Nose-to-brain peptide delivery – the potential of nanotechnology

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GRAPHICAL ABSTRACT

Nose-to-Brain Peptide Delivery

Simple Protein/Peptide solution
- Co-administration with permeation enhancers

Polymer-based nanosized drug delivery systems
- PLA, PLGA, Gelatin or Chitosan NPs
- PEGylated NPs
- Chitosan/P80 coated NPs
- Protein

Lipid-based nano-sized drug delivery systems
- Liposomes
- Nanospheres
- Nanostructured lipid carriers (NLCs)
- Cubosomes

The contribution of nanotechnology
- Protection from proteolytic degradation
- Improved uptake by the olfactory mucosa
- Facilitated access to the CNS based either on passive or active targeting
- Extended half-life times and higher concentrations in the CNS
Abstract

Nose-to-Brain (N-to-B) delivery offers to protein and peptide drugs the possibility to reach the brain in a non-invasive way. This article is a comprehensive review of the state-of-the-art of this emerging peptide delivery route, as well as of the challenges associated to it. Emphasis is given on the potential of nanosized drug delivery carriers to enhance the direct N-to-B transport of protein or peptide drugs. In particular, polymer- and lipid- based nanocarriers are comparatively analyzed in terms of the influence of their physicochemical characteristics and composition on their in vivo fate and efficacy. The use of biorecognitive ligands and permeation enhancers in order to enhance their brain targeting efficiency is also discussed. The article concludes highlighting the early stage of this research field and its still unveiled potential. The final message is that more explicatory PK/PD studies are required in order to achieve the translation from preclinical to the clinical development phase.

Keywords:
Nose-to-brain delivery; intranasal drug administration; therapeutic peptides; protein drugs; olfactory; nanomedicine; nanoparticles; polymer-based nanocarriers; lipid-based nanocarriers.

1. Introduction

Neurological disorders, such as Alzheimer’s disease, Parkinson’s disease, Multiple sclerosis etc., but also diseases like obesity, behavior disorders and sexual dysfunction have been directly associated to different modalities of brain dysfunction. These debilitating diseases are nowadays continuously growing, affecting more and more people worldwide. In addition to the social burden and the individual suffering that they cause, the treatment of these diseases is also associated with very high costs. Up to date, most of the drugs intended to treat CNS disorders and other brain related diseases are administered systemically and, for this, a prerequisite is that they are able to cross the blood–brain barrier (BBB). Unfortunately, there is a significant number of drugs, notable peptide and protein drugs which could potentially have a powerful effect in the CNS provided that they could overcome the BBB or acquire other routes of access to the brain.

Nose-to-brain (N-to-B) delivery may represent a non-invasive method that enables the delivery of complex drugs to the CNS, while avoiding the BBB. This route is based on the principle that drugs can access the CNS following a “shortcut” from the nose directly to the brain along the trigeminal or olfactory nerves, located at the upper part of the nasal cavity. The increasing numbers of peptide and protein drugs which may be of interest to treat chronic CNS diseases and the recent identification of important brain functions have stimulated research in the nose-to-brain delivery field. Within this field, the level of evidence of the value of nanotechnology for the direct CNS targeted peptide
delivery is still limited, however the knowledge generated over the last decade about this specific
topic has raised some expectancies.\textsuperscript{4,5}

Based on this background information, the main objective of this article is to focus on the potential
of nanotechnology-mediated peptide delivery to the brain via the nose. More precisely, this review
will provide the reader with a view of the challenges associated to this modality of administration,
followed by the current status of the nose-to-brain peptide transport, and it will end with a critical
analysis of the value of nanotechnology as compared to that of penetration enhancers for helping
peptide drugs to reach brain targets.

2. Challenges and barriers to N-to-B peptide delivery

In early 1937, Faber reported for the first time the possibility of a direct passage from the nose to the
brain, after administering a dye in the nostrils of rabbits.\textsuperscript{6} Still, it was only in the late 90s, that the
growing interest in the field of brain delivery motivated the scientific community to start exploring
this alternative route.\textsuperscript{7–13} Mechanistic studies in animal models have proven that N-to-B drug
transport takes place either by extracellular or transcellular transport mechanisms along the olfactory
epithelium or via the trigeminal nerve, after administration of the drug into the nasal cavity (Fig.
1).\textsuperscript{14–19} Despite this increasing interest, the mechanisms underlying this direct N-to-B pathway are
not fully elucidated yet.

