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Research paper

# Sodium hyaluronate as a mucoadhesive component in nasal formulation enhances delivery of molecules to brain tissue

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ABSTRACT

Intranasal administration of molecules has been investigated as a non-invasive way for delivery of drugs to the brain in the last decade. Circumvention of both the blood–brain barrier and the first-pass elimination by the liver and gastrointestinal tract is considered as the main advantages of this method. Because of the rapid mucociliary clearance in the nasal cavity, bioadhesive formulations are needed for effective targeting. Our goal was to develop a formulation containing sodium hyaluronate, a well-known mucoadhesive molecule, in combination with a non-ionic surfactant to enhance the delivery of hydrophilic compounds to the brain via the olfactory route. Fluorescein isothiocyanate-labeled 4 kDa dextran (FD-4), used as a test molecule, was administered nasally in different formulations to Wistar rats, and detected in brain areas by fluorescent spectrophotometry. Hyaluronan increased the viscosity of the vehicles and slowed down the in vitro release of FD-4. Significantly higher FD-4 transport could be measured in the majority of brain areas examined, including olfactory bulb, frontal and parietal cortex, hippocampus, cerebellum, midbrain and pons, when the vehicle contained hyaluronan in combination with absorption enhancer. The highest concentrations of FD-4 could be detected in the olfactory bulbs, frontal and parietal cortex 4 h after nasal administration in the mucoadhesive formulation. Intravenous administration of a hundred times higher dose of FD-4 resulted in a lower brain penetration as compared to nasal formulations. Morphological examination of the olfactory system revealed no toxicity of the vehicles. Hyaluronan, a non-toxic biomolecule used as a mucoadhesive in a nasal formulation, increased the brain penetration of a hydrophilic compound, the size of a peptide, via the nasal route.

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## 1. Introduction

Targeting of drugs to the central nervous system (CNS) is still a difficult task to fulfill because the blood–brain barrier (BBB), segregating the brain interstitial fluid from the circulating blood, and the blood–cerebrospinal fluid barrier, separating the blood from the cerebrospinal fluid, prevents the influx of hydrophilic compounds with a molecular weight above 600 Da from the systemic circulation into the brain. Hence, these barriers actively controlling cellular and molecular trafficking prevent the utilization of many novel therapeutic agents for treating CNS disorders such as Parkinson's and Alzheimer's diseases [1,2]. In order to enhance the blood–brain transport and to deliver drugs to the brain in an effective concentration, several approaches have been attempted. These methods include the manipulation of BBB (hyperosmotic shock, vasoactive substances, inhibition of efflux transporters) and the modification of the drug molecules (lipophilic or cationic molecules, prodrugs, binding of drugs to carriers, for example, transferrin, or targeted vesicle systems) [1–8]. However, the increased transport of drugs to the central nervous system can be carried out not only by the modification of the BBB function or the drug molecule itself, but also via an alternative route by the selection of an application site circumventing the BBB.

In recent years, nasal route for delivery of drugs to the brain via the olfactory region has received a lot of attention [9–11]. Olfactory sensory neurons are the only first-order neurons whose cell bodies are located in a distal epithelium. Their dendritic processes are directly exposed to the external environment in the

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upper nasal passage, while their axons project through perforations in the cribriform plate of the ethmoid bone to synaptic glomeruli in the olfactory bulb. These unique anatomic and physiologic properties of the olfactory region provide both extracellular and intracellular pathways into the CNS that bypass the BBB [9–13]. Intranasal delivery of drugs offers several advantageous properties. This method is non-invasive, essentially painless, and can be easily and readily administered by the patient or a physician. Furthermore, it ensures rapid absorption, the avoidance of first-pass metabolism in gut and liver and does not require sterile preparation [12,14,15].

During the formulation of a dosage form intended for intranasal application, several aspects should be taken into consideration [16]. The olfactory region in man is situated in the upper part of the nasal cavity, an area that is difficult to reach with presently available nasal spray or powder devices. Furthermore, the nasally administered drugs will normally be cleared rapidly from the nasal cavity into the gastrointestinal tract by the mucociliary clearance system [9–11]. Another important factor limiting the nasal absorption of large molecular weight or polar drugs is the low membrane permeability. The epithelial cells of nasal mucosa are closely connected on the apical surface by intercellular junctions [17], which hinder the paracellular transport of polar drugs between the cells. Moreover, the nasal mucosa has a metabolic capacity as well, which can contribute to the low transport of peptides and proteins across the nasal membrane [12,14–16].

Considering the above-mentioned aspects, our aim was to formulate a carrier system, which combines bioadhesive and absorption enhancer properties to prolong retention time and to increase membrane permeability of a test molecule [18,19].

Polyethoxylated 40 hydrogenated castor oil (Cremophor RH40) is a non-ionic solubilizing and emulsifying agent. This surfactant can be used to increase bioavailability of drugs by solubilizing of poorly soluble compounds and by increasing of cell membrane fluidity. Furthermore, Cremophors have been shown to inhibit P-glycoprotein activity, therefore, they increase the bioavailability of drugs which are known substrates of this efflux transporter [20–23].

