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Brain Transport of Neurotoxin-I with PLA Nanoparticles through Intranasal Administration in Rats: A Microdialysis Study

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ABSTRACT: The purpose of this study was to encapsulate neurotoxin-I (NT-I) within polylactic acid (PLA) nanoparticles (NPs) and to evaluate their transport into the brain after intranasal administration (i.n.) using a microdialysis sampling technique. NT-I-NPs (NT-I radiolabeled with sodium [^{125}I]iodide) were prepared and characterized. Then, NT-I-NPs were administered i.n. or i.v. to rats and the radioactivities in the olfactory bulbs were monitored for up to 240 min. The nanoparticles prepared were spherical with a homogenous size distribution. The mean particle size, zeta potential and entrapment efficiency were -28.6 ± 2.3 mV, 65 nm and $35.5 \pm 2.8\%$, respectively. The brain transport results showed that the time to reach the peak level (T_{max}) of NT-I-NPs (i.n.) was 65 min, shorter than NT-I-NPs (i.v.) (95 min) or NT-I (i.v.) (145 min). The concentration at peak level (C_{max}) and the total area under the concentration–time curves from zero to 4 h ($AUC_{0-4\,h}$) of each group followed the following order: NT-I-NPs (i.n.)>NT-I-NPs (i.v.)>NT-I (i.v.). The corresponding absolute bioavailabilities (Fabs) of NT-I-NPs (i.n.) were about 160%, 196% with NT-I-NPs (i.v.) and NT-I (i.v.) as reference preparations, respectively. The brain delivery of NT-I could be enhanced with PLA nanoparticles either through i.n. or i.v. administration. Furthermore, the enhancement was more significant for i.n. than for i.v. administration. Nanoparticles as carriers would be a potential way to improve the brain transport for centrally active peptides. Copyright © 2008 John Wiley & Sons, Ltd.

Key words: neurotoxin-I; brain transport; nanoparticles; intranasal administration; microdialysis

Introduction

The delivery of drugs to the central nervous system (CNS) remains a challenge despite a significant improvement in the mechanism governing the maintenance of brain homeostasis. Specifically, the blood–brain barrier (BBB), featuring tight continuous circumferential junctions between the brain micro-vessel endothelial cells (BMECs), hinders water-soluble molecules and those with a molecular weight above 500 Da, such as therapeutic peptides, proteins, genes and antibiotics being delivered from the circulation system to the brain [1,2]. The routine approaches applied for the enhancement of drug concentrations in the brain involve craniotomy-based drug delivery, such as intraventricular drug diffusion, local intracerebral implants, or disruption of the BBB by infusion of hyperosmotic solutions or vasoactive agents prior to systemic administration of the drugs. Being highly invasive, these

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approaches are most appropriate for short-term treatments, where a single or infrequent exposure to a drug is required [3], but not an ideal method for long-term treatments.

Due to the formidable obstacle imposed by the BBB, there has been increased interest in developing strategies to overcome the BBB barrier. It has been a widely held view that intranasal (i.n.) administration provides a means of circumventing the BBB and thus may allow increased CNS penetration of compounds that otherwise display limited CNS exposure [4]. In general, there are three pathways that a drug administered into the nasal cavity may travel. These routes include entry into the systemic circulation direct from the nasal mucosa, entry into the olfactory bulb via axonal transport along neurons, and direct entry into the brain via the olfactory epithelium. A drug that enters into the systemic circulation should be absorbed through the nasal mucosa. Administration via this route avoids the hepatic/gastrointestinal first-pass effects and therefore may provide more extensive systemic exposure for substrates that have poor oral bioavailability (such as proteins or peptides etc.) [5]. Large molecules such as nerve growth factor (NGF) were reported to achieve significantly higher brain concentrations by i.n. administration than that by systemic administration at the same dose. The effectiveness of this route for enhancing the brain delivery of large molecules, including vasopressin, cholecystokinin, insulin and insulin-like growth factor-1, was demonstrated in both humans and animals. These results suggest that the nasal pathway functions for the direct delivery of drugs into the brain [6].

