Evaluation of the Safety and Brain-Related Tissues Distribution Characteristics of TAT-HaFGF *via* Intranasal Administration

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Disabilities triggered by neurodegeneration mainly result in mortality in the elderly, and patients with neurodegenerative disease also display deficits in olfactory function. Therefore drug distribution to the brain through intranasal administration has become one of the most difficult challenges in the treatment of central nervous system (CNS) diseases. TAT-human acidic fibroblast growth factor (HaFGF) is a new fused protein retaining the neuroprotective activities of HaFGF, and is a promising prospect in the treatment of neurodegenerative diseases. TAT (a cell-penetrating peptide) contains a high relative abundance of positively charged amino acids such as lysine and arginine, which have a powerful attraction to the negatively charge on the nasal epithelial membrane. The present study focused on the evaluation of the safety and absorption characteristics of TAT-HaFGF following intranasal administration. After TAT-HaFGF intranasal administration (100, 300, 600 $\mu\text{g}/\text{kg}$) for 5 weeks, hematoxylin–eosin (HE) staining showed no pathology in any of the investigated tissues and organs. The expression of olfactory marker protein (OMP) was observed with immunohistochemical staining, which showed no altered expression in the sensory neurons of the nasal epithelium. Nasal ciliotoxicity studies carried out using an *in situ* palate model and optical microscope showed that TAT-HaFGF had no nasal ciliotoxicity. The distribution of the TAT-HaFGF following intranasal administration was assessed using a radioisotopic tracing method. Radioactivity was observed in the brain after 15 min. This became stronger at 30 min and weaker at 1 h. All of the results confirmed the *in vivo* safety of TAT-HaFGF *via* intranasal administration.

Key words TAT-human acidic fibroblast; intranasal administration; mucosal adsorption; safety evaluation

Neurodegenerative disease occurs in specific regions of the brain, and is classically associated with memory problems, olfactory deficits and movement disorders.^{1–3} Intranasal drug administration has been shown to increase the delivery of peptides of a particular size and charge to the central nervous system (CNS) for the treatment of CNS diseases.^{4,5} For example, intranasal administration of the 26.5 kDa recombinant human nerve growth factor in rats resulted in CNS concentrations 10–45-fold higher (depending on the brain region) than concentrations observed following intravenous administration.⁶ Similarly, intranasal delivery of hypocretin-1 increased targeting to various brain regions by 7–13-fold.⁷ Enhancement of delivery and targeting to the brain has been demonstrated with the intranasal delivery of a wide range of compounds, including galanin-like peptide,⁸ insulin-like growth factor,⁹ insulin,¹⁰ interferon- β -1b,¹¹ transforming growth factor- β 1,¹² and hypocretin-1.⁷

Human acidic fibroblast growth factor (HaFGF) is involved in the regulation of synaptic plasticity and learning and memory processes through the improvement of cholinergic nerve functions. Furthermore, recombinant polypeptide fibroblast growth factor has been applied in clinical settings for the treatment of cardiovascular and neurodegenerative diseases.¹³ Delivery of bioactive peptides across the blood–brain barrier (BBB) is generally restricted. Recent research showed that a 9-amino acid motif from the protein transduction domain of human immunodeficiency virus-1 (HIV) Tat, has a power-

ful attraction to the negatively charge on the nasal epithelial membrane.¹⁴ This feature was attributed to a 9-amino acid stretch of basic amino acids, with the sequence Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg.^{15–17} When partially denatured, the chimeric protein is capable of crossing the BBB of adult mice.¹⁸ Our previous study has shown that modified TAT-HaFGF could penetrate the BBB when administered intravenously,¹⁹ however, this method of injection is not specifically targeted to the brain. With intranasal administration, drugs can directly reach the CNS for the treatment of CNS diseases *via* nose-to-brain pathways, minimizing systemic exposure and decreasing side effects.²⁰ In our previous work, intranasal administration of TAT-HaFGF was found to significantly increase the concentration of HaFGF in the brain. Importantly, TAT-HaFGF enhanced the learning and memory abilities of senescence-accelerated mouse prone-8 (SAMP8) mice (a mouse model of Alzheimer's disease), as demonstrated by the Morris water maze test, and promoted the function of the cholinergic system shown by the measurement of acetylcholine (ACh), acetylcholinesterase (AChE) and choline acetyltransferase (ChAT). TAT-HaFGF also significantly reduced amyloid-beta ($A\beta$)_{1–42} deposits as well as the levels of soluble $A\beta$ forms in the mice brains and prevented apoptosis in the neurons.²¹ However it is unclear whether repeated delivery of fluid into the nares will increase the risk of damage to the nasal cavity and olfactory bulbs.

