College of Pharmaceutical Science[1], Hebei Medical University; Institution of Pharmacology and Toxicology[2], Academy of Military Medical Sciences, Beijing, China

Pharmacodynamics and toxicity of vasoactive intestinal peptide for intranasal administration

Xu Cui[1], De-Ying Cao[1], Zhi-Min Wang[1], Ai-Ping Zheng[2]

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Xu Cui, Hebei Medical University, College of Pharmaceutical Science, 27 Beijing Taiping Road, 100850 Bejing, China
cuixu5693255@163.com

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The aim of this work was to study the nasal route for the delivery of vasoactive intestinal peptide (VIP) to the brain and to evaluate the toxicity of VIP nasal spray. Mice were injected intracerebroventricularly with the aggregated $A\beta_{25-35}$ to mimic Alzheimer's disease. Following administration, different groups of mice were treated over one week, and their spatial learning and memory capacities were evaluated by the Morris water maze test. The toxicity of VIP nasal spray was evaluated by examining the morphology of individual rat nasal mucosa cilia and the pathology of rat nasal mucosa. Rats receiving intranasal VIP (40 μg/ml) showed good spatial memory relative to the $A\beta_{25-35}$ model group, but the escape latency did not show any statistically significant difference. Intranasal administration of VIP nasal spray (200 μg/ml) improved deficits in spatial memory to the point that test animals receiving intranasal VIP showed no statistically significant differences from the normal control group in escape latency. This indicated that the nasal spray method could increase the quantity of VIP entering the brain and protect the central nervous systems of mice. Toxicity evaluation showed that the preparation could cause minor irritation, which resolved spontaneously within a week at the end of treatment. In conclusion, VIP can be delivered successfully to the brain using the intranasal route.

1. Introduction

Delivering large, hydrophilic drug molecules (peptides and proteins) to the brain requires overcoming or sidestepping the blood–brain barrier (BBB) (Begley 1996). It is well known that 98% of the drugs hardly be able to enter the brain because of the blood-brain barrier (BBB) (Cummings 2004). Intranasal drug delivery systems offer a non-invasive means of delivery, which effectively bypasses the blood-brain barrier. The olfactory receptor cells are in contact with both the nasal cavity and the central nervous system (CNS), and this connection constitutes a direct pathway to the brain (Dejda et al. 2005). After intranasal administration, some of the drugs are absorbed by the nasal mucosa into the olfactory bulbs or cerebrospinal fluid. Intranasal administration also allows drugs to avoid the hepatic first-pass effect and degradation in the blood compartment. Intranasal administration is particularly relevant to peptide drugs.

Vasoactive intestinal peptide (VIP) is a member of the secretin/glucagon family. VIP is a cationic 28-amino-acid peptide (Delobette et al. 1997), widely distributed within the body. It can be found in the brain, lungs, gastrointestinal tract, kidneys, and heart (Ganea et al. 2002). It has various peripheral biological functions, such as smooth muscle relaxation, pulmonary bronchodilation, and anti-inflammatory and immunomodulatory effects (Gololobov et al. 1998; Gozes et al. 1996; Groneberg et al. 2006). It also functions as a neurotransmitter and neuromodulator. VIP plays a significant role in promoting memory and intelligence, and may have applications in the treatment of various neurological disorders, such as Alzheimer's disease (AD) (Hardy and Selkoe 2002), which is the most common neurodegenerative disorder of the central nervous system (Hassan et al. 1994). Deposition of Aβ in the brain is believed to be the critical step in the onset of AD, but the precise mechanism remains unknown (Igna et al. 2007). A lipophilic VIP analogue was proposed for such a therapeutic application. VIP performs its biological effects through specific, G-protein-coupled membrane receptors, namely $VPAC_1$, $VPAC_2$, and PAC_1 (Mathison et al. 1998; Petkov et al. 2003). However, like most endogenic peptides, its potential therapeutic applications are limited by its poor ability to cross the blood-brain barrier and rapid elimination after intravenous administration. Its half-life *in vivo* in blood has been estimated to be less than 1 minute in both rats and humans (Refai et al. 1999). We propose that intranasal administration will allow VIP to reach the brain tissue intact via a direct nose-to-brain pathway.