Another possibility of delivering drugs to the brain is by the use of nanoparticles. Nanoparticles for pharmaceutical and chemical use are defined as polymeric particles made of natural or artificial polymers ranging in size between about 10 and 1000 nm [7]. Drugs may be bound in the form of a solid solution or dispersion, or adsorbed to the surface or chemically attached. Loaded by nanoparticles, the drugs will be released at an appropriate rate and dose at specific sites in the body during a certain time to achieve accurate delivery, which will enhance the therapeutic efficacy and reduce toxicity and side effects [8]. It has been reported that nanoparticles overcoated with polysorbates (especially polysorbate 80) were capable of transporting the loaded drugs across the BBB after administration, which functioned as a tool to deliver drugs to the brain. Up to now, most studies have focused on nanoparticles of poly (butylcyanoacrylate) (PBCA), a polymer that is not authorized for use in humans [9–12]. However, PBCA will be degraded rapidly by esterases present in biological fluids and its toxic products will stimulate or damage the CNS [13]. At present, brain targeting is mainly characterized by the physiological or pharmacological reactions of tested animals that were administered model drugs mediated by nanoparticles [3,10,14]. No study on concentration–time profiles of brain transport of drug-loaded nanoparticles has yet been reported.

The objective of this work was to evaluate the brain transport of large molecular weight drug-loaded nanoparticles after i.n. administration. A new microdialysis sampling technique was used in the elementary work. Neurotoxin-I (NT-I), an analgesic peptide with limited permeability across the BBB which was separated from the venom of *Naja naja atra* [15,16], was selected as a model drug for this study. PLA, a typical biodegradable polyester currently employed in the clinic approved by Food and Drug Agency of USA, was employed instead of PBCA to avoid the toxic effects induced by the matrix material.

Materials and Methods

Chemicals

NT-I (m.w. of 6900 Da; purity of 99.3%) was provided by Kunming Institute of Zoology, the Chinese Academy of Sciences (Jiaochang East Road No. 32, Kunming City in Yunnan Province). [^{125}I]NT-I (3.8 mCi/0.90 mg; 800 μl) was supplied by Fudan University (Medical Road No. 138, Shanghai City, China). PLA (m.w. of 11 KDa) and sodium cholate were purchased from Sigma-Aldrich Corporation (St Louis, MO, USA). Ethyl acetate (analytical grade) and polysorbate-80 were products from Huadong Medicine Group Co. (Qingtai Road No. 350, Hangzhou City, China). Other reagents were analytical grade.

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Animals

The protocol for this study was approved by the Care and Use of Animals of the Zhejiang University (Yugu Road No. 20, Hangzhou City, China). Twenty male adult Sprague-Dawley rats, 13 weeks old and weighing 350 g were purchased from the Laboratory Animal Center of Zhejiang Academy of Medical Sciences (Zhejiang, China). They were maintained in a climate controlled room acclimatized for at least 5 days kept at a temperature of $22 \pm 1^{\circ}C$ with 12 h light–dark cycles and a relative humidity of $60 \pm 10\%$. Water and standard laboratory food (Zhejiang Academy of Medical Sciences, Tianmushang Road No. 182, Hangzhou City, China) were available *ad libitum*.