The present study focused on the safety evaluation of repeated delivery of fluid into the nares and the absorption characteristics of TAT-HaFGF following nasal administration.

The authors declare no conflict of interest.

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MATERIALS AND METHODS

Animals Eight male and 8 female bufo bufo gargarizans weighing 60–80 g and 20 male and 20 female Sprague-Dawley (SD) rats weighing 200–220 g were purchased from the Medical Experimental Animal Center of Guangdong Province (Guangzhou, China).

All rats were kept under conditions of controlled temperature ($24 \pm 1^\circ\text{C}$), humidity (50–60%) and a light/dark cycle of 12/12 h. Animals were allowed free access to a standard rodent diet and water. All experiments were conducted according to the Chinese Guidelines for Animal Care and Use, and were approved by the Animal Ethics Committee of the Chinese Academy of Medical Science.

Materials TAT-HaFGF recombinant fusion proteins were expressed and purified in our own lab by using the previously described method,²²⁾ stored at -80°C and were dissolved in 0.9% normal saline to create an experimental dosage solution for use in drug administration. Ephedrine hydrochloride was obtained from the National Institute for the Control of Pharmaceutical and Biological Products. Deoxysodium cholate was provided by Sinopharm Chemical Reagent Corporation Limited. An SABC immunohistochemical kit was purchased from Boster Biotech. A Rabbit monoclonal anti-olfactory marker protein (OMP) antibody was purchased from Abcam. Anti-TAT was purchased from Cell Application. A kit for the ^{125}I labeling of proteins was provided by PerkinElmer, Inc.TM

Safety Evaluation of TAT-HaFGF by Intranasal Administration. Nasal Ciliotoxicity Nasal ciliotoxicity studies were carried out using an *in situ* palate model.¹⁸⁾ In brief, after pithing bufo bufo gargarizans bull toads, their palates were excised and blood on the palate was cleaned. The isolated palates were placed on a glass slide and treated with 0.5 mL of normal saline, TAT-HaFGF (1.4 mg/mL), ephedrine hydrochloride (10 mg/mL) and deoxysodium cholate (10 mg/mL). To evaluate the influence of TAT-HaFGF drops on the movement of toad palate mucosa cilia, we continuously observed the mucosa for 12 h and recorded the time span of continuing segmental motion of the toad palate cilia after sufficient effect by drugs under an optical microscope (IX71, Olympus).²³⁾

Effects of Repeated Intranasal Administration of TAT-HaFGF on SD Rats **Animal Grouping and Drug Treatment:** Rats were anesthetized with intraperitoneal (i.p.) pentobarbital sodium (40 mg/kg) and were placed in a supine position and a pad was inserted under the dorsal neck to extend the head back toward the supporting surface. An average of 50 μL of total TAT-HaFGF (100, 300, 600 $\mu\text{g/kg}$) and 0.9% saline was then administered by pipette in 5 μL drops, alternating between each naris every 2 min, over a total of 15 min.²⁴⁾ Drugs were delivered intranasally to rats every 2 d for 5 weeks. The details of animal groups and drug treatments are shown in Table 1.

Table 1. Animal Grouping and Drug Treatment

Group	Treatment	Dosage ($\mu\text{g/kg}$)	Number of rat
Control	Saline	—	5
T100	TAT-HaFGF	100	5
T300	TAT-HaFGF	300	5
T600	TAT-HaFGF	600	5

Sample Processing: After 5 weeks, rats were fasted overnight after the last delivery, then anesthetized and flushed *via* cardiac perfusion with saline. The heart, liver, spleen, lung, kidney, olfactory bulbs, and noses of the animals were collected. The main organs were immersion-fixed in 4% paraformaldehyde for 24 h. The noses were immersion fixed and decalcified in 20% formic acid and formaldehyde decalcified fluid for 12 h, and then placed in a new decalcified fluid for 12 h to ensure complete decalcified inside the nares. Tissue was paraffin embedded, and 3 mm horizontal sections were cut using a paraffin slicing machine and stored at 4°C for later use. Then the slides prepared for immunohistochemistry and HE staining.

