD3) (Jansson and Bjork, 2002).

As a central active anti-ED drug, the induction of penile erection APO was through releasing oxytocin in the paraventricular nucleus of the hypothalamus or surrounding structures. Therefore, the concentration of APO in CNS has a good correlation with its pharmacological effect. Examination of the data obtained in rats confirms the sexual stimulant activity of intranasal APO had a dose–response effect. The optimized intranasal APO dose at 200 μg/kg can cause the same effect as those through subcutaneous route at the equal dose. Intranasal APO appeared faster onset compared with subcutaneous route. In addition, assuming that ML, IL, PEI and III reflect arousal/motivation (Clark et al., 1987; Everitt, 1999), in the intact animals categorized as SE and NE, APO exerted sexual stimulation involving both ejaculation and arousal, because PEI and III were strongly affected in SE rats, and III was reduced in NE.

5. Conclusion

The paper proved there exists a nose-to-brain pathway for APO intranasal delivery, which significantly increased brain transport of APO. The bioequivalence of brain drug content compared with its subcutaneous route had good correlation with its sexual stimulant activity. Current results in rats also explained the reason why the intranasal application of APO could be an effective alternative to subcutaneous and oral formulations.

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