Preparation of nanoparticles

The procedures used for preparing the nanoparticles were similar to the reported double emulsification solvent evaporation method [17,18]. Briefly, a 50 µl aliquot of the above [^{125}I]NT-I solution containing 56 µg of toxin was emulsified in 1 ml of PLA in ethyl acetate (50 mg/ml) by sonication (JY92-IID, Ningbo Scientz Biotechnology Co. Qianfeng Road No. 9, Ningbo City in Zhejiang Province, China) on an ice-bath for 30 s (40 w). Then, 2 ml of aqueous sodium cholate solution (1%, w/v) was added and the resulting (w/o)/w emulsion was sonicated for 20 s (40 w). The double emulsion was diluted in 100 ml aqueous sodium cholate solution (0.3%, w/v) and the solvent was rapidly eliminated by evaporation under vacuum. Finally, the nanoparticles were isolated by centrifugation [$22000 \times g$, 30 min (J2-HS, Beckman Coulter, 4300 N. Harbor Blvd. Fullerton, CA 92834-3100)], washed three times with water and lyophilized. Polysorbate 80 was added at a ratio of 1:1 (w/w) to reconstitute nanoparticles and incubated for 30 min.

Physicochemical characterization of nanoparticles

The morphological examination of nanoparticles was performed using a transmission electron microscope (TEM, JEM-1200EX, Jeol, Tokyo, Japan) following negative staining with sodium phosphotungstate solution (0.2%, w/v). The particle size distribution and zeta potential of nanoparticles were determined using a Zetasizer 3000HSA (Malvern, England).

Determination of encapsulation efficiency

Nanoparticles containing [^{125}I]NT-I were prepared using the procedure indicated above, centrifuged and the supernatants were assessed in a SN-682 gamma-counter (Rihuan Instrument Factory of Shanghai Atomic Nuclide Institute, Shanghai City, China). The amount of NT-I encapsulated into the nanoparticles was calculated by the difference between the total amount used to prepare the nanoparticles and the amount of NT-I present in the aqueous phase.

Surgical procedures

Rats were anesthetized with intraperitoneal ketamine (90 mg/kg)+xylazine (10 mg/kg) and mounted on a stereotaxic frame (Bioanalytical Systems, West Lafayette, IN, USA), as reported [19]. Briefly, a 0.5 mm hole was drilled in the rat skull separately (right olfactory bulb: −1.0 mm lateral to the midsagittal suture and 6.0 mm anterior to bregma). One chronic brain microdialysis guide cannula (MD-2251, BAS, West Lafayette) with styles in place was inserted into the brain aimed at the right olfactory bulb identical to a depth of 4.6 mm ventrally from the dura according to the *Rat Brain Atlas* of Paxinos and Watson [20]. The guide cannula was fastened to the cranium with skull screws and dental acrylic cement. After surgery, the animals were allowed to recover for 6 days in single cages under standard conditions, with free access to food and water until 24 h before administration, at which time only food was withdrawn.

Recovery determination

The retrodialysis recoveries of the drug [^{125}I]NT-I *in vitro* and *in vivo* were determined as described below prior to probe implantation. The retrodialysis recoveries *in vivo* by the loss of [^{125}I]NT-I were used for further recalculations of the true extracellular drug concentrations at the sampling site of the olfactory bulb.

Both recoveries *in vitro* obtained by the difference between gain and loss (retrodialysis)

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before the rat experiments of three probes of the same type for use in brain (MD-2200, BAS, West Lafayette) with characteristics of 0.5 mm (o.d.), 2 mm (membrane length), and nominal 38 kDa [mw cut-off of four concentration levels (1, 5, 10 and 15 ng/ml)], were investigated at a flow rate of 2.0 μl/min. Thus, the recoveries *in vitro* (gain and loss) for [^{125}I]NT-I were 25.4% and 28.7%, respectively; they were not significantly different ($p>0.05$). No difference among the three individual probes was shown ($p>0.05$). The recoveries *in vitro* (gain and loss) were used only to evaluate the functional properties of each probe.