Pathways related to the transport of peptides from the nose directly to the brain

The olfactory region is located at the top part of the nasal cavity under the cribiform plate in close
proximity to the olfactory bulb, interlocking the nose with the brain (Fig. 1). More specifically, the
olfactory epithelium consists of three types of cells, namely the basal epithelial cells, sustentacular
cells, and the olfactory neurons with their cilia extending towards the nasal cavity (Fig. 2).\textsuperscript{16,20} More
detailed information about nasal physiology can be found in previous reviews.\textsuperscript{16,18,19,21}

\textbf{Figure 1.} Olfactory and Trigeminal nerve position in the nasal cavity
After administration of the drug into the nasal cavity, N-to-B drug transport may occur through the olfactory epithelium, either (i) by axonal transport after internalization into the neurons, (ii) by paracellular transport across the spaces between cells and, notably across the channels next to the olfactory nerves, or (iii) by transcellular transport across the basal epithelial cells (Fig. 2).14-17,22,23 The paracellular pathway is considered to be the dominant transport mechanism based on animal studies, and it allows a more rapid drug transport (usually <30 minutes) than the others, which can last from a few hours up to days.16,24 This could be explained by the slow regeneration of the olfactory neurons (every ~1 month) and the coexistence of mature and newly formed neurons, resulting in the absence of tight junctions in some parts of the olfactory epithelium.24 This leakiness, in combination with the bulk flow of the cerebrospinal fluid (CSF) into the brain, enables the transport of the intranasally administered drugs to the CNS.16 However, the predominance of one specific transport mechanism vs. the others depends on the properties of the drug or the delivery system used.25 Depending on the pathway, the drug may reach the olfactory bulb by intraneuronal uptake and, from there, it may go into the brain regions connected to the olfactory tract (i.e., the piriform cortex, hypothalamus, amygdala) and finally disseminate through the CNS, and/or it may diffuse directly from the CSF into the whole CNS (Fig. 2).26

**Figure 2.** Schematic representation of the olfactory pathway and possible uptake mechanisms involved in the transport of peptides from the nose directly to the brain. (CSF: cerebrospinal fluid; DDS: drug delivery system)
Based on studies in different animal models, some authors have proven that direct N-to-B drug delivery can also take place along the less explored trigeminal nerve, parts of which extend from the brainstem through the nasal respiratory epithelium, and provide thus a direct passage to the caudal and the rostral parts of the brain (Fig. 3). Still, the contribution of the trigeminal pathway is not fully understood and is considered to be less relevant than the olfactory track.

![Figure 3. Schematic representation of the Trigeminal nerve pathway. (CSF: cerebrospinal fluid)](image)

**Challenges encountered in the transport of peptides from the nose directly to the brain**

Despite the potential of this patient-friendly drug delivery route to the CNS, there are significant challenges associated to this modality of administration. Nose-to-brain transport is significantly affected by the surface and structural properties of the administered biomolecules (e.g., size and lipophilicity, degree of ionization). Proteins, because of their larger size (>1000 Da) and hydrophilicity, are transported in a far less extent than smaller lipophilic molecules. Another important factor is the presence of metabolic enzymes (cytochrome P450, esterases and transferases) in the mammalian olfactory mucosa. On the other hand, from an anatomical point of view, the localization of the olfactory epithelium in the roof of the nasal cavity makes it difficult for drugs to gain access to the targeted region. To address these pitfalls and enhance the bioavailability of the protein molecules, different approaches have been suggested, such as the use of permeation enhancers, cell penetrating molecules, mucoadhesives or nano-based drug delivery systems. The last ones have the additional advantage of protecting the therapeutic load, while improving its interaction with the olfactory region. However, a limitation of this route, is related to the low volumes that can be administered (maximal dosing volume in humans is 0.4 mL), which implies the need of designing nanocarriers with a high drug loading capacity. Lastly, it is worth mentioning
that so far, the mechanistic studies have been mainly performed in animals, whereas the studies in humans have focused on the evaluation of the drugs therapeutic effects, or, in some exceptional situations the measurement of the drug concentrations in the CSF, or the use of positron emission tomography (PET) scanning. Despite the above listed limitations, the potential benefits of this pain-free and direct approach are clear and, hence, N-to-B transport of peptides is becoming a promising alternative to the other established methods for CNS delivery.

3. Direct transport of P/P drugs from nose-to-brain

Owing to the aforementioned challenges, there has been much discussion during the last decades about whether peptides can efficiently employ the nose-to-brain pathway. Nowadays, after numerous studies published and patents filed, we can say that peptides and proteins can be transported to the CNS directly through the nose. Fig. 4 depicts the growing interest in this scientific area, as found in a PubMed database search that selected exclusively articles reporting in vivo studies (172 articles). Interestingly, almost one third of the registered studies were published in the last 3 years.

The first study describing the direct delivery of a protein molecule to the brain through the olfactory nerve pathway was published by Frey et al. in 1995. This affirmation was based on the observed accumulation of the radiolabeled nerve growth factor (NGF) in the olfactory bulb shortly after its intranasal administration in rats. Years later, a breakthrough study was published, in which the authors showed that insulin and other peptides like melanocortin (4–10) could efficiently reach the CSF, with undetectable serum levels, after their intranasal administration in 36 healthy human volunteers. Their study was considered to be the first proof of the existence of the olfactory track in humans, even if Merkus and van den Berg pointed out the necessity of comparing the nose-to-brain and the intravenous routes in order to have a clear understanding of the distribution of drugs into the brain.