The aim of the current study was to investigate the potential of VIP nasal spray as a delivery system. In this study, we evaluated and characterized the effects of VIP nasal spray in a mouse model of impaired memory established by intracerebroventricular (i.c.v.) injection of $A\beta_{25-35}$. The toxicity of VIP nasal spray was also evaluated. This was carried out by evaluating the irritation of the morphology of individual rat nasal mucosa cilia and pathology of rat nasal mucosa.

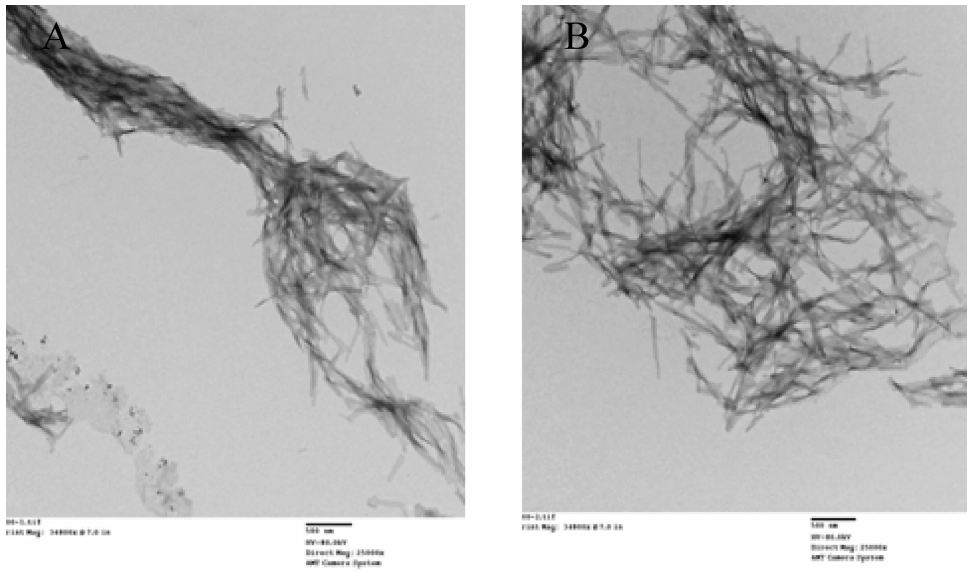

Fig. 1: Transmission electron micrograph of aggregated $A\beta_{25-35}$ at two different concentrations. A (50 μg/ml), B (100 μg/ml) magnification: 25,000 × ; Scale bar: 500 nm

2. Investigations and results

2.1. Feasibility of the nasal administration

The brain delivery of large hydrophilic drug molecules (peptides, proteins) is severely hindered by the presence of the blood–brain barrier (BBB) that strictly controls the access of solutes to the brain. Several strategies, such as drug lipidization, conjugation to ligands or incorporation in BBB-targeting liposomes have been proposed to cross the BBB. In addition, the intranasal route offers a much simpler approach for brain drug delivery. The olfactory receptor cells are in contact with both the nasal cavity and the central nervous system (CNS) and this neuronal connection constitutes a direct pathway from peripheral to the brain, in which the drugs can reach the brain parenchyma mainly by a paracellular route.

2.2. Examination of incubated $A\beta_{25-35}$

Freshly prepared $A\beta_{25-35}$ was in an amorphous state. After incubation for 7 days at 37 °C, $A\beta_{25-35}$ aggregated into characteristic fibrils (Fig. 1) in clear mimicry of AD.

2.3. Morris water maze task

2.3.1. Training trial

In the Morris water maze training session, the control group mice rapidly learned the location of the platform, but the mice that had received 1 mM aggregated $A\beta_{25-35}$ exhibited significantly longer escape latency relative to the control group. This showed that the mouse AD model had been successful. In contrast, the sham control group had significantly shorter latencies than the model group, suggesting that the surgery itself had no effect on the spatial learning and memory ability, but i.c.v. injection of $A\beta_{25-35}$ caused severe impairment (Fig. 2).

The SC-VIP (40) and IN-VIP (40) groups did not show improvement in spatial learning or memory performance. The IN-VIP(200) group showed improved spatial learning and memory performance but there was no significant effect on latency relative to the model group ($P > 0.05$) (Fig. 2).