Sodium hyaluronate is the sodium salt of the hyaluronic acid, a naturally occurring linear polysaccharide composed of alternating disaccharide units of *N*-acetyl-D-glucosamine and D-glucuronic acid [24]. Hyaluronic acid, a non-sulfated glycosaminoglycan, can be found in the extracellular tissue matrix of vertebrates, including connective tissue, synovial fluids, vitreous humour and aqueous humour [25]. It plays a critical role as a signaling molecule in cell motility, cell differentiation, and wound healing. This natural anionic polysaccharide has an excellent mucoadhesive capacity [24,26] and many important applications in formulation of bioadhesive drug delivery systems [27]. Besides its mucoadhesive properties, it was found that this biopolymer may enhance the absorption of drugs and proteins via mucosal tissues [26,28,29]. While hyaluronan is used in diverse drug delivery systems e.g. ophthalmic, pulmonary and vaginal [24,26,30], it has not been widely exploited for nasal drug delivery to the nervous system.

In this study, the nose-to-brain transport was investigated in case of different carrier systems. Fluorescein isothiocyanate (FITC)-labeled dextran, a hydrophilic compound with an average molecular weight of 4.4 kDa (FD-4), the size of a peptide, with no transporter at the BBB, was selected as a test molecule. Vehicles containing absorption enhancer polyethoxylated 40 hydrogenated castor oil or both mucoadhesive polymer sodium hyaluronate and absorption enhancer were formulated and tested in rats.

## 2. Materials and methods

### 2.1. Materials

All reagents, including FITC-labeled dextran (Mw = 4.4 kDa) were purchased from Sigma-Aldrich Chemical Co. (MO, USA), unless otherwise indicated. Polyethoxylated 40 hydrogenated castor oil (Cremophor RH40) was obtained from BASF (Germany). Sodium hyaluronate (Mw = 1400 kDa) was obtained as a gift from Gedeon Richter Ltd. (Hungary). All other materials were of reagent grade.

### 2.2. Preparation of dosing solutions

Cremophor RH40 was dissolved in the physiological saline solution (PhS; 0.9% w/v sodium chloride in sterile distilled water). In case of sodium hyaluronate-containing samples the mucoadhesive polymer was added in small amounts to the solution. In order to ensure the complete solvation of polymers, samples were rehomogenized after 24 h. The FD-4 was dissolved in the prepared vehicles. The concentration of the test molecule was 1 mg/ml for intranasal and 8 mg/ml for intravenous administration. The compositions of the dosing solutions were as follows: (i) physiological saline (PhS) 100% w/w, (ii) absorption enhancer solution (AE) 10% w/w Cremophor RH40 and 90% w/w physiological saline, (iii) hyaluronate solution (HA) 1.5% w/w sodium hyaluronate and 90% w/w physiological saline, (iv) mucoadhesive solution (MA) 1.5% w/w sodium hyaluronate, 10% w/w Cremophor RH40 and up to 100% w/w physiological saline.

### 2.3. Rheological measurements

Rheological measurements were carried out with a Rheostress 1 Haake instrument. A cone-plate measuring device was used in which the cone angle was 1°, and the thickness of the sample was 0.048 mm in the middle of the device. The flow and viscosity curves of the samples were determined by changing the shear rate between 0.01 and 100 $s^{-1}$ at 37 °C.

### 2.4. In vitro drug release studies

In case of samples with 1 mg/ml FD-4 content, in vitro drug release experiments were performed as well. The dissolution studies were carried out using ointment cells and small volume dissolution vessels in a Hanson SR8plus dissolution apparatus (Chatsworth, CA). Samples, 0.4 g each, were placed as donor phase on the Porafil membrane filter (pore diameter 0.45 μm). The effective diffusion surface area was 1.767 $cm^2$. Phosphate-buffered saline solution (PBS; pH = 7.4, 100 ml) was used as dissolution medium at a temperature of 37 °C and a paddle speed of 50 rpm. Samples (3 ml each) were taken and immediately replaced with fresh dissolution medium at 15, 30, 60, 120, 180, 240, 300, 360, 420 and 480 min, and further analyzed by spectrofluorometry. Six parallel measurements were performed in case of each dosing solution. Dissolution profiles were compared by using difference factor $f_1$ and similarity factor $f_2$. For two similar preparations, the value of $f_1$ must be between 0 and 15 and that of $f_2$ must be in the range of 50–100. The factors can be calculated according to the following equations: [31–33],

$$f_1 = \left\{ \left[ \sum_{t=1}^{n} |R_t - T_t| \right] \Big/ \left[ \sum_{t=1}^{n} R_t \right] \right\} \times 100 \quad (1)$$

$$f_2 = 50 \times \log \left\{ \left[ 1 + (1/n) \sum_{t=1}^{n} (R_t - T_t)^2 \right]^{-0.5} \times 100 \right\} \quad (2)$$

where $n$ is the number of time points, $R$ is the dissolution value of the reference batch at time $t$, and $T$ is the dissolution value of the test batch at time $t$.