![Figure 4](image-url)

**Figure 4.** Number of publications on nose-to-brain peptide delivery, reporting in vivo studies [PubMed database].
Table 1 illustrates the studies that reported *in vivo* data showing the nose-to-brain transport of protein or peptide molecules, administered either as simple aqueous solution, with permeation enhancers, or in the form of drug delivery systems. Overall, the most explored protein delivered to the brain via this direct route is insulin, followed by oxytocin and hypocretin-1. These well-characterized peptides have crucial regulating functions in the CNS, owing to the wide distribution of their receptors in the CNS, and numerous clinical trials have shown their potential in the treatment against several disorders, such as cognitive and behavior disorders, narcolepsy, and other important neurological disorders, i.e. Alzheimer’s disease and Parkinson’s disease. With regard to the preclinical data, the limited pharmacokinetic data (37 publications) make very difficult the drawing of clear conclusions regarding the bioavailability of the different peptides in the brain after their intranasal administration. Still, while most of the studies have reported enhanced brain delivery of the protein molecules after their intranasal administration in comparison to their intravenous administration, the bioavailability of these molecules remained low, generally lower than 1%. Intranasal co-administration or conjugation with permeation enhancers, such as cell permeating peptides (i.e. penetratin, low molecular weight protamine, Pz-peptide, Tat), polyethylenimine, chitosan, lauroyl carnitine, cyclodextrins, Pluronic P85 or peppermint oil, has been a frequent strategy to enhance the bioavailability of the intranasally (i.n.) administered protein molecules. For example, some authors reported that the co-administration of chitosan (0.25%) with NGF led to an increase of up to 13 times in the brain bioavailability of the intranasally administered NGF, relative to the i.n. free peptide solution. Similarly, other authors found a 60% enhanced olfactory bulb uptake of exendin after its co-administration with cyclodextrin, compared to the intravenously administered free peptide.

On the other hand, there have been attempts to obtain direct visual evidence of the route that the proteins follow after their intranasal administration, employing mainly radioactivity-based imaging techniques. These studies provided undeniable evidence of the peptides’ ability to reach the CNS following the olfactory and/or trigeminal pathway.