OMP Immunohistochemical Staining: Olfactory marker protein (OMP) is a 19 kDa intracellular protein expressed at high levels in OSNs in many vertebrate species, with very limited expression elsewhere in the nervous system. The molecular genetics and structure of OMP are now well understood. OMP is expressed late in cellular development and is widely used as a marker of mature OSNs, which can reflect the integrity of olfactory sensory neurons.²⁵⁾ Immunohistochemical staining of OMP was performed to detect the distribution of olfactory nerves in the nasal mucosa and olfactory bulbs of mice. Briefly, paraffin-embedded brain sections were stained according to the protocols of the SABC immunohistochemical kit (Boster Biotech, China). Paraffin sections were dewaxed, rehydrated, and treated in a 3% H_2O_2 solution for 20 min in order to eliminate endogenous peroxidase activity. Then, sections were placed in a 70% formic acid solution for 20 min for antigen retrieval. After being rinsed in phosphate buffered saline (PBS), sections were incubated in blocking solution (5% bovine serum albumin) for 1 h at 37°C . They were then incubated overnight with rabbit OMP monoclonal antibody (1:500, Abcam, U.S.A.) in a humidified chamber. The sections were incubated with the secondary antibody, horseradish peroxidase-conjugated-goat anti-rabbit immunoglobulin G (IgG) (1:200), for 1 h at 37°C . After 3,3'-diaminobenzidine (DAB) and hematoxylin staining, the sections were dehydrated, cleared in xylene, and covered with neutral balsam. Sections from all experimental groups were photographed through a light microscope (IX71, Olympus), and micrographs of five sections per group were analyzed using Image-Pro plus 6.0 software to measure the number of OMP positive plaques in the nasal mucosa and olfactory bulb. The percentage of the total area of epithelium and lamina propria of the nasal mucosa or olfactory glomerular layer of the olfactory bulb in the micrograph that contained OMP deposit areas was also semi-quantitatively analyzed by the above software.

Mucosal Absorption Characteristics of Intranasal Administration **TAT-HaFGF Radiolabeling and Purification:** The distribution of TAT-haFGF following intranasal administration was assessed using a radioisotopic tracing method. TAT-HaFGF was radiolabelled using a Na^{125}I iodogen method described previously.²⁶⁾ Briefly, 1 mCi of Na^{125}I was added into an iodogen-coated tube with 1 mL of 1.47 mg/mL TAT-HaFGF to react for 5 min at $27\text{--}29^\circ\text{C}$. The radiolabelled TAT-HaFGF was collected using Sephacryl S-100 HR (Pharmacia). The radioactivity was measured with a γ -counter (WALLAC 1470 WIZARD® Gamma Counter, PerkinElmer, Inc.).

Intranasal Administration of ^{125}I -TAT-HaFGF: An average of 50 μL of total ^{125}I labeled TAT-HaFGF (300 μg TAT-

HaFGF/kg body weight; ratio of unlabeled TAT-HaFGF to ^{125}I -TAT-HaFGF, 3:1) and 0.9% saline was then administered according to the above methods of administration.

Sampling and Measurement of ^{125}I -TAT-HaFGF Content in Tissues: Rats were administered ^{125}I -TAT-HaFGF intranasally, but allowed to recover from initial pentobarbital anesthesia and then reanesthetized prior to final blood sampling and transcardial perfusion (15, 30, 60 min following the start of administration). After gross dissection of the thymus, olfactory bulb, brainstem, hypophysis, cerebellum, spinal cord, and nasal cavity, each sample was placed in a 5 mL tube and the wet weight was determined with a microbalance prior to gamma counting. Tissue concentrations were calculated using a γ counter (WALLAC 1470 WIZARD Gamma Counter, PerkinElmer, Inc.).