### 2.5. Animal experiments

Male Wistar rats (316 ± 48 g, 3-month-old) were used for the study. The number of rats used for each treatment group was 4. The experiments performed conform to European Communities "Council directive for the care and use of laboratory animals" and were approved by local authorities (XVI./03835/001/2006). The experimental groups, the dosing solutions and treatments, the applied volumes, concentration and dose of FD-4 are summarized in Table 1.

#### 2.5.1. Treatments

Nasal administration was performed as follows: The rats were anesthetized ip. with Avertin solution (1.25%, 10 ml/kg b.w.) and they were placed in supine position. An average of 40 μl solution was then administrated by micropipette positioned 5 mm deep into the right naris to achieve the longest possible residency time of the vehicle in the nasal cavity. In case of intravenous treatment 500 μl solution was injected into the tail vein of anesthetized animals. There were five treatment groups as shown in Table 1, and five different time points, 30, 60, 120, 240 and 480 min, during the experiments.

#### 2.5.2. Collection of plasma and brain samples for the measurement of FD-4 levels

In deep anesthesia 30, 60, 120, 240, 480 min after the treatments, 200 μl blood was taken by cardiac puncture into heparinized tubes from all four rats in each treatment group and at each time point, then the animals were transcardially perfused (100 ml PBS, pH = 7.4). The brains were removed and 10 brain regions including the olfactory bulb, frontal and parietal cortex from the left and the right side, the hippocampus, the midbrain, the pons and the cerebellum were excised. The weight of the brain samples was measured. Blood samples were immediately centrifuged (10 min, 3000$g$), and plasma samples were transferred to new tubes. All samples were kept frozen until further investigations.

#### 2.5.3. Ultrathin section electron microscopy

Rats were transcardially perfused with 2.5% glutaraldehyde buffered in 0.1 M cacodylate buffer (pH 7.4). Thereafter, the olfactory tissue was dissected out and postfixed in the identical fixative for further 4 h, and then stored in pure cacodylate buffer without glutaraldehyde. The tissues were postfixed in 1% $OsO_4$ in 0.1 M cacodylate buffer and then dehydrated in an ethanol series (50, 70, 96, 100%). The 70% ethanol was saturated with uranyl acetate for contrast enhancement. Dehydration was completed in propylene oxide. The specimens were embedded in Araldite (Serva, Heidelberg, Germany). Ultrathin sections were produced on a FCR Reichert Ultracut ultramicrotome (Leica, Bensheim, Germany), mounted on pioloform-coated copper grids, contrasted with lead citrate and analyzed and documented with an EM10A electron microscope (Carl Zeiss, Oberkochen, Germany).

**Table 1**
The composition of the experimental groups.

| Vehicle | Treatment | $n$ | $V_{FD-4}$ | $C_{FD-4}$ | $m_{FD-4}$ |
|---|---|---|---|---|---|
| PhS | Intravenous | 4 | 500 μl | 8 mg/ml | 4000 μg |
| PhS | Intranasal | 4 | 40 μl | 1 mg/ml | 40 μg |
| AE | Intranasal | 4 | 40 μl | 1 mg/ml | 40 μg |
| HA | Intranasal | 4 | 40 μl | 1 mg/ml | 40 μg |
| MA | Intranasal | 4 | 40 μl | 1 mg/ml | 40 μg |

$n$, number of animals/group; $V$, volume of the dosing solution given for treatment; $C$, concentration of FD-4 in dosing solutions; $m$, quantity of FD-4 given in dosing solutions/animal; PhS, physiological saline solution; AE, vehicle containing absorption enhancer Cremophor RH40; HA, vehicle containing hyaluronan alone; MA, vehicle containing mucoadhesive hyaluronan and absorption enhancer Cremophor RH40.

### 2.6. Determination of FD-4 concentration of samples

Brain samples were homogenized with 1 ml of 7.5% w/v trichloroacetic acid solution in Potter–Elvehjem tissue grinder. Homogenized samples were centrifuged at 9500$g$ for 10 min at 4 °C. 800 μl of supernatant was neutralized by addition of 2N NaOH solution. In case of samples obtained from the in vitro drug release experiment, the above-mentioned pre-treatment was not required. The FD-4 content of samples was determined by a PTI spectrofluorometer (Photon Technology International Inc., South Brunswick, NJ, USA) at excitation wavelength of 492.5 nm and emission wavelength of 514.5 nm. The sensitivity of the measurement was 1 ng/ml FD-4 in the samples. Linearity of the measurement of FD-4 was $r^2 \geqslant 0.9986$, while the precision was found to be RSD $\leqslant$ 9.19% across all the concentration ranges used in the study.

### 2.7. Statistical analysis

All data presented are means ± SEM or SD The values were compared using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). The analysis of variance was followed by Newman–Keuls multiple comparison test. Changes were considered statistically significant at $P < 0.05$.