The mice in the IN-VIP-NS(40) group that were given VIP nasal spray showed improved spatial learning and memory performance but there was no significant effect on latency relative to the model group ($P > 0.05$). The IN-VIP-NS (200) group showed a significant effect on latency relative to the model group ($P < 0.05$). The latency of the IN-VIP-NS (200) group was not statistically different from that of the control group ($P > 0.05$). These results suggest that a nasal drug delivery system can increase the amount of VIP that reaches the brain.

The IN blank NS group did not show improved spatial learning or memory, proving that the blank adjuvant had no effect on spatial learning or memory (Fig. 3).

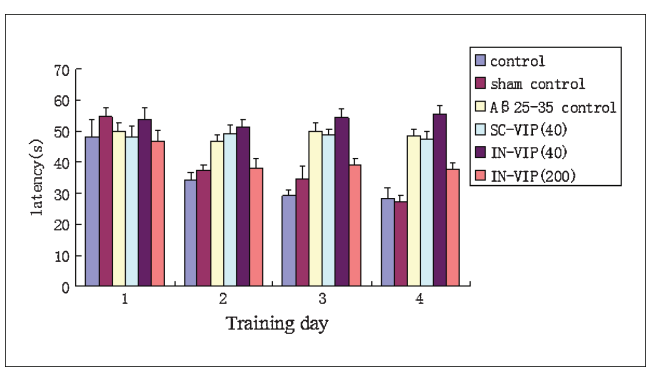

Fig. 2: Escape latency times of the control, sham control, $A\beta_{25-35}$ control, SC-VIP(40), IN-VIP (40), and IN-VIP (200) groups

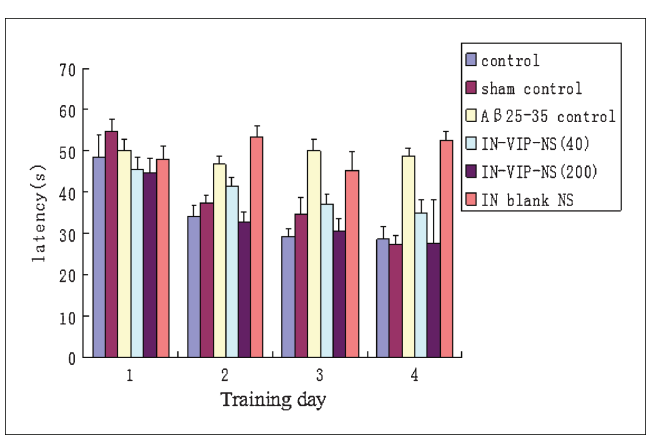

Fig. 3: Escape latency times of control, sham control, $A\beta_{25-35}$ control, IN-VIP-NS(40), IN-VIP-NS (200), and IN blank NS (200) groups

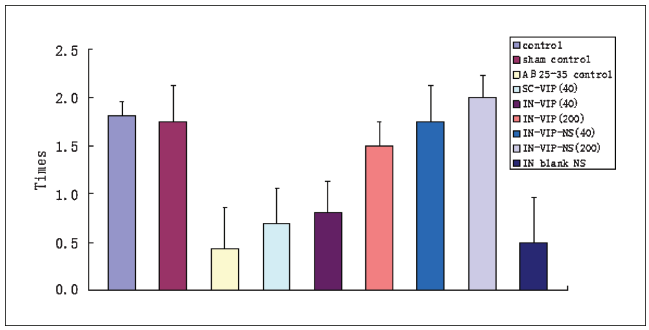

Fig. 4: Number of times mice from each group swam across the previous location of the platform

2.3.2. *Probe trial*

Similarly, on the day of the probe trial, the number of times the mice crossed the platform's previous location decreased more in the model group mice than in the control or sham control groups. The number of times mice from the SC-VIP (40), IN-VIP (40), and IN blank NS groups crossed the previous location of the platform were not statistically different from that of mice in the model group ($P > 0.05$). However, the number of times mice from the IN-VIP (200), IN-VIP-NS (40), and IN-VIP-NS (200) groups crossed the previous platform was statistically significantly different from that of the model group ($P < 0.05$) (Fig. 4).