Transport Visualization of TAT-HaFGF: Rats were administered 50 μL of TAT-HaFGF (300 μg TAT-HaFGF/kg body weight) intranasally for once using the previously described method. Thirty minutes after intranasal administration of TAT-HaFGF, rats were anesthetized and perfused with saline, the olfactory bulbs and noses were dissected out and prepared for immunofluorescence. The remainder of the nose was immersion fixed and decalcified in 20% formic acid and formaldehyde decalcified fluid for 24 h. Tissue was embedded with O.C.T compound (McCormick, U.S.A.), and 10 mm horizontal sections were cut with a cryomicrotome (Leica, Germany) and stored at -20°C for later use. Sections were immersed in a PBS solution for 10 min in order to eliminate O.C.T compound. Then, sections were placed in a 0.2% triton solution for 10 min. After being rinsed in PBS, sections were

incubated in 5% BSA solution for 1 h at 37°C . They were then incubated overnight with rabbit OMP monoclonal antibody (1:500, Abcam, U.S.A.) and anti-TAT antibody (1:500, Cell Application, U.S.A.) in a humidified chamber. The sections were incubated with the secondary antibody, DyLight[®] 488-goat anti-rabbit IgG (1:200, Abcam, U.S.A.) and DyLight[®] 594 -donkey anti-mouse IgG (1:200, Abcam, U.S.A.), for 1 h at 37°C . Confocal microscopy (LSM 700, Germany) was used to localize the TAT-HaFGF (anti-TAT, Cell Application).

Statistical Analysis All data are expressed as mean $\pm$ S.E.M. Statistical differences were determined using one-way ANOVA, followed by *post hoc* analysis of Dunnett for multi-group comparison and $p < 0.05$ was considered statistically significant.

RESULTS

Safety Evaluation of Intranasal Administration. Nasal Ciliotoxicity Deoxysodium cholate, used here as a positive control, inhibits cilia movement when acting on toad mucosa cilia for ten minutes. Ephedrine hydrochloride cannot weaken the continuing movement of toad mucosa cilia *in vitro* so was therefore used as a negative control. Optical microscopic observation of toad palates showed that there were a great number of cilia with a fast rate of beating on the edge of mucosa treated with TAT-HaFGF for 420 min (Fig. 1), and that the duration of ciliary movement lasted for 638 min, which is comparable with those treated with ephedrine hydrochloride (650 min) and far longer than those treated with the deoxysodium cholate (only 10 min) (Table 2). The results suggest that

Fig. 1. Optical Microscopic Images of (A) Saline, (B) Negative Control (Ephedrine Hydrochloride, Cilia on the Mucosa Intact 7 h after Treatment), (C) TAT-HaFGF (Similar Phenomenon with That of Saline Visualized 7 h after Treatment), (D) Positive Control (Deoxysodium Cholate Solution, no Cilia on the Mucosa Observed but a Few Exfoliated Cilia 10 min after Treatment)

Cilia are indicated by arrows (40 $\times$, $n=4$ for each group).

TAT-HaFGF drops caused no remarkable injury to the function of toad mucosa cilia.

Effect of TAT-HaFGF on the Nasal Cavity and Olfactory Bulbs of SD Rats Since intranasal administration results in a high concentration of TAT-HaFGF in the nose and olfactory bulbs, damage to the integrity of the nasal cavity and olfactory bulbs and the number of olfactory sensory neurons is possible. To address this problem, noses and olfactory bulbs from intranasal treated animals were processed as described in Materials and Methods. Sections were then visualized with a light microscope to ascertain the morphology of the nasal epithelium. Figures 2a and 3a demonstrate that there were no gross morphological changes or outward damage visible in any of the groups examined. Images were processed by Image-pro plus. The numbers of OMP-positive cells in the epithelium and lamina propria of the nasal mucosa or olfac-

tory glomerular layer of the olfactory bulb were counted and compared in all intranasal treatment groups using a one-way ANOVA. No significant difference was found in the number of olfactory sensory neurons between all intranasal treatment groups (Figs. 2b, 3b). Additionally, HE stained sections from each treatment group demonstrated excellent tissue structure and histology (Fig. 4). No obvious pathological lesions were detected in the heart, liver, spleen and lung in any of the groups of rats following administration of TAT-HaFGF formulations (images not shown), which were in accordance with the condition recorded in the saline control group.