The recovery *in vivo* by the loss of [^{125}I]NT-I for each probe at the olfactory bulb was calculated by the following equation

$$\text{Recovery}(^{125}\text{I}-\text{NT}-\text{I}) = \frac{(C_{\text{NT-T.dialysate}} - C_{\text{NT-I.perfusate}})}{(C_{\text{NT-I.BIF}} - C_{\text{NT-I.perfusate}})}$$

in which $C_{NT\text{-}I}$ and the [^{125}I]NT-I concentration, given for dialysate, perfusate and BIF (brain interstitial fluid), respectively. During the retrodialysis period, the concentration of [^{125}I]NT-I in BIF was assumed to be zero because of its relative high diffusion coefficient in brain tissues. Three samples (20 μl per sample) from each period were collected and analysed over 10 min intervals (first two and last samples discarded). The recovery *in vivo* (n=12) of ^{125}I-NT-I (loss) was also conducted on the same type probes as those in *in vitro* recovery experiments with four concentration levels (1, 5, 10 and 15 ng/ml). All statistical analyses (ANOVA) were performed using Origin 6.0 (Microcal Software, Inc., Northampton, MA, USA).

Brain transport of nanoparticles

At the beginning of the experiment, potassium iodide (40 mg/kg) was orally administered in case of deposition in the thyroid gland. Then the rats were randomly divided into four groups (n=5, each group); group 1 and 2 rats were administered intranasally with NT-I-NPs (45 mg lyophilized nanoparticles was dissolved in 0.15 ml PBS with addition of 1:1 (w/w) polysorbate 80, 17 μg/kg) and free NT-I solution, respectively. Each animal was dosed intranasally by instilling 80 μl in each nostril (15 μl three times in 3 min time-intervals) of the preparations. A supine-70 position was employed in rats controlled using a home-made patent device when the nasal formulations were dosed. Nasal preparations were administered using a soft PE-10 tubing (Becton Dickinson and Co., Sparks, Maryland, USA) fitted to a 50 μl Hamilton microsyringe, inserting 15 mm into the cavity in the right nostril [21]. Groups 3 and 4 were treated intravenously with the same formulations as groups 1 and 2 (dose, 115 μg/kg). All microdialysis experiments were performed in the awake state (freely moving) kept in an awake animal caging system (Stand-Alone Raturn and Rodent Bowl kit, BAS, West Lafayette), with no anaesthesia used throughout the experiment.

On the day of the experiment, the brain microdialysis probe (MD-2200) was inserted into the guide cannula, 30 min before sampling was begun. The inlet of the probe was attached to a BAS syringe driver (MD-1001, BAS, West Lafayette, USA) connected as a controller (240V/50 Hz MD-1000 K, BAS, West Lafayette), filled with a modified Ringer's solution as perfusion fluid (145 mM NaCl, 3.7 mM KCl, 1.0 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM $NaHCO_3$, 0.1 mM ascorbic acid, pH 7.4). The brain microdialysis probe was simultaneously perfused at a flow rate of 2 μl/min for 30 min (delay time) and all microdialysates collected were discarded in order to eliminate the void volume in tubing and to stabilize solute levels around the dialysis membrane before starting to collect samples. After this equilibrium period, brain microdialysate samples were collected using a refrigerated fraction collector (MD-1201, BAS, West Lafayette) into 300 μl vials at a 10 min sampling regime automatically continued for 240 min (sample volume of 20 μl per vial).

After each experiment, the animals were anesthetized with excessive amount of chloral hydrate and transcardially perfused with 4% paraformaldehyde in 0.1M Sørenson buffer as described previously [22]. The brains were dissected out, immediately frozen and stored at 20°C. Coronal 40 μm thick slices were later cut with a cryo-microtome and the unstained sections were observed under magnification to localize the probe tract. Only animals with the probe located in the olfactory bulb and cerebellar nuclei were included in the present study.

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Data analysis

Data are presented as mean ± SD. A two-tailed Student's *t*-test was used to evaluate the statistical significance of differences between experimental groups. In all cases, $p < 0.01$ was used as a criterion for statistical significance.

The time to reach peak level (T_{max}) and peak concentrations (C_{max}) were directly obtained from the observed data of the concentration–time curves. The total area under the concentration–time curve from time zero to 5 h ($AUC_{0-4\,h}$) was calculated using the linear trapezoidal rule method.