2.4. *Toxicity of VIP nasal spray*

2.4.1. *Toxicity of nasal cilia*

After intranasal administration of various formulations of VIP for 7 consecutive days, the scanning electron micrographs of rat nasal mucosal cilia were recorded (Fig. 5). The cilia of the physiological saline group, i.e. the negative control, were all arranged on the surface of the mucosa in an orderly fashion. Similar phenomena were observed in the VIP solution groups. In contrast, the cilia in the sodium deoxycholate (1%) group showed shedding and obvious erosion. Slight cilial erosion was found on the nasal mucosa after administration of VIP nasal spray, but the cilia were not broken. In mice that were allowed to recover for a week, the cilia were found to have the same

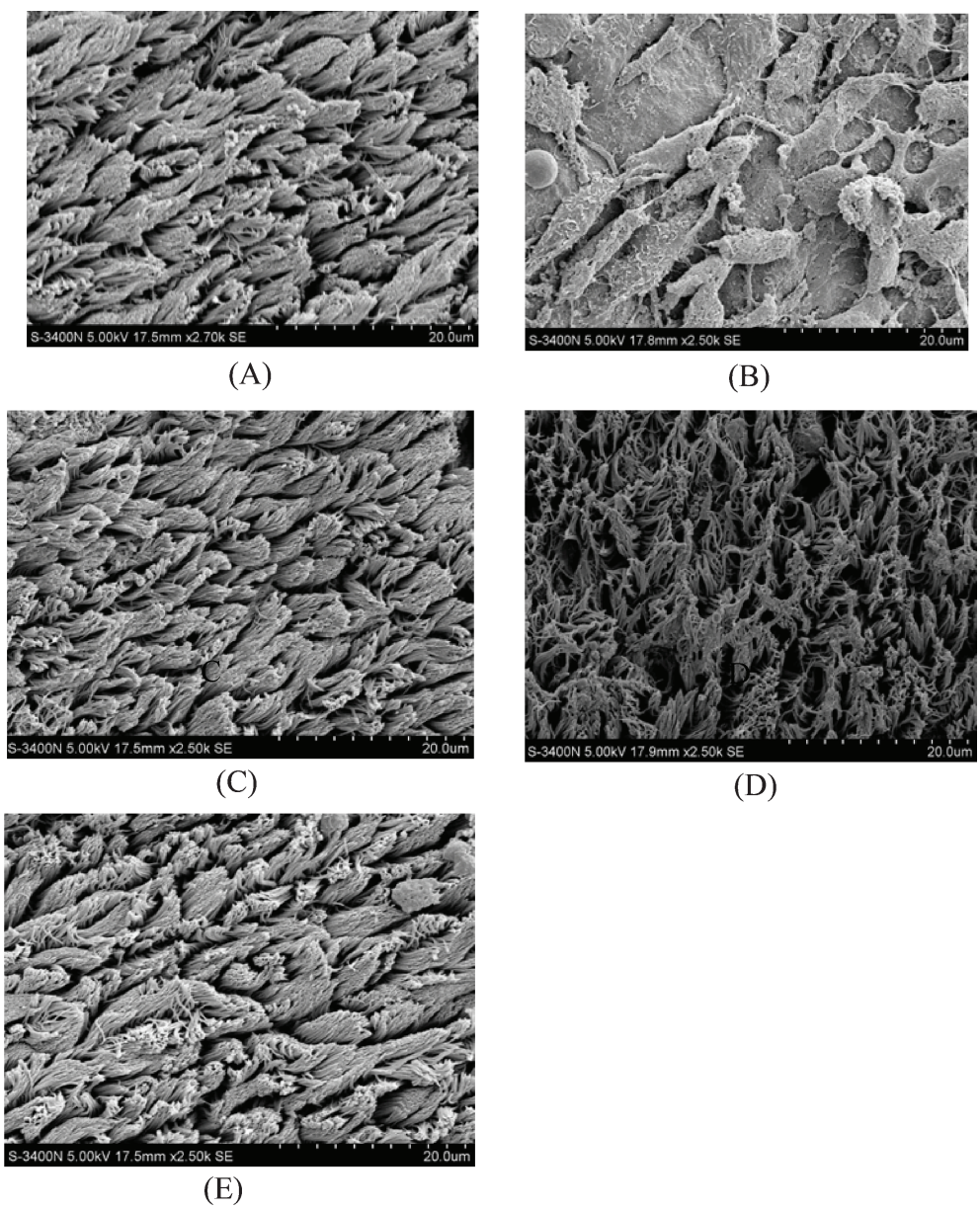

Fig. 5: Scanning electron microscope examination (×2500) of nasal mucocilia of rats after intranasal administration of (A) physiological saline, (B) 1% sodium deoxycholate, (C) VIP solution, (D) VIP nasal spray, and (E) VIP nasal spray plus one week of recovery. Magnification: 2,500 × ; scale bar: 20 μm