**Table 1.** Peptide/protein drugs transported directly from the nose to the brain

<table>
<thead>
<tr>
<th>P/P drug</th>
<th>MW (Da)</th>
<th>Disease</th>
<th>Animal Model</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>5,800</td>
<td>AD</td>
<td>Mice, rats, rabbits, sheep</td>
<td>46,47,75,94,96,104</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>1,000</td>
<td>CBD, ASD, PTSD, SD, SCZD</td>
<td>Rats, pigs, vampire bats, <em>monkeys, macaques</em></td>
<td>105-109</td>
</tr>
<tr>
<td>Erythropoietin</td>
<td>30,400</td>
<td>AD, CI, Epilepsy</td>
<td>Mice, rats</td>
<td>85,88,93,110-114</td>
</tr>
<tr>
<td>Human nerve growth factor</td>
<td>26,500</td>
<td>AD</td>
<td>Mice, rats</td>
<td>8-10,39,73,115-120</td>
</tr>
<tr>
<td>Basic fibroblast growth factor</td>
<td>18,000</td>
<td>PD</td>
<td>Rats</td>
<td>74,121-124</td>
</tr>
<tr>
<td>Neuropeptide</td>
<td>Fold Change</td>
<td>Conditions</td>
<td>Species</td>
<td></td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>-------------</td>
<td>---------------------</td>
<td>------------------</td>
<td></td>
</tr>
<tr>
<td>NAP neuropeptide (NAPVSIPQ)</td>
<td>825</td>
<td>AD, MCI, SCZD, FTD</td>
<td>Mice, rats</td>
<td></td>
</tr>
<tr>
<td>Vasoactive intestinal peptide</td>
<td>2,800</td>
<td>AD</td>
<td>Mice, rats</td>
<td></td>
</tr>
<tr>
<td>Insulin-like growth factor I</td>
<td>7,650</td>
<td>AD, HD, CI</td>
<td>Mice, rats</td>
<td></td>
</tr>
<tr>
<td>Glucagon-like peptide I</td>
<td>4,100</td>
<td>OB</td>
<td>Mice</td>
<td></td>
</tr>
<tr>
<td>Exendin (9-39)</td>
<td>3,400</td>
<td>CHI</td>
<td>Mice, rats</td>
<td></td>
</tr>
<tr>
<td>Leptin</td>
<td>16,000</td>
<td>OB</td>
<td>Rats</td>
<td></td>
</tr>
<tr>
<td>Interferon – β1B</td>
<td>18,500</td>
<td>MS</td>
<td>Rats, monkeys</td>
<td></td>
</tr>
<tr>
<td>Brain derived neurotrophic factor</td>
<td>26,900</td>
<td>AD, HD, ASD</td>
<td>Mice, rats</td>
<td></td>
</tr>
<tr>
<td>Neurotoxin I</td>
<td>6,900</td>
<td>Pain management</td>
<td>Mice, rats</td>
<td></td>
</tr>
<tr>
<td>Insulin-like growth factor I</td>
<td>15,000</td>
<td>PD</td>
<td>Mice, rats</td>
<td></td>
</tr>
<tr>
<td>Hypocretin-I (orexin A)</td>
<td>3,500</td>
<td>Narcolepsy</td>
<td>Rats, monkeys</td>
<td></td>
</tr>
<tr>
<td>Glial cell-derived neurotrophic factor</td>
<td>66,500</td>
<td>-</td>
<td>Mice</td>
<td></td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>25,000</td>
<td>PD, CI</td>
<td>Mice, rats</td>
<td></td>
</tr>
<tr>
<td>Vascular endothelial growth factor</td>
<td>38,200</td>
<td>AD</td>
<td>Rats</td>
<td></td>
</tr>
<tr>
<td>Pituitary adenylate cyclase-activating peptide</td>
<td>4,500</td>
<td>PD, CI</td>
<td>Mice</td>
<td></td>
</tr>
<tr>
<td>Thyrotropin-releasing hormone</td>
<td>362</td>
<td>Epilepsy</td>
<td>Rats</td>
<td></td>
</tr>
<tr>
<td>Substance P</td>
<td>1,347</td>
<td>PD</td>
<td>Rats</td>
<td></td>
</tr>
<tr>
<td>NEMO-binding domain peptide</td>
<td>2,841</td>
<td>HIE</td>
<td>Rats</td>
<td></td>
</tr>
<tr>
<td>Osteopontin</td>
<td>35,423</td>
<td>CI</td>
<td>Rats</td>
<td></td>
</tr>
<tr>
<td>Arginine-vasopressin</td>
<td>1,080</td>
<td>CBD, pro-social effects</td>
<td>Rats, monkeys</td>
<td></td>
</tr>
<tr>
<td>Glucagon-like peptide II</td>
<td>3,766</td>
<td>Depression</td>
<td>Mice</td>
<td></td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>45,000</td>
<td>-</td>
<td>Rats</td>
<td></td>
</tr>
<tr>
<td>Leucine-enkephalin</td>
<td>555</td>
<td>Pain management</td>
<td>Mice</td>
<td></td>
</tr>
<tr>
<td>Neurotrophin-4</td>
<td>22,400</td>
<td>MS</td>
<td>Rats</td>
<td></td>
</tr>
<tr>
<td>interleukin-1 receptor antagonist</td>
<td>17,000</td>
<td>CI</td>
<td>Rats</td>
<td></td>
</tr>
<tr>
<td>Ciliary neurotrophic factor</td>
<td>22,700</td>
<td>AD, HD</td>
<td>Rats</td>
<td></td>
</tr>
<tr>
<td>Transforming growth factor –a</td>
<td>5,000-35,000</td>
<td>CI</td>
<td>Rats</td>
<td></td>
</tr>
<tr>
<td>Growth differentiation factor 5</td>
<td>27,400</td>
<td>PD</td>
<td>Rats</td>
<td></td>
</tr>
<tr>
<td>Galamin-like peptide</td>
<td>6,500</td>
<td>OB</td>
<td>Mice</td>
<td></td>
</tr>
<tr>
<td>Calcitonin gene-related peptide</td>
<td>3,800</td>
<td>Migraine</td>
<td>Rats</td>
<td></td>
</tr>
<tr>
<td>Exendin-4</td>
<td>4,186</td>
<td>CI</td>
<td>Mice</td>
<td></td>
</tr>
<tr>
<td>Urocortin</td>
<td>4,700</td>
<td>PD</td>
<td>Rats</td>
<td></td>
</tr>
<tr>
<td>Hexarelin</td>
<td>887</td>
<td>GHD</td>
<td>Rabbits</td>
<td></td>
</tr>
<tr>
<td>Neuropeptide Y</td>
<td>4,253</td>
<td>PTSD, Depression</td>
<td>Rats</td>
<td></td>
</tr>
</tbody>
</table>