Mucosal Absorption Characteristics of Intranasal Administration. Distribution of TAT-HaFGF in Tissues Distribution of ^{125}I -TAT-HaFGF following intranasal administration was measured in SD rats and the levels of radioactivity in blood, nasal cavity, thymus, and brain tissues are shown in Table 3.

Nasal absorption of ^{125}I -TAT-HaFGF into the systemic circulation was rapid, and the plasma level remained almost constant until 1 h post application (Fig. 5). At 15 min after intranasal administration of ^{125}I -TAT-HaFGF, concentrations of radioactivity were observed in the hypophysis, olfactory bulb, brainstem and cerebellum. At 30 min, stronger radioactive signals were observed in the brainstem, cerebrum, and cervical spinal cord (CSpC) and the signals became weak at 60 min in the brainstem, cerebellum, cerebrum and spinal cord.

Table 2. The Time Span of Toad Palate Cilium Continuing Segmental Motion

Group	Time of swing/min	Relative to normal saline
Normal saline	655 ± 22	100%
Deoxysodium cholate	10 ± 0.3	1.5%
Ephedrine hydrochloride	650 ± 30	99%
TAT-HaFGF	638 ± 30	97%

$n=4$.

Fig. 2. The Level and Distribution of Olfactory Sensory Neurons (OSNs) in Nasal Mucosa Following Intranasal Administration of TAT-HaFGF to SD Rats for Five Weeks

(A) Morphology of the olfactory nerve in the nasal mucosa (200 $\times$). (B) The percentage of the sum of OMP deposit areas as compared with the total area of epithelium (OE) and lamina propria (LP) of the nasal mucosa. Five sections per group were analyzed using Image-Pro plus 6.0 software to measure the number of OMP positive plaques in the nasal mucosae.

Fig. 3. The Level and Distribution of OSNs in Olfactory Bulb Following Intranasal Administration of TAT-HaFGF to SD Rats for Five Weeks

(A) Morphology of the olfactory nerve in the olfactory bulb (100 $\times$). (B) The percentage of the sum of OMP deposit areas as compared with the total area of olfactory glomerular layer (GL) of olfactory bulb in the micrograph. $n=5$ for each group.

The intensity of ^{125}I -TAT-HaFGF radioactivity in the whole brain, olfactory bulb, brainstem, cerebellum and cervical spinal cord reached its maximum at 30 min and then decreased at 60 min (Fig. 5).

Transport Visualization of TAT-HaFGF: In order to obtain direct evidence about the olfactory transfer of TAT-HaFGF, TAT-HaFGF were stained red on the nasal slice after 30 min following intranasal administration. DAPI, a nucleus-specific dye that emits blue fluorescence, was used to detect individual cells. Since olfactory cells are the only sensory neurons with their cell bodies in the nasal epithelium, OMP positive staining in the epithelia (shown in green in Fig. 6) were considered as olfactory epithelia.

The olfactory cells in the olfactory mucosa are bipolar sensory neurons, projecting a dendrite to the surface of the nasal epithelium and a long axon toward the olfactory bulb, which is beneficial for transport of TAT-HaFGF across the epithelium. Furthermore, red fluorescence (TAT-HaFGF) was observed in the connective tissue around the olfactory nerve bundles (Figs. 6A, B). In the nasal mucosa near the cribriform plate (Fig. 6C), only small doses of TAT-HaFGF were colocalized within nerve bundles, and more of the TAT-HaFGF were distributed around the olfactory nerve bundles. These phenomena indicated that TAT-HaFGF was transferred into the olfactory nerve bundles or their surrounding connective tissue.