shape as those of the physiological saline group, also arranged in an orderly fashion on the surface of the mucosa. These results indicate that VIP alone did not affect the mucosal cilia. The enhancers in the nasal spray exhibited a slight toxicity to nasal mucosal cilia, but it was reversible (Fig. 5).

2.4.2. Morphological examination of nasal cilia

The nasal respiratory epithelium is normally ciliated, pseudostratified, and columnar. The entire nasal membrane is covered by a protective layer of mucus, which is produced by the goblet cells and serous glands within the epithelium. The nasal mucosa of physiological saline group was covered by the pseudostratified columnar epithelium cell. The cells were packed tightly and the cilia were intact. In contrast, the cilia of sodium deoxycholate (1%) group had been completely shed, and the nasal mucosa was bare. Similar phenomena were observed in the VIP solution group. Slight damage to the epithelia did occur in the VIP nasal spray group, but the cilia had not been shed. The epithelia of mice that had been allowed to recover for a week were similar to those of the physiological saline group (Fig. 6).

3. Discussion

Our studies show that VIP nasal spray (especially at a dose of 200 μg/ml) enhanced swimming performance in the Morris water maze test of mice that had been intracerebroventricularly injected with aggregated $A\beta_{25-35}$. As shown in Fig. 2, the $A\beta_{25-35}$ control group had significantly longer escape latency than the normal control mice, indicating potentially successful establishment of AD model. In spite of multiple etiological factors, it is generally accepted that a close correlation exists between $A\beta_{25-35}$ and the neurodegenerative process of AD. Aggregation and deposition of $A\beta_{25-35}$ in the brain are a key step in the pathogenesis of AD, eliciting a cascade of cellular events leading ultimately to neuron loss and dementia.

Figure 3 shows that intranasal administration of VIP nasal spray (200 μg/ml) improved deficits in spatial memory relative to the normal control group but there was no significant difference in escape latency. This suggested that this method of administration could increase the quantity of VIP entering into the brain and protect the central nervous system. The main reason for this is probably that chitosan can open tight junctions, thus enhancing the quantity of VIP that enters the brain. The use of the enhancer HP-β-CD had a synergistic effect with the chitosan, also increasing the quantity of VIP entering the brain. The SC-VIP (40) group did not show improved spatial learning or memory performance. The study also proves that the VIP neuropeptide is unable to cross the blood-brain barrier and undergoes a rapid degradation in blood *in vivo*. Figures 2 and 3 show that mice in the IN-VIP (200) and IN-VIP-NS (40) groups showed improved spatial learning and memory but there was no significant effect on latency ($P > 0.05$). After nasal administration of VIP, some VIP was cleared by the nasal cilia, and some VIP was absorbed into the blood and eliminated quickly. Only the remainder entered the brain. Results show that spatial learning and memory in test mice can be improved by either increasing the dosage or choosing proper enhancers. That is why the IN-VIP (200) and IN-VIP-NS (40) groups showed improvements in spatial learning and memory but not in latency.

To provide a basis for the application of these preparations, the toxicity of VIP nasal spray was evaluated in the morphology of rat nasal mucosa cilium and pathology of rat nasal mucosa. According to Figs. 5 and 6, after one week of treatment, some inflammatory cells and loss of integrity were observed. Mucosa from the physiological saline group and VIP solution group appeared similar. This suggested that the adjuvant, not VIP, damaged the nasal mucosa. This showed that the preparation caused a little irritation, but mucosa morphology was found to recover one week after treatment ceased.