## 3. Results

### 3.1. Rheological measurements

Rheological parameters like viscosity of the vehicle may influence the diffusion speed of the incorporated drug and play an important role in the drug release process. The flowcurves obtained in PhS, AE, HA and MA samples are shown on Fig. 1. Low shear stress values and a linear correlation between shear stress and shear rate were measured for both the physiological saline solution and the absorption enhancer-containing system, typical for Newtonian flow behavior. The addition of the surface active agent in AE slightly increased the slope, reflecting the viscosity of the sample. The viscosity values of HA and MA vehicles containing sodium hyaluronate are two and three order of magnitude higher,

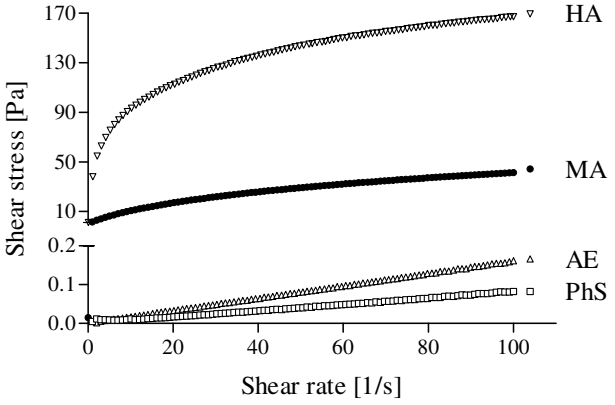


**Fig. 1.** Flowcurves of the physiological saline solution (PhS), the absorption enhancer Cremophor RH40 containing solution (AE), vehicle containing hyaluronan alone (▽) HA, and vehicle containing mucoadhesive hyaluronan and absorption enhancer Cremophor RH40 (MA), $n = 4$.

respectively, than for PhS and AE. HA and MA solutions can be characterized by pseudoplastic flow behavior. The presence of the surfactant Cremophor RH40 in MA has decreased the viscosity of the vehicle to one-tenth as compared to HA (Fig. 1).

### 3.2. In vitro drug release studies

The in vitro dissolution profiles of FD-4 from different vehicles are shown in Fig. 2a. Carriers PhS and AE, not containing biopolymer, showed similar dissolution kinetics and profiles, but in the case of vehicles containing the mucoadhesive hyaluronan, HA and MA, different dissolution profiles could be detected (Fig. 2a, Table 2).

The addition of hyaluronan slowed the release of FD-4 between 30–240 min. This effect can be related to the viscosity-increasing effect of the polymer in agreement with the data of the rheological measurements (Fig. 1). Despite the differences in the dissolution profiles, the same amount of FD-4 was released from the vehicles at 8 h (Fig. 2b).

### 3.3. Quantitative investigation of FD-4 transport to the systemic circulation and to brain

#### 3.3.1. Kinetics in plasma

Intravenous injection of 4 mg FD-4 resulted in a high concentration, 8.25 μg/ml in the plasma at 30 min (Fig. 3) was still higher than 100 ng/ml 4 h after the injection. When FD-4 was administered nasally (for doses see Table 1), three orders of magnitude smaller plasma concentrations were measured. The highest plasma concentration, 20 ng/ml was measured in the MA group at 2 h time point. FD-4 levels in all groups were lower than 10 ng/ml at 8 h.

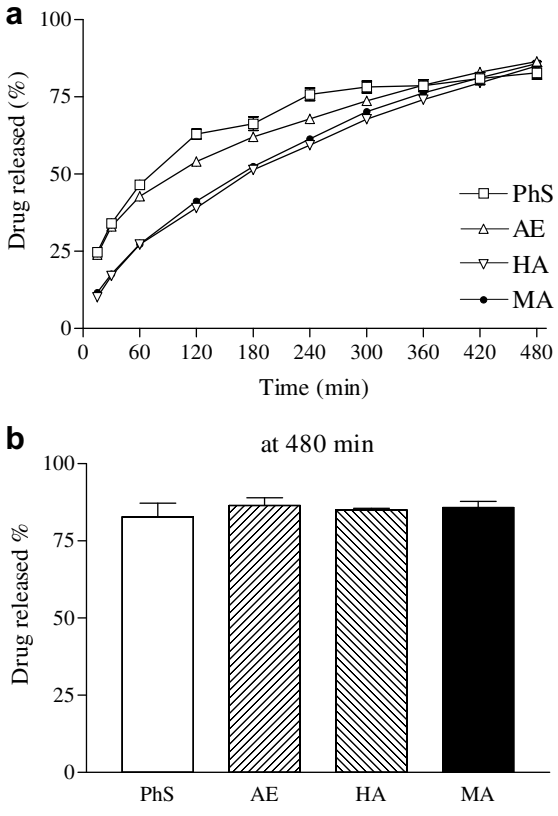


**Fig. 2.** Results of in vitro drug release studies. Dissolution profiles of FD-4 from different vehicles. (□) PhS, (△) AE, (▽) HA, (●) MA, (a). The amount of released FD-4 after 480 min of dissolution time (b). Data are presented as mean ± SEM, $n = 12$).