Given the still limited number of studies in animal models, it is not surprising that the clinical development of the peptide nose-to-brain formulations is still at an early stage. Nevertheless, as shown in Table 2, it is encouraging to realize that several peptides are making their way along the clinical development path, with some of them, i.e. insulin and oxytocin, having reached Phase IV clinical studies.\(^\text{59,67,176-178}\) In the case of insulin, the indication selected in the phase IV clinical studies focused on its role in energy metabolism.\(^\text{178}\) So far the disclosed studies have shown that a single dose of intranasal insulin can increase the peripheral insulin sensitivity and reduce the hepatic fat in healthy humans.\(^\text{179,180}\) Apart from this, intranasal insulin is currently being evaluated for its memory-ameliorating effect in Alzheimer’s suffering patients, as well as its action against different CNS disorders in humans, like Parkinson’s disease, multiple system atrophy and psychiatric conditions such as schizophrenia and major depressive disorder.\(^\text{64}\) With regard to oxytocin, it is known that it works as a brain neurotransmitter, thereby influencing the social behavior and emotional function in different species. Oxytocin has been tested against several disorders, such as stress disorders, social dysfunction, cognitive and behavior disorders, Autism Spectrum Disorder (ASD) and sexual dysfunction in clinical studies.\(^\text{65,181}\) The most advanced indication (Phase IV clinical studies) is the control of satiety in people with schizophrenia, as well as its effect on pain threshold.\(^\text{182,183}\) The first results of these on-going studies have shown that a single dose of intranasal oxytocin can significantly increase the satiety feeling and, subsequently, reduce food consumption in schizophrenia patients. No results have been posted yet on its effect on pain sensitivity.

Table 2. P/P drugs under clinical studies for N-to-B delivery
<table>
<thead>
<tr>
<th>P/P drug</th>
<th>Disease</th>
<th>Stage of development</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>AD, OB, PD, MS, SCZD, MDD</td>
<td>Phase I</td>
<td>56,59,184–192</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phase II</td>
<td>52,184,186,188,193–199</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phase III</td>
<td>191,198,200</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phase IV</td>
<td>1/8</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>CBD, ASD, PTSD, SD, SCZD, Pain management</td>
<td>Phase I</td>
<td>37,201–211</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phase II</td>
<td>212–217</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phase III</td>
<td>218–220</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phase IV</td>
<td>182,183</td>
</tr>
<tr>
<td>Arginine-vasopressin</td>
<td>CBD, pro-social effects</td>
<td>Phase I</td>
<td>56,97,203,206,221,222</td>
</tr>
<tr>
<td>Melanocortin (4–10)</td>
<td>OB</td>
<td>Phase I</td>
<td>56,184,223,224</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phase II</td>
<td>184</td>
</tr>
<tr>
<td>Cholecystokinin</td>
<td>OB, AD, CBD</td>
<td>Phase I</td>
<td>221,225,226</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phase II</td>
<td>227</td>
</tr>
<tr>
<td>NAP neuropeptide</td>
<td>AD, SCZD</td>
<td>Phase I</td>
<td>228</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phase II</td>
<td>229,230</td>
</tr>
<tr>
<td>Hypocretin-I (orexin A)</td>
<td>OB, AD, PD, narcolepsy</td>
<td>Phase I</td>
<td>231–233</td>
</tr>
<tr>
<td>Hexarelin</td>
<td>GHD</td>
<td>Phase I</td>
<td>234</td>
</tr>
<tr>
<td>Neuropeptide Y</td>
<td>OB</td>
<td>Phase I</td>
<td>184</td>
</tr>
<tr>
<td>Insulin-like growth factor-I</td>
<td>DB, OB</td>
<td>Phase I</td>
<td>195</td>
</tr>
</tbody>
</table>


Another interesting point to note is that most of the peptide drugs presented in Table 2, have already been tested in numerous clinical trials, using systemic administration routes and, thus, their safety and pharmacological/toxicological effects have been assessed. Still, a new evaluation of the toxicity and safety of these drugs would be required in the case of intranasal delivery. These studies may, additionally, include histological assessments of local tissues and potentially affected brain areas. Apart from this, the majority of the P/P drugs in Table 2 have been administered intranasally to humans with the help of liquid delivery devices, such as spray pumps or nebulizers/atomizers. Detailed description of the principles and the types of these devices can be found elsewhere. This fact introduces additional regulatory requirements for the development of an intranasal product, such as droplet size distribution, dose uniformity, plume geometry and spray pattern studies.