In summary, the results of this study and all other reported properties of VIP nasal spray strongly suggest its usefulness in the

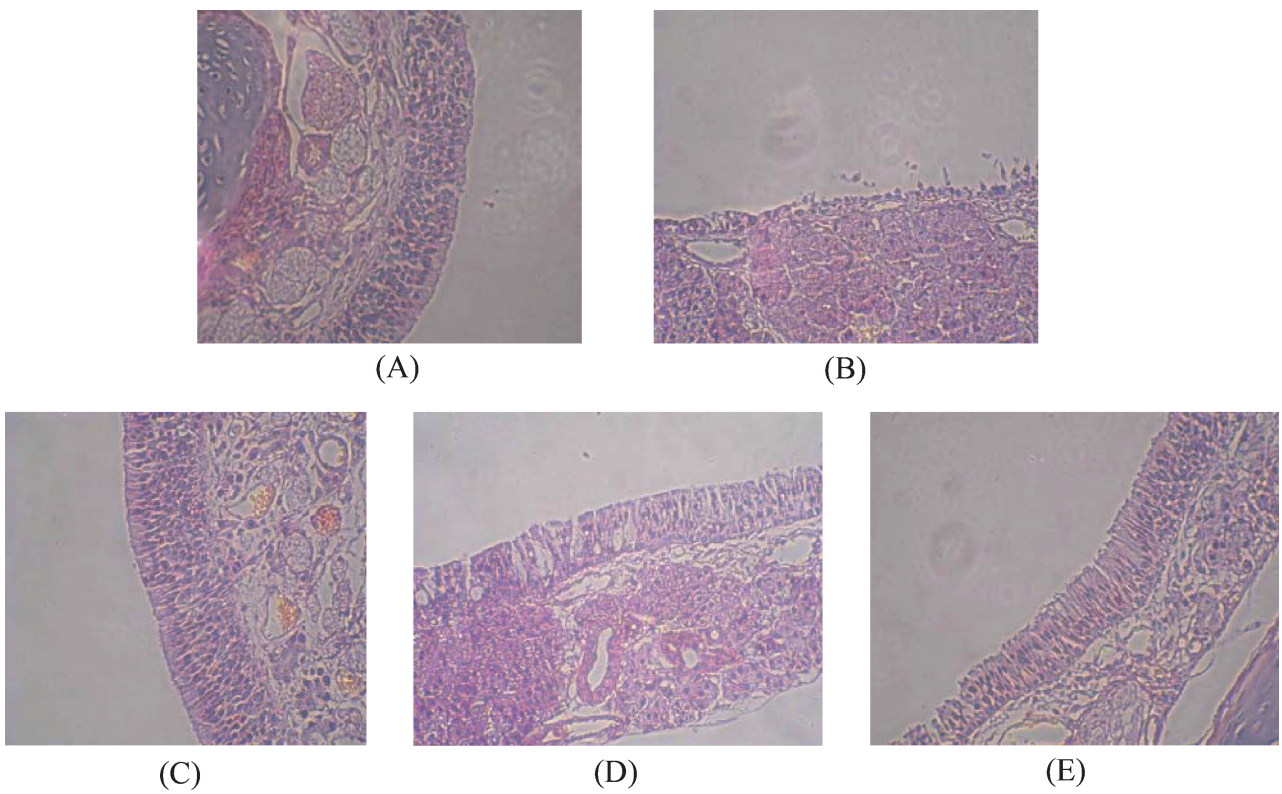

Fig. 6: Light microscope examination of nasal septum of rats after intranasal administration of (A) physiological saline, (B) 1% sodium deoxycholate, (C) VIP solution, (D) VIP nasal spray, and (E) VIP nasal spray plus one week of recovery

treatment of the memory impairment observed in AD. In particular, the study of pharmacodynamics of the nasal spray of VIP shows that its curative effect is reliable, and the safety evaluation shows that the nasal spray of VIP has little ciliotoxicity and the damage is reversible without further intervention. The nasal spray promotes VIP transport into the brain.

4. Experimental

4.1. Materials and animals

VIP was obtained from Beijing Aoke Corporation (Beijng, China). HP-β-CD was provided by Xian Deli Biology Chemical Corporation (Xian Shanxi Province, China). Chlorobutanol was obtained from Beijing chemical agent Corporation (Beijng, China). Chitosan oligosaccharide was purchased from Zhejiang Aoxing agent Corporation (Zhejiang, Province, China). $A\beta_{25-35}$ was purchased from Beijing Biosynthesis Biotechnology Corporation (Beijng, China). All the other materials were of analytical grade.