**Table 2**
Comparison of dissolution profiles.

| | PhS | | AE | | MA | |
|---|---|---|---|---|---|---|
| AE | $f_1$ = 6.11 Similar | $f_2$ = 66,85 | | | | |
| MA | $f_1$ = 19.96 Different | $f_2$ = 43.84 | $f_1$ = 15.38 Different | $f_2$ = 50.47 | | |
| HA | $f_1$ = 22.36 Different | $f_2$ = 42.03 | $f_1$ = 18.02 Different | $f_2$ = 48.22 | $f_1$ = 3.13 Similar | $f_2$ = 86.08 |

PhS, physiological saline solution; AE, vehicle containing absorption enhancer Cremophor RH40; HA, vehicle containing hyaluronan alone; MA, vehicle containing mucoadhesive hyaluronan and absorption enhancer Cremophor RH40.

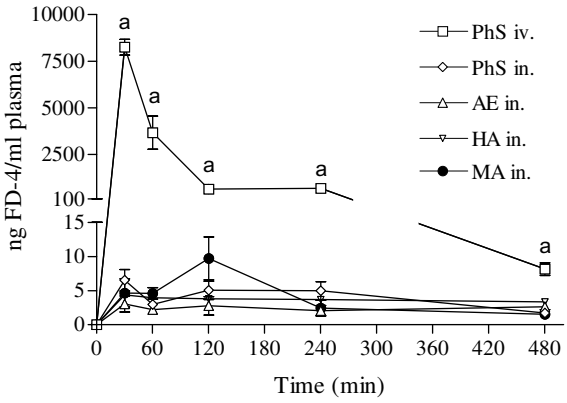


**Fig. 3.** Plasma concentrations of FD-4, in vivo experiments. (□) PhS iv., (■) PhS in., (△) AE in., (▽) HA, (●) MA in., [a]Concentrations measured in PhS iv. group were significantly ($P < 0.001$) higher at each time point than those in the other three groups. Plasma FD-4 levels did not differ significantly in the four intranasal groups at any time point. Data are presented as mean ± SEM, $n = 4$.

The plasma area under the concentration-versus-time curve (AUC) value of the intravenous injection group (PhS iv., Table 3) is about 3–500 times higher than the AUC values in the nasal treatment groups, which do not differ from each other statistically. Taking into account the 100 times difference in the doses administered intravenously or nasally, the relative bioavailability (BA) of the FD-4 given in nasal vehicles was about the fifth or third of the relative BA of the intravenous FD-4.

#### 3.3.2. Regional distribution in brain

The highest concentration of FD-4 was measured at 240 min in the olfactory bulb and in the frontal cortex in case of vehicle MA, containing both hyaluronate and Cremophor (Fig. 4). In case of other carriers, smaller peaks can be observed and the corresponding *t*-max values are smaller. FD-4 concentration in different brain regions is compared at 4 h after the treatments (Fig. 5). The highest values were obtained in case of MA mucoadhesive formulation. Although the concentration of the FD-4 decreases with the increasing distance from the nasal cavity, this vehicle significantly enhances the nose-to-brain transport of the test molecule even in the pons and the cerebellum.

The pharmacokinetic parameters in the brain obtained after intranasal and intravenous administrations of FD-4 are summarized in Table 4. The largest AUC value for all brain regions measured was obtained in case of the MA vehicle. To calculate the relative bioavailability, the one hundred times higher dose of FD-4 applied intravenously was considered one. The highest relative BA value was 561.5 in case of MA formulation. Furthermore, the calculated relative bioavailability values were two orders of magnitude higher after the intranasal treatments as compared to the intravenous administration. AUC values in various brain regions were also determined, and the MA group had significantly higher

**Table 3**
Pharmacokinetic parameters in plasma.

| Vehicle | $c_{max}$ [ng FD-4/ml plasma] mean ± SD | $t_{max}$ [min] | AUC mean ± SD | Relative BA mean ± SD |
|---|---|---|---|---|
| PhS iv. | 8253 ± 848 | 30 | 592411 ± 82638 | 1.00 ± 0.14 |
| PhS in. | 6.52 ± 3.16 | 30 | 1887 ± 730 | 0.32 ± 0.12 |
| AE in. | 3.04 ± 2.35 | 30 | 1129 ± 567 | 0.19 ± 0.10 |
| HA in. | 4.36 ± 0.53 | 30 | 1719 ± 79 | 0.29 ± 0.01 |
| MA in. | 9.73 ± 6.27 | 120 | 1847 ± 1060 | 0.31 ± 0.18 |

AUC, area under the concentration-versus-time curve; BA, bioavailability compared to the PhS iv. group; PhS, physiological saline solution; AE, vehicle containing absorption enhancer Cremophor RH40; HA, vehicle containing hyaluronan alone; MA, vehicle containing mucoadhesive hyaluronan and absorption enhancer Cremophor RH40; $n = 4$.