DISCUSSION

Nasal cilia are mobile hair like appendages extending from

the surface of the nasal epithelial cells that move in a well organized and coordinated way to propel the overlying mucus layer toward the throat. This contributes to the body's primary nonspecific defense mechanism by propelling potentially hazardous substances inside the nasal cavity. This function of the cilia emphasizes that the constituents of preparations intended for nasal delivery should not adversely affect the clearance system, especially in the chronic therapy. In the TAT-HaFGF groups, the morphology and integrity of the cilia on the toad palate, were similar to those of the normal saline group, with intact, dense cilia (Fig. 1). These results suggest that the nasal ciliotoxicity of TAT-HaFGF is negligible.

OMP immunohistochemical staining was done for long-term drug safety evaluation. OMP is expressed late in cellular development and is widely used as a marker of mature OSNs.¹⁴⁾ OMP immunohistochemical staining demonstrated that intranasal TAT-HaFGF does not cause damage to tissue structure or the number of OSNs (Figs. 2–4). HaFGF, a neurotrophin-like growth factor in the brain, plays important roles in the development, differentiation and regeneration of brain neurons.²⁷⁾ Olfaction disorder almost is the accompaniment of ND,^{28,29)} this result may associated with the damage and apoptosis of OSNs. Although there is no significant difference in OSNs level between all groups, it was likely higher than the trends in OSNs level in TAT-HaFGF-treated groups than that in the control group as shown in Figs. 2b and 3b. This result is expecting to improving the olfactory functions of ND. The two facts that HaFGF could improve the functions of cholinergic nerve²¹⁾ and the cholinergic system modulates several as-

Fig. 4. SD Rats Were Treated with Intranasal Administration of TAT-HaFGF for Five Weeks

The olfactory bulbs and nasal cavity were prepared for HE staining and demonstrated excellent tissue structure and histology of nasal cavity (NC) and olfactory bulbs (OB), $n=5$.

Table 3. Amount–Time Profiles of Radioactivity in the Blood, Brain and Immune Glands

Tissue	Amount of ^{125}I -TAT-HaFGF in tissue (cpm · h/g tissue/mL blood)		
	15 min	30 min	60 min
Blood	601.2±223.1	1296.6±135.8	1454.2±287.7
Olfactory bulb	3027.8±1368.1	3255.0±1950.2	2200.4±1304.3
Cerebrum	—	249.2±13.8	—
Brainstem	380.4±53.4	1194.6±1270.2	—
Hypophysis	12620.2±1337.2	9526.3±195.7	4772.7±102.3
Cerebellum	606.0±83.8	—	—
Cervical spinal cord	—	261.2±1.4	—
Thymus	291.9±135.4	327.2±80.2	363.7±95.9
With thyroid's throat	18539.4±6250.2	35245.7±10759.0	78711.1±30145.5
Nasal cavity	37394.8±22985.6	42746.6±25905.5	39984.8±27791.8

$n=5$.

pects of central olfactory functions were well-known. The absence of olfactory behavior experiment is the main deficiency of the study. Another possibility is that, although the structure and morphology of the olfactory epithelium remained intact, the functional cellular transduction machinery within the olfactory sensory neurons, or the general olfactory processing might be damaged and cause general anosmia in the animal.

Early evidence demonstrating protein transport within olfactory sensory neurons to the olfactory bulb and other CNS areas, led to the suggestion that these nerves, or perineuronal compartments associated with them, might facilitate the initiation of some CNS diseases.³⁰⁾ The present study demonstrates that TAT-HaFGF can bypass the blood–brain barrier to reach multiple sites within the brain and spinal cord likely by olfactory and trigeminal pathways.

The olfactory pathway has been reported to play a major role in the delivery of therapeutics directly to the CNS following intranasal administration.³¹⁾ The nasal barrier to the brain might be weakened because the olfactory cells regenerate every 3–4 weeks due to exposure to external toxins.³²⁾ In addition, the continuous perineurial channels generated by the olfactory ensheathing cells that envelop the axons of olfactory cells are kept open.³³⁾ As a result, intranasally administered TAT-HaFGF may reach the brain *via* extracellular transport along olfactory nerves (Fig. 6). Furthermore, neural projections of the olfactory bulb extend into multiple rostral brain tissues such as the olfactory tract, anterior olfactory nucleus, and piriform cortex⁹⁾ (Fig. 7). Therefore, the significant TAT-HaFGF distribution in rostral brain areas neighboring the olfactory bulb is mainly due to the olfactory pathway.