Sprague-Dawley rats weighing 230–280 g and Kunming mice weighing 25–30 g were obtained from the Experimental Animal Center of Beijing Institute of Pharmacy and Toxicity (Beijing, China) and maintained at $22 \pm 2\,^{\circ}C$ on a 12-hour light-dark cycle with access to food and water *ad libitum*. All animal experiments adhered to the principles of care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of Beijing Institutute of Pharmacy and Toxicity.

4.2. Preparation of nasal spray of vasoactive intestinal peptide

Nasal spray containing vasoactive intestinal peptide was composed of the following five materials: VIP, chitosan oligosaccharide, HP-β-CD, chlorobutanol and NaCl. The procedure for preparing the formulation is as follows. Briefly, the chitosan oligosaccharide was dissolved in 0.7% (w/v) sodium chloride distilled water solution, and then allowed to swell overnight at room temperature. The sodium chloride solution kept the system isotonic. HP-β-CD and chlorobutanol were slowly added to the solution above with stirring to final concentrations of 5% (w/v) and 0.3% (w/v), respectively. After the HP-β-CD and chlorobutanol had completely dissolved, sufficient quantum VIP was added to the mixture. The final concentrations of VIP were 0.08 mg/ml and 0.4 mg/ml, respectively, for the two nasal spray formulations used. Formulations were stored at $-20\,^{\circ}C$.

4.3. Pharmacodynamics

4.3.1. Intracerebroventricular injection and administration

$A\beta_{25-35}$ is the active portion of the neurotoxic Aβ peptide (Sethi et al. 2005). It was dissolved in physiological saline solution at 2 mg/mL. The $A\beta_{25-35}$ solution was sealed and incubated for 7 days at $37\,^{\circ}C$ in order to transform Aβ into an aggregated phase before use. It is generally believed that the insoluble aggregated peptide aids learning and memory deficits in several tasks (Pardridge 1999). After incubation, the peptide formation was observed under a transmission electron microscope. Five microliters of the $A\beta_{25-35}$ solution was placed on the 300-mesh carbon-coated copper grids and negatively stained with 2% (w/v) uranyl acetate for 1 minute. Grids were examined under a transmission electron microscope at 80 kV.

Kunming mice were randomly divided into nine groups: (1) control, (2) sham control, (3) model, (4) subcutaneous injection of VIP solution, dose: 40 μg/kg (SC-VIP 40), (5) intranasal administration of VIP solution, dose: 40 μg/kg (IN-VIP 40), (6) intranasal administration of VIP solution, dose: 200 μg/kg (IN-VIP 200), (7) intranasal administration of VIP nasal spray, dose: 40 μg/kg (IN-VIP-NS 40), (8) intranasal administration of VIP nasal spray, dose: 200 μg/kg (IN-VIP-NS 200), (9) intranasal administration of blank nasal spray (IN blank NS). The mice in groups 3–9 were intracerebroventricularly (i.c.v.) injected with aggregated $A\beta_{25-35}$. The mice in the sham control group received the same volume of axenic physiological saline solution, and the mice in the control group did not undergo any surgical operation. Intracerebroventricular injection was performed under pentobarbital sodium 50 mg/kg anesthesia. An injection cannula was inserted stereotaxically at a site 0.8 mm posterior and 1.5 mm lateral to the bregma and 4.0 mm below the surface of the cranium. Five μl $A\beta_{25-35}$ (1 mM) or the same volume axenic physiological saline solution was gradually injected into the left ventricle with a microinjector. All injection sites were pre-confirmed by injection of trypan blue in place of peptide in preliminary experiments. After surgery, animals were returned to their cages.

VIP was dissolved in physiological saline to 0.08 and 0.4 mg/ml for nasal administration and to 4 μg/ml for subcutaneous injection. The treatments were performed after i.c.v. injection of $A\beta_{25-35}$. The mice in groups 4–8 received VIP via subcutaneous injection or nasal administration daily once during the test. The mice in the control and sham control groups were given physiological saline solution via nasal administration. The mice in the blank NS groups were given the blank nasal spray via nasal administration (Table 1).

4.3.2. Morris water maze task

The Morris water maze test was performed 7 days after i.c.v. injection of $A\beta_{25-35}$. The water maze was a circular pool 90 cm in diameter and 45 cm in height with a black inner surface. The tank was placed in a dimly lit soundproof room with several visual cues. The pool was divided into 4 quadrants of equal area and filled to a depth of 30 cm with water at $20 \pm 2\,^{\circ}C$. A black platform 6 cm in diameter and 1 cm below the surface of the water was placed in one of the pool quadrants.