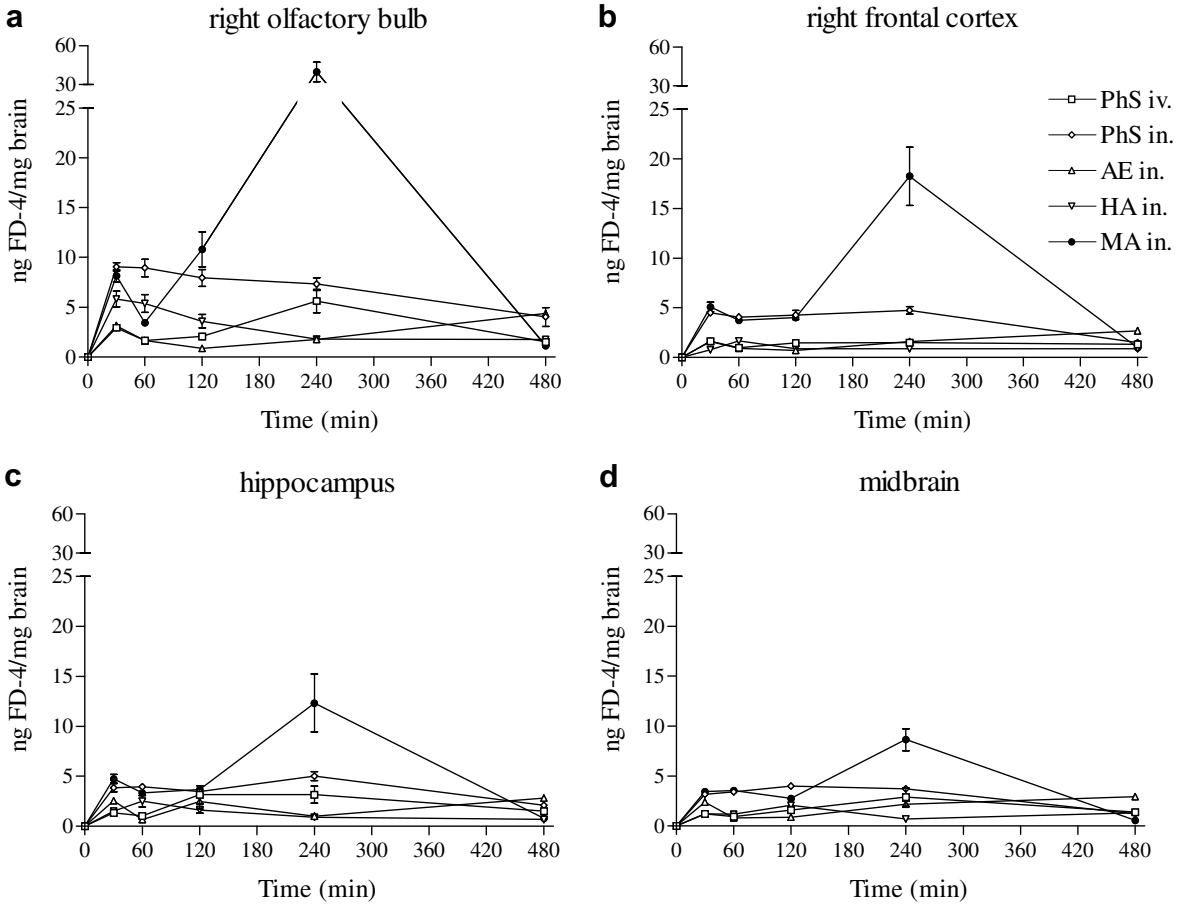


**Fig. 4.** Brain concentrations of FD-4, in vivo experiments. (□) PhS iv., (■) PhS in., (△) AE in., (▽) HA, (●) MA in., (a) Olfactory bulb from the right side, (b) frontal cortex from the right side, (c) hippocampus, (d) midbrain. Data are presented as mean ± SEM, $n = 4$.

AUC in the right olfactory bulb (OBR) and frontal cortex (FCR) than the other four treatment groups (OBR: PhS. iv. 1548 ± 865, PhS. in. 3197 ± 1066, AE 1096 ± 144, HA 1278 ± , 430, MA 8693 ± 5214, $P < 0.021$; FCR: PhS. iv. 653 ± 85, PhS. in. 1738 ± 136, AE 766 ± 129, HA 465 ± 112, MA 4087 ± 1067, $P < 0.009$).

In case of MA, nose-to-brain transport of the test molecule was also confirmed by fluorescent microscopy of frozen brain sections (data not shown). In agreement with the quantification data, high fluorescent intensity could be observed in the olfactory bulb and the frontal cortex, while no signal was detected in sections of control animals treated with solutions without FD-4 treated.

### 3.4. Electron microscopy

To test for toxic side-effects of the solutions, the olfactory system was investigated by electron microscopy (Fig. 6). The olfactory epithelium, the epithelial cells and the apical microvilli of rats treated with MA solution did not show any pathological alteration in comparison with the untreated tissue (Fig. 6A and B), indicating that the formulation has no toxic effect in the olfactory tissue. No morphological change could be observed in the olfactory fila within the lamina propria underneath the olfactory epithelium, showing again that the MA treatment did not induce any pathological effect on the ultrastructure of the sensory axons when compared with the untreated nerve (Fig. 6C and D).