4. The potential of nanotechnology for nose-to-brain peptide delivery

The contribution of nanotechnology to this field is crucial since not only it allows the protection of the delicate therapeutic cargo from degradation, but most importantly it improves the uptake by the olfactory mucosa and the access to the CNS, based either on passive or active targeting. As a result, the use of drug delivery nanocarriers has led to enhanced drug concentrations and extended half-life
times with the subsequent improved therapeutic effect of the delivered molecules. According to our records, Gao et al. was the first group to prove in 2007 that poly(ethylene glycol)-poly(lactic acid) nanoparticles (PEG-PLA NPs) functionalized with wheat germ agglutinin (WGA) could deliver a neuropeptide against Alzheimer’s disease directly from the nose to the brain.\textsuperscript{131} Since then, a variety of nano-based drug delivery systems have been developed with the objective of improving the nose-to-brain delivery of a variety of peptide drugs, most of them summarized in Tables 3 and 4. These delivery carriers made of either polymers (Table 3), mainly polyesters and chitosan, or lipids (Table 4), were engineered to promote the transport across the neural pathway of different drug molecules. Nevertheless, the number of research articles in this field is limited, a fact that explains the lack of nose-to-brain potential nanomedicines in clinical development.

We have analyzed the tendency in the nose-to-brain nanomedicine field, and the results are summarized in Figure 5. It should be noted that 87\% of the 172 publications analyzed, refer to the administration of drugs in the form of a simple solution or after co-administration with permeation enhancers to facilitate the drugs transport across the olfactory epithelium. Nevertheless, the trend is changing now and the number of nano-based drug delivery systems explored to nose-to-brain peptide/protein delivery increases year by year. The majority of these delivery carriers are either polymeric nanoparticles, mainly made of PLA and derivatives, and lipid-based nanocarriers, with a predominance of liposomes and lipid nanoparticles. A tendency is also observed towards the functionalization of the nanocarriers.

Figure 5. Representation of the publication trends in N-to-B peptide delivery. (NPs: nanoparticles, NLCs: nanostructured lipid carries, GNLs: gelatin core lipid carriers)
4.1 Importance of the chemical and physicochemical characteristics of the nanocarriers

Although it has been generally accepted that the particle size is a key factor in the capacity of nanocarriers to overcome mucus barriers and well-organized epithelia, the recent work by Ahmad et al. provided a good illustration of this effect. The authors studied the biodistribution of nanoemulsions of different sizes after intranasal administration in rats, by fluorescence imaging, and concluded that nanocarriers with a particle size around 100 nm were able to be transported along the olfactory or trigeminal route, whereas nanoemulsions with a larger droplet size were not able to follow the olfactory pathway. Apart from this, a number of studies have also reported the efficiency of nanocarriers with a size up to 200 nm in terms of facilitating the transport of molecules in animal models.

These results are aligned with the reported morphological studies of the olfactory epithelium of different species, which have revealed that the average diameter of olfactory axons is around 200 nm, with many axons to have diameters even less than 100 nm, while in humans this range goes from 100 nm up to 700 nm. This fact alone sets a size constraint for the effective transcellular transport of the nanocarriers via the olfactory axons to the brain. In agreement with this morphological constraint, the size of the majority of the nanocarriers developed for the peptide delivery through the intranasal route is in the range of 70-150 nm (Table 3&4).

On the other hand, the effect of the zeta potential on the performance of the nanocarriers has not been clearly elucidated yet. Apart from the already known fact that the use of positively charged nanocarriers will likely adhere to the mucus layer, due to the presence of negatively charged mucus proteins in the region, the potential benefit of this adhesive/retention behavior remains to be clarified. In a recent study, the authors sought to describe this effect by tracking the localization of fluorescently labeled poly(lactic-co-glycolic acid) nanoparticles (negative) and chitosan-coated PLGA NPs (positive) in different brain areas, following their intranasal administration in rats. The conclusion of this study was that while both type of carriers were found in the brain, the surface charge and composition of the nanocarriers had a significant effect on their transport pathway, with negative NPs showing a preference for the olfactory pathway, while the positive nanoparticles for the trigeminal pathway. While these conclusions remain to be confirmed, it should be highlighted that the comparison of these nanocarriers was not done in a rigorous manner as they had a different particle size (118 nm for PLGA nanoparticles and 213 nm, for chitosan-coated PLGA nanoparticles).

Irrespective of the differences in the surface charge, a critical parameter that determines the fate of the nanocarriers after their intranasal administration is their composition. For example, in a study gelatin-nanostructured lipid carriers (GNLs) stabilized with Poloxamer 188 (size of ~170 nm and zeta potential of −27 mV) were compared with gelatin nanoparticles (GNPs), (size of ~120 nm and zeta potential of −17 mV), with regard to their capacity for the nose-to-brain transport of bFGF in
hemiparkinsonian rats. The results showed the superiority of the Poloxamer 188-containing formulation, which was attributed to the known permeation and mucodiffusive properties of the surfactant. Other studies have evaluated the effect of various coatings, i.e. chitosan and polysorbate 80 around the nanoparticles on their olfactory transport. The results showed that the presence of polysorbate-80 or chitosan could potentially enhance the nanocarriers’ interaction with the olfactory mucosa. As in the case of poloxamer, this positive behavior of polysorbate 80 was attributed to its permeation and mucodiffusive properties, whereas chitosan apart from increasing the hydrophilicity on the surface of the carrier, can significantly prolong also its retention time in the olfactory area. However, these mechanistic behaviors remain to be elucidated.