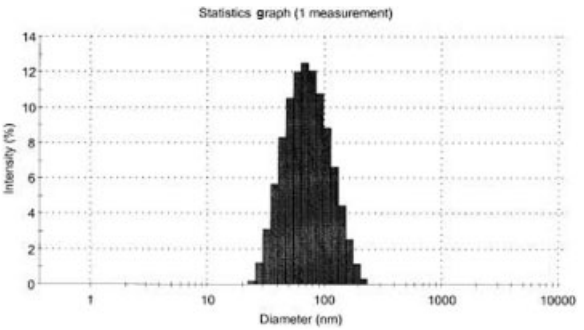

Figure 2. Particle size distribution of NT-I-NPs

Results

Physicochemical characteristics of nanoparticles

Nanoparticles were initially characterized for morphology, particle size distribution, zeta potential and encapsulation efficiency. As can be seen in Figures 1 and 2, nanoparticles were spherical with a homogenous size distribution and had a sub-micron particle size (65 nm). The zeta potential of the nanoparticles was −28.6 ± 2.3 mV. NT-I could be encapsulated efficiently in nanoparticles (35.5 ± 2.8% encapsulation efficiency). Nanoparticles were stable by lyophilization and in various buffers.

Recovery determination of microdialysis probes

Average recovery by loss (retrodialysis) *in vivo* (n=12) of three brain probes (MD-2200) with four concentrations of [^{125}I]NT-I used in the right olfactory bulb of the rat was 14.1% (± 1.2%) (p>0.05). This was used to correct the actual concentration of NT in dialysates sampled from the right olfactory bulb in rats.

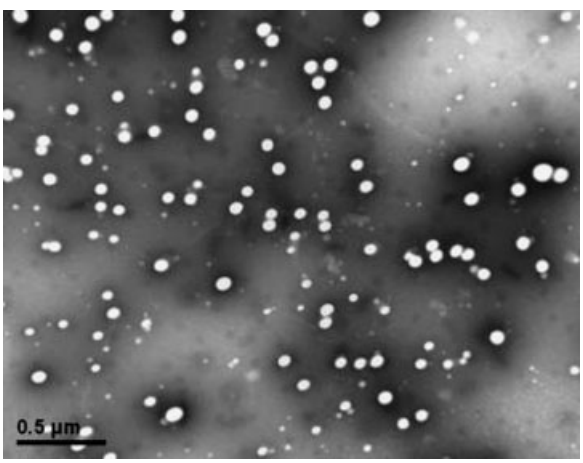

Figure 1. Transmission electron micrography of NT-I-NPs

Brain transport of nanoparticles

The present results showed that the brain delivery of NT-I could be enhanced with PLA nanoparticles either via i.n. or i.v. administration. The enhancement was more significant for i.n. administration than for i.v. administration. The concentration–time curves from different groups are shown in Figure 3.

The transport parameters, T_{max}, C_{max} and $AUC_{0-4\,h}$ from different groups are shown in Table 1. These results suggest that PLA nanoparticles play an important role in the transport of NT-I to the brain. These results are in good agreement with previously reported results [9–12].

Discussion

Centrally active proteins and peptides with poor bioavailability and severe limitation of BBB penetration have been increasingly developed, which makes the administration mode more challenging. Recent developments have gradually revealed that i.n. administration is a promising alternative for brain delivery for large molecular weight drugs [23]. A drug administered by the nasal route may primarily enter the blood of the general circulation and then pene-