## 4. Discussion

Despite the potential of the nasal route, several factors limit the intranasal absorption of drugs [9–11]. Mucociliary clearance, enzymatic activity, and the epithelium combined with the mucus layer constitute barriers to the nasal absorption especially of high-molecular-weight and hydrophilic drugs [16]. Therefore, the use of absorption enhancers and the design of suitable dosage formu-

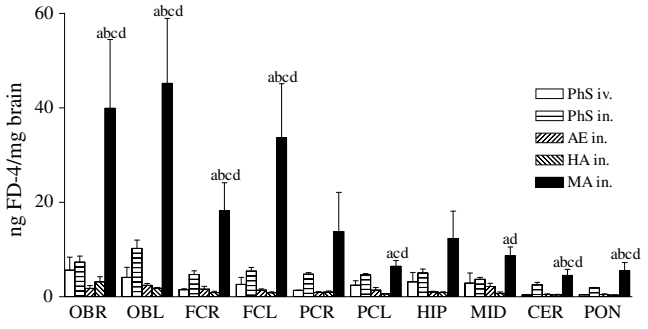


**Fig. 5.** FD-4 concentrations in different brain regions measured 240 min after different treatments (OBR: olfactory bulb from right side; OBL: olfactory bulb from the left side; FCR: frontal cortex from the right side; FCL: frontal cortex from the left side; PCR: parietal cortex from the right side; PCL: parietal cortex from the left side; HIP: hippocampus; MID: midbrain; CER: cerebellum; PON: pons). Statistically significant differences ($P < 0.05$) were detected in the MA group compared to the values measured in [a]PhS iv. group; [b]PhS in. group; [c]AE group; [d]HA group. Data are presented as mean ± SEM, $n = 4$.

lations, such as mucoadhesive delivery systems, are necessary to enhance the nasal bioavailability of these drugs [18,34].

The main finding of the study is that the combination of the surfactant Cremophor, as an absorption enhancer, and hyaluronic acid, as a viscosifying and bioadhesive polymer, could significantly increase the nose-to-brain transport of the test molecule FD-4, especially in the olfactory bulb and frontal cortex regions. These data are in accordance with the results of Thorne et al., [13] who found similar brain distribution for insulin-like growth factor following intranasal administration. We hypothesize that peptides or peptide fragments in the size range of 1–4 kDa acting on neuropeptide or hormone receptors in the nervous system, e.g. peptides regulating appetite and body weight, could be targeted with the MA formulation for therapeutical purposes.

In this study, the surfactant Cremophor RH40 was used as an absorption enhancer. Although surfactants, which are non-specific permeation enhancers, can improve the absorption of drugs by increasing their paracellular and transcellular transports via the modification of the cell membrane, no increase in the brain FD-4 levels was observed after application of the AE vehicle compared to that of the PhS. A possible explanation can be the fast removal of both vehicles from the nasal cavity by the mucociliary activity.

Because of its favorable characteristics, natural biocompatibility, bioadhesiveness, and lack of immunogenicity, hyaluronic acid and its derivatives are used in ophthalmic, rheumatologic, tissue engineering, cosmetic and veterinary products [24,27]. Although viscosity and mucoadhesion are key factors in nasal drug delivery [16], and hyaluronates have excellent mucoadhesive properties, only one study tested viscous sodium hyaluronate solutions in nasal absorption so far [35]. Hyaluronate solutions enhanced the nasal absorption of vasopressin in rats measured by a bioassay [35]. However, the vehicle contained no other components, and the concentration of the peptides in the blood or in the CNS were not determined.

The incorporation of sodium hyaluronate alone as a viscosifying agent and as mucoadhesive component into the vehicle HA in our experiments did not increase the amount of FD-4 transported to the brain in contrast to the combination of hyaluronan with Cremophor RH40, MA vehicle. The effect of MA is not related to elevation in plasma levels of FD-4, since FD-4, injected intravenously, resulted in a high AUC in the plasma, and a low AUC in brain, due to the highly selective and strictly regulated transport processes of the BBB [2,36]. The nasal formulations led to negligible plasma AUC, while the brain AUC of FD-4 in MA group exceeded that of the intravenously administered FD-4. This clearly indicates the circumvention of the BBB and a direct access to the CNS.

While the exact mechanism of this effect is not known, it is well documented that mucoadhesive additives can reduce the mucociliary clearance rate, increase the residence time of the drug formulation in the nasal cavity, and hence prolong the period of contact with the nasal mucosa, which may improve drug absorption [37,16]. Furthermore, some mucoadhesive polymer-containing systems may directly change epithelial tight junctions and increase drug absorption and bioavailability [18,19]. The difference between the efficacy of nasal vehicles HA and MA may indicate that optimal viscosity, supposed mucoadhesivity, longer residence time and absorption enhancement induced by the non-ionic surfactant all contributed to the increased brain penetration of the test molecule.

Despite advances in nasal mucoadhesive drug delivery, the application of mucoadhesive agents in nasal vehicles may not work for all compounds [16]. In a recent paper a pectin formulation resulted in lower systemic absorption and lower brain uptake of a drug compared to a solution formulation in rats [38]. Differences in the experimental design, short vs. long treatment time intervals, presence vs. absence of absorption enhancer, and different physico-chemical properties of the test molecule may explain the different results.