4.2 Polymer-based nanocarriers

Polymeric nanocarriers, made either of natural or synthetic polymers, have recently attracted significant research attention for N-to-B peptide delivery, since they offer a plethora of advantages, like enhanced stability, the ability to protect and control the release of their therapeutic payload and multiple possibilities for surface modification. However, so far only few examples have been reported in the literature, including polylactic/glycolic acid (PLGA) nanoparticles, PEG-PLGA nanoparticles, PLA NPs modified with chitosan or polysorbate 80, PEG-poly (ε-caprolactone) (PEG-PCL) nanoparticles, chitosan nanoparticles, and gelatin nanoparticles. Table 3 summarizes the physicochemical characteristics and the PK/PD behavior of the different polymer-based carriers employed so far in the literature. The performance of these nanocarriers, after their i.n. administration, was further enhanced in some cases via their surface modification with targeting moieties, which will be discussed below.

The advantages of using polymer-based nanocarriers for the N-to-B delivery of small hydrophobic drugs have been explored by different authors since 2000. This know-how was then transferred to N-to-B peptide delivery, with Gao et al. being the first to prove the capacity of PEG-PLA NPs functionalized with WGA, to facilitate the transport of a peptide drug, the vasoactive intestinal peptide, to the brain in 2007. In their study, an almost 4-fold increase in the concentration of the peptide in the brain was reported, when delivered associated to PEG-PLA NPs, reaching a 7-fold increase with the help of the active targeting functionalization, in comparison to the peptide solution after its intranasal administration in mice. Following this pioneering study, several authors have investigated the effect of the surface composition of PLA/PLGA particles in their capacity to overcome the N-to-B barriers. Interestingly, the potential of PLA or PLGA nanoparticles for peptide/protein delivery, as well as the effect of the particle size and the PEGylation on the transport of nanoparticles across the nasal mucosa was first reported by our group almost 2 decades ago. Years later, in 2009, the effect of the particle size on the performance of PLA particles for N-to-B peptide delivery for the N-to-B transport of Thyrotropin-releasing hormone (TRH) was also reported. The evaluation of the uptake of the particles by fluorescent microscopy led to the
conclusion that 100 nm PLA nanoparticles could gain access to different brain regions through the olfactory epithelium, whereas the larger ones (~560 nm) could not. This explained the enhanced therapeutic effect of the neuropeptide in terms of suppressing stage IV seizures in epileptic rats.\textsuperscript{150,151} Moreover, different authors employed PEG-PLGA of a 120 nm size in order to protect Urocortin (UCN) and basic fibroblast growth factor (bFGF) respectively, from degradation and facilitate their intranasal delivery to the CNS.\textsuperscript{122,167} Additionally, Zhang et al. presented PK results, showing an almost 1.5-fold increase of the AUCs of bFGF in different brain areas in the case of the i.n. administration of the bFGF-loaded NPs, relative to i.n. administered bFGF solution in rats.\textsuperscript{122} This increase reached up to 3 times more in the case of the i.n. administration of lectin modified bFGF-NPs, and will be further discussed below.

Another tendency observed is the surface modification of PLA NPs with either PEG-containing surfactants, like polysorbate 80 (P80), or chitosan. This trend, in the case of P80, originated from the reported improved performance of the P80-coated nanocarriers in the CNS drug delivery, which was later adopted in N-to-B peptide delivery.\textsuperscript{254-256} Accordingly, P80-coated PLA NPs with a particle size of 60 nm were employed for the nose to brain administration of Neurotoxin-I in rats, resulting in an 1.5/1.8-fold increase in the peptide bioavailability relative to i.v. administration of the same nanoparticles and peptide solution, respectively.\textsuperscript{140} In a similar study, the N-to-B transport of the same peptide was evaluated upon its incorporation into P80-coated PLA nanoparticles (size was not mentioned in the report) and the brain uptake showed a 2.2/3 times higher drug transport in comparison to the i.v. administration of the same particles and the i.n. administration of the free peptide in mice, respectively. This enhanced delivery could potentially be attributed to the PEG-containing surface coating of the NPs that facilitates their diffusion through the olfactory mucosa since it raises the hydrophilicity of the carrier, or to its general surfactant-like properties and its interaction with the endothelial cells.\textsuperscript{141}