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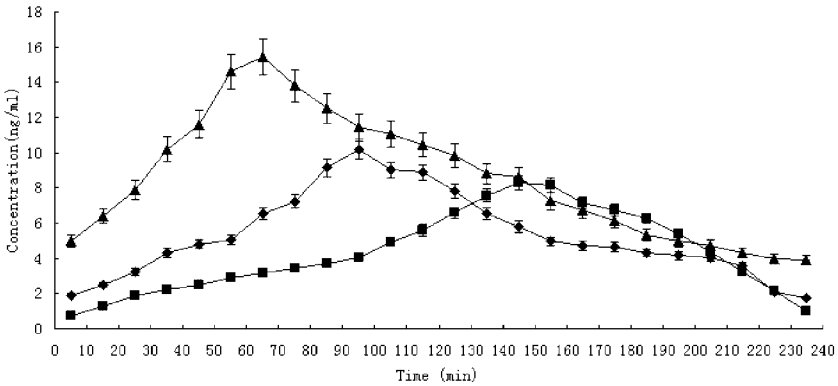

Figure 3. Mean concentration–time curves in the olfactory bulbs from rats of NP-I-NPs (▲ i.n., ◆ i.v.) and NT-I (■ i.v.). Data are mean ± SD (n=5)

Table 1. Brain transport parameters of NT-I-NPs or NT-I through i.n. or i.v. administration. Data are mean ± SD (n=5)

Parameter	NT-I-NPs (i.n.)	NT-I-NPs (i.v.)	NT-I (i.v.)
T_{max} (min)	65	95	145
C_{max} (ng/ml)	15.41 ± 1.11	10.22 ± 1.03	8.30 ± 0.67
$AUC_{0-4\,h}$ (ng min/ml)	2000 ± 52.11[ab]	1250 ± 31.41	1020 ± 30.83

[a]$p<0.01$ compared with NT-I-NPs (i.v.); [b]$p<0.01$ compared with NT-I (i.v.).

trate across BBB into the CNS. However, this mode of administration may also allow circumvention of the BBB, thereby delivering drugs directly into the brain. With the advance of nanotechnology, polymer nanoparticles can be employed as carriers to transport the entrapped or adsorbed drugs across the BBB. Since Kreuter *et al*. first reported that polysorbate 80-coated PBCA nanoparticles could deliver the peptide dalargin into CNS to exert its analgesic effect in 1995 [24], several drugs have been successfully delivered into the brain by this colloidal carrier. Our present findings suggest that nanoparticles could exert enhanced delivery of NT-I into the brain. The enhancement was more significant after i.n. administration.

The present results on brain transport showed that the T_{max} (approximately 65 min) of NT-I-NPs (i.n.) was shorter than that of NT-I-NPs (i.v.) (95 min) and NT-I (i.v.) (145 min). It seemed that the brain delivery speed was quicker for NT-I-NPs (i.n.) than that for NT-I-NPs (i.v.) and NT-I (i.v.). The difference in either C_{max} or $AUC_{0-4\,h}$ among these three groups were significant ($p<0.01$). The corresponding absolute bioavailability (F_{abs}) of NT-I-NPs (i.n.) were about 160%, 196% with NT-I-NPs (i.v.) and NT-I (i.v.) as reference preparations respectively. It implied that the brain delivery of NT-I could be enhanced with NPs as carriers and being administered intranasally, this being consistent with Zhang's conclusion. Zhang *et al*. demonstrated that MPEG-PLA nanoparticles showed a potential for improving the efficacy of the direct nose–brain transport for drugs [25]. These results indicate that although the majority of drugs would be absorbed into the systemic circulation through the nasal mucosa, and a small amount of drug would be delivered direct from the nasal cavity. Several studies have shown a direct route of transport from the olfactory region to the CNS in animal models, without prior absorption into the circulating blood [26–28]. For instance, in our previous study, with borneol–menthol eutectic mixture as an absorption enhancer, NT-I could be transported direct from the nasal cavity to the brain [29]. Although i.n. administration has been used for brain delivery of some drugs, a comprehensive understanding of the mechanisms governing this pathway is necessary in

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order to investigate the potential use of nasal administration as a practical means of delivery of agents to the brain. There are three nose–brain pathways proposed as modes for a drug to reach the CNS from the nasal cavity: transcellular pathway, paracellular pathway and intracellular axonal transport [30]. However, whether or not the NT-I-NPs could be delivered to the brain through the direct nose–brain pathway needs to be studied further.