Toxicity is a major issue in the development of formulations for the nasal route [16]. It is very important to note that in our study, the vehicle containing sodium hyaluronate and Cremophor RH 40 did not induce tissue damage, epithelial or subepithelial toxicity, or ciliotoxicity. Our observations are in agreement with previous data that acute and chronic toxicity tests in animals have shown Cremophors to be essentially non-toxic and non-irritant materials [39]. Hyaluronate solution did not affect the ciliary beat frequency of rabbit nasal mucosal membranes in vitro [35], further supporting the non-toxic properties of this biomolecule. The excellent properties and safety profile of hyaluronic acid [24] could be better exploited for nasal drug delivery systems.

In conclusion, combination of absorption enhancer materials and bioadhesive polymers such as Cremophor RH 40 and sodium hyaluronate can potentially increase the delivery of drugs, including peptide size molecules, into the CNS via the olfactory pathway. Further experiments are in progress to test the efficacy of the MA system for targeting biologically active peptides to the brain.

**Table 4**
Pharmacokinetic parameters in brain.

| Vehicle | $c_{max}$ [ng FD-4/mg brain] mean ± SD | $t_{max}$ [min] | AUC mean ± SD | Relative BA mean ± SD |
|---|---|---|---|---|
| PhS iv. | 1.72 ± 0.90 | 240 | 598 ± 178 | 1.0 ± 0.3 |
| PhS in. | 4.49 ± 0.14 | 120 | 1523 ± 97 | 254.7 ± 16.3 |
| AE in. | 2.07 ± 0.21 | 30 | 554 ± 83 | 92.7 ± 13.9 |
| HA in. | 1.39 ± 0.09 | 60 | 431 ± 52 | 72.2 ± 22.7 |
| MA in. | 15.21 ± 9.11 | 240 | 3358 ± 1713 | 561.5 ± 286.5 |

AUC, area under the concentration-versus-time curve; BA, bioavailability compared to the PhS iv. group; PhS, physiological saline solution; AE, vehicle containing absorption enhancer Cremophor RH40; HA, vehicle containing hyaluronan alone; MA, vehicle containing mucoadhesive hyaluronan and absorption enhancer Cremophor RH40; $n = 4$.

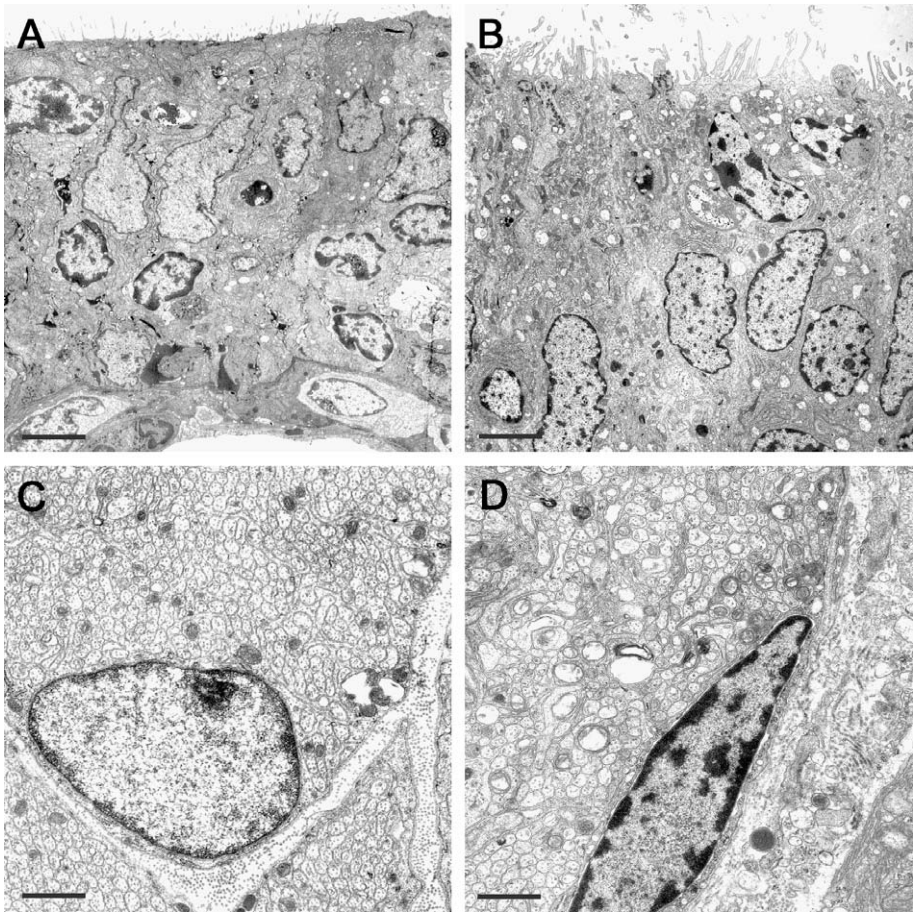


**Fig. 6.** Electron microscopical investigation of the olfactory system after treatment with MA solution (B and D) and without treatment (A and C). A and B olfactory epithelium showing epithelial cells with apical microvilli; C and D olfactory fila within the lamina propria underneath the olfactory epithelium. MA treatment (B and D) did not induce any pathological effect on the ultrastructure of the epithelium or sensory axons when compared with the untreated tissue (A and C). Bars: in A and B: 5 μm, in C and D: 1 μm.

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