Fig. 5. Distribution of ¹²⁵I-TAT-HaFGF in Blood, Olfactory Bulb (OB), Cerebrum, Brainstem, Hypophysis, Cerebellum, Cervical Spinal Cord (CSpC), Thymus, with Thyroid's Throat (TT), and Nose Cavity (NC)

Fig. 6. Distribution of TAT-HaFGF (Red) in Slices A, B (the Nasal Mucosa near the Wing of Nose), and C (the Nasal Mucosa near the Cribriform) after 30 min Following the Intranasal Administration of TAT-HaFGF to Rats at the Dose of 300 μ g/kg, Fluorescence Was Observed in the Olfactory Epithelium (OE) and Lamina Propria (LP)

Cell nuclei were stained with DAPI (blue). Olfactory nerves were immunolabeled with OMP (green). Co-location (yellow) of the green and red fluorescence is shown by arrows. BG: Bowman gland, NB: nerve bundle (200 $\times$). (Color images were converted into gray scale.)

Fig. 7. Extracellular Pathways into the CNS Following Intranasal Administration

Intranasally applied proteins are rapidly transported into CNS tissue by a formerly identified pathway associated with the peripheral olfactory system (shown in red), connecting the nasal passages and olfactory bulb (OB)/rostral brain, and a newly identified pathway associated with the peripheral trigeminal system (shown in blue), connecting the nasal passages and the brainstem. Cisternal sampling in rats (asterisk) has demonstrated that some molecules, mostly smaller molecular weight solutes, can rapidly enter the CSF after intranasal administration (shown in black). (Color images were converted into gray scale.)

The trigeminal nerve enters the brainstem at the pons and some of its nerve endings terminate beneath the nasal epithelial surface, which provides a possible entrance for intranasally administered TAT-aFGF into the brain (Fig. 7). In the present study, high concentrations of TAT-HaFGF were detected in the cervical spinal cord and cerebellum (Fig. 5), demonstrat-

ing the involvement of the trigeminal nerves. Nevertheless, considering the rapid delivery shown by high radioactivity in the cervical spinal cord 15 min after intranasal administration (Fig. 5), an extracellular mechanism is most likely to be the predominant mode of transport into the CNS *via* the trigeminal nerve pathway.³⁴⁾

Generally, neurodegenerative diseases occur in specific regions of the brain, therefore efficient drug delivery is required, not only to the brain in general, but to these specific regions. For example, Alzheimer's disease (AD), a neurodegenerative disease afflicting *ca.* 35 million people worldwide in 2009,³⁵⁾ has been demonstrated to damage the cerebral cortex and hippocampus; two areas near to the olfactory bulb. In addition, over the past 30 years, studies have shown early olfactory deficits in AD that spread across the olfactory spectrum with as the disease worsens.^{36,37)} A β alters the activity of the OB network, resulting in a later inability to process olfactory information, as occurs in patients with AD and in transgenic animals.^{38,39)}

HaFGF plays significant roles in the development, differentiation and regeneration of brain neurons, and have similar functions to neurotrophin, such as nerve growth factors (NGF) and brain-derived neurotrophic factor (BDNF), which have been selected as new potential drugs for the treatment of AD.^{40,41)} TAT-HaFGF recombined in our lab retains the neuroprotective activities of HaFGF. TAT, a protein transduction domain with a strong positive charge, could combine with the negatively charged nasal mucosa.¹⁴⁾ It is expected that positively charged TAT can interact electrostatically with the nasal mucosal layer, and therefore retain the entrapped TAT-HaFGF on the mucosal surface, resulting in a high local concentration for absorption. Therefore, for the treatment of AD, a new molecular designing to target the olfactory mucosa such as TAT-HaFGF, which has a high affinity for the olfactory epithelium, may be rational. Similarly, a drug delivery system designed to target the trigeminal nerve may be beneficial to deliver therapeutics for the treatment of the brain diseases in caudal regions, such as cerebellar atrophy. Our findings may provide valuable information for the rational design of peptides to improve the treatment of neurological and psychiatric diseases.

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