From the current results, it was found that both the C_{max} and $AUC_{0-4\,h}$ of the three groups was as follows: NT-I-NPs (i.n.)>NT-I-NPs (i.v.)>NT-I (i.v.). These results suggest that nanoparticles could significantly improve the drug transport to the brain either through i.n. or i.v. administration. This conclusion was demonstrated by many other investigators. For example, Gao *et al*. found that nanoparticles overcoated by polysorbate 80 could notably enhance the drug level into both the brain tissues and cerebrospinal fluids after i.v. administration compared with uncoated ones and simple solution [31]. The first drug that was transported across the BBB with nanoparticles as carriers was dalargin. After adsorption to the nanoparticles and i.v. injection, dalargin led to a dose- and time-dependent antinociceptive effect using the tail-flick test as well as in the hot plate test [10,14,24,32]. Other drugs that have been transported across the BBB using the polysorbate 80-coated PBCA nanoparticles including kytorphin (dipeptide), loperamide, tubocurarine, MRZ 2/576 and MRZ 2/596 (NMDA-receptor antagonists), doxorubicin, methotrexate [7,24,31], etc.

A number of possibilities exist that could explain the mechanisms of the delivery of the above mentioned substances across the BBB [7,12,33]: (1) An increased retention of the nanoparticles in the brain blood capillaries combined with an adsorption to the capillary walls. This could create a higher concentration gradient that would enhance the transport across the endothelial cell layer and delivery into the brain. (2) A general surfactant effect characterized by a solubilization of the endothelial cell membrane lipids that would lead to membrane fluidization and an enhanced drug permeability through the blood–brain barrier. (3) The nanoparticles could lead to an opening of the tight junctions between the endothelial cells. The drug could then permeate through the tight junctions as a free form or together with the nanoparticles in bound form. (4) The nanoparticles may be endocytosed by the endothelial cells followed by the release of the drugs within these cells and delivery to the brain. (5) The nanoparticles as bound drugs could be transcytosed through the endothelial cell layer. (6) The polysorbate 80 used as the coating agent could inhibit the efflux system, especially P-glycoprotein (Pgp). All these mechanisms also could work in combination. In the present work, the mechanism of NT-I transport into the brain with NPs as carriers after i.n. administration remains to be further studied.

Microdialysis, originally developed to monitor neurotransmitter concentrations in the brain, has been proven by those who have used it, to be a valuable method for studying drug delivery to the CNS. The method saves animals, since many data can be obtained within one experiment and since it is possible to perform crossover experiments in animals. The technique also provides data on temporal aspects of drug delivery to the CNS. With microdialysis it is possible to calculate the ratios of drug concentrations in the brain ECF to those in the blood, quantitating the influence of active transport mechanisms at the BBB, in order to estimate influx and efflux clearances that are independent of binding in blood or brain tissue, and to estimate the amount of binding that takes place in blood and brain tissue.

Another advantage of microdialysis is that it makes it possible to study hydrophilic drugs, which is difficult with other *in vivo* methods. It is also possible to estimate drug distribution within brain tissue, i.e. whether the drug is distributed mainly into brain ECF or whether it is also transported intracellularly or bound to extracellular components [34]. The results showed that microdialysis is quite suitable for the brain delivery research of drugs [34].

In conclusion, the brain delivery of NT-I could be enhanced with PLA nanoparticles either through i.n. or i.v. administration. The enhancement is more significant for i.n. than for i.v. administration. With nanoparticles as carriers, i.n. administration would be a potential way to deliver centrally active peptides to improve their brain transport. Microdialysis is quite a

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good technique for the study of drug delivery to the brain.

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