All mice in each group were given four daily trials in the presence of the platform. For three training trial sessions, mice were placed into the water facing the pool wall in one of the quadrants. The groups of mice were tested in a different order each day to balance the data. During these sessions, the time taken to find the hidden platform (escape latency) was recorded. When a mouse located the platform, it was allowed to remain on it for 20 s. If the mouse did not locate the platform within 60 s, it was placed on the platform for 10 s. The animal was then taken to its cage and allowed to dry under a warm towel after each trial. On the fifth day, mice were subjected to a probe trial session in which the platform was removed from the pool. The mice were allowed to swim for 60 s to search for it, and the time spent looking for the platform in the correct quadrant and the number of times each mouse swam across its original location were recorded.

4.4. Cilia toxicity of VIP nasal spray

4.4.1. Groups and treatments

The Sprague-Dawley rats were separated into five groups at random: (1) physiological saline group, (2) 1% sodium deoxycholate group, (3) VIP solution group, (4) VIP nasal spray group, and (5) VIP nasal spray one-week recovery group (Table 2).

4.4.2. Toxicity of nasal cilia

After the above treatments were completed, the rats were killed. The nasal septum mucosa were removed for examination according to established literature. The nasal septum mucosa were washed with cold saline, fixed with 2.5% glutaraldehyde solution and then with 1% osmic acid. The samples were dehydrated by a series of concentration ethanol solutions, replaced by n-amyl acetate, dried at critical pointer of carbon dioxide, and coated with gold by an ion coater. Each processed nasal mucosa sample was examined with a scanning electron microscope.

Table 1: Division of Kunming mice for performance in Morris water maze task

Group	Treatment
Control	Intranasal administration of physiological saline, 10 μl/day for 7 days
Sham control	Intranasal administration of physiological saline, 10 μl/day for 7 days
Model	Intranasal administration of physiological saline, 10 μl/day for 7 days
SC-VIP (40)	Subcutaneous administration of VIP solution (4 μg/ml), 0.2 ml/day for 7 days
IN-VIP (40)	Intranasal administration of VIP solution (0.08 mg/ml), 10 μl/day for 7 days
IN-VIP (200)	Intranasal administration of VIP solution (0.4 mg/ml), 10 μl/day for 7 days
IN-VIP-NS (40)	Intranasal administration of VIP of nasal spray (0.08 mg/ml), 10 μl/day for 7 days
IN-VIP-NS (200)	Intranasal administration of VIP of nasal spray (0.4 mg/ml), 10 μl/day for 7 days
IN blank NS	Intranasal administration of blank nasal spray, 10 μl/day for 7 days

Table 2: Division of Sprague-Dawley rats for toxicity treatments

Groups	Treatments
Physiological saline group	Intranasal administration of physiological saline, 20 μl/day for 7 days
Sodium deoxycholate group	Intranasal administration of sodium deoxycholate (1%), 20 μl/day for 7 days
VIP solution group	Intranasal administration of VIP solution (0.25 mg/ml), 20 μl/day for 7 days
VIP nasal spray group	Intranasal administration of VIP nasal spray group (0.25 mg/ml), 20 μl/day for 7 days
VIP nasal spray one-week recovery group	Intranasal administration of VIP nasal spray group (0.25 mg/ml), 20 μl/day for 7 days followed by 7 days of recovery

4.4.3. Morphological examination of nasal cilia

After the treatments were completed, the rats were killed. The nasal septum mucosa were removed for morphological examination according to the reports. The nasal septum mucosa were fixed with 10% formalin. After fixation, the nasal septum mucosa were immersed in 20% formic acid and decalcified for a minimum of 7 days. The nasal septum mucosa samples were then dehydrated and embedded in paraffin wax. Sections (5–7 mm) were cut and stained with haematoxylin and eosin. The processed nasal mucosa were examined with a light microscope.

4.5. Statistical analysis

All data are expressed as mean ± SEM. The SPSS software package was used to plot and analyze data by one-way analysis of variance (ANOVA) across groups. $P < 0.05$ was considered statistically significant.

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