Chitosan, on the other hand, is considered to be an attractive polymer due to its biocompatibility and bioadhesion and penetration enhancing properties.\textsuperscript{257} It is worth mentioning that our group pioneered in the development of CS nanoparticles and nanocapsules for nasal drug delivery in the mid-90s.\textsuperscript{258,259} Regarding its use in N-to-B peptide drug delivery, chitosan-coated PLA nanoparticles, having a particle size of 140 nm and a positive surface charge of +34 mV, were shown to significantly enhance the brain uptake of the neurotoxin-I peptide (~1.8-fold relative to the uncoated NPs), an effect that was attributed to the mucoadhesive and cell permeating properties of chitosan.\textsuperscript{142} In another study, Kumar et al. investigated the performance of trimethyl chitosan nanoparticles (TMC NPs) with a mean size of 440 nm and a positive zeta potential of +15 mV for the intranasal delivery of Leucine-enkephalin (Leu-Enk). The authors reported a high peptide uptake in different brain regions, however this conclusion was simply based on qualitatively microscopic observation.\textsuperscript{160} Both of the studies mentioned did not provide a systemic comparison though, and
thereby proof that the delivery of the respective peptides is only due to direct passage of the therapeutic molecules via the olfactory or trigeminal nerves to the brain and not through the nasal epithelium also.

Another biodegradable and FDA approved biomaterial that has received minor attention is gelatin. Joachim et al. used gelatin nanoparticles (GNPs) to study their ability to transfer Osteopontin (OPN) to the brain after their intranasal application. The selection of gelatin was based on the authors claim that this protein can passively target several brain areas in case of ischemic stroke.\textsuperscript{155,260} According to the expectations, the results of this work showed an enhanced response for the peptide associated to the nanoparticles as compared to the free peptide administered intranasally.

Moreover, nanoparticles made of PEG-poly (ε-caprolactone) and surface-modified with lactoferrin, with mean diameter less than 90 nm, have been used to study the delivery of NAP, a model octapeptide, from the nose directly to the brain of Alzheimer’s disease animal models. Throughout this study, a rapid accumulation of the NPs in various brain areas was showed based on fluorescent microscopy, whereas the enhanced memory amelioration effect of the Lf-NPs was further proven by a Morris water maze experiment.\textsuperscript{129}

Depending on the polymer chosen and the composition of the nanocarriers, different properties can be attributed to the systems. A general characteristic of the polymer-based carriers employed so far for the N-to-B peptide delivery is that their diameter is within the range of 70-200 nm. Overall, the surface functionalization of the carriers either with PEG, pegylated surfactants, chitosan and/or targeting moieties makes a significant contribution to the enhancement of the peptide bioavailability in the brain. However, a strict comparison among these nanocarriers is difficult to make due to the different nature of the studies performed to evaluate them.

**Table 3.** Overview of polymer-based nanocarrier systems for N-to-B peptide delivery.

<table>
<thead>
<tr>
<th>Nanocarrier</th>
<th>Drug</th>
<th>Size (nm)</th>
<th>Z-pot (mv)</th>
<th>Targeting molecule</th>
<th>Animal</th>
<th>Pharmacokinetics outcome/ Therapeutic effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>D,L- PLA NPs</td>
<td>Thyrotropin-releasing hormone</td>
<td>~100</td>
<td>-</td>
<td>-</td>
<td>Rats</td>
<td>Significant suppressed seizures in epileptic rats Enhancement of neuroprotective effect 1.8-fold increased bioavailability compared to i.v. peptide solution</td>
</tr>
<tr>
<td>PLA NPs coated with polysorbate 80</td>
<td>NT-I</td>
<td>65</td>
<td>-29</td>
<td>-</td>
<td>Rats</td>
<td>3-fold increased brain concentration of NT-I relative to the i.n. solution 9.5-fold increased brain concentration relative to the i.n. solution and 1.8-fold to unmodified PLA-NPs</td>
</tr>
<tr>
<td>PLA NPs coated with polysorbate 80</td>
<td>NT-I</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Mice</td>
<td>1.8-fold increased bioavailability compared to i.v. peptide solution</td>
</tr>
<tr>
<td>PLA NPs modified with chitosan</td>
<td>NT-I</td>
<td>140</td>
<td>+34</td>
<td>-</td>
<td>Rats</td>
<td>3-fold increased brain concentration of NT-I relative to the i.n. solution 9.5-fold increased brain concentration relative to the i.n. solution and 1.8-fold to unmodified PLA-NPs</td>
</tr>
</tbody>
</table>

\textsuperscript{150,151,140,141,142}
<table>
<thead>
<tr>
<th>PEG-PLA NPs</th>
<th>Vasoactive intestinal peptide</th>
<th>100 - 120</th>
<th>WGA</th>
<th>Mice</th>
<th>~7-fold increased bioavailability relative to i.n. peptide solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG–PLGA NPs</td>
<td>Urocortin</td>
<td>120</td>
<td>-20</td>
<td>OL</td>
<td>Rats</td>
</tr>
<tr>
<td>PEG-PLGA NPs</td>
<td>bFGF</td>
<td>120</td>
<td>-32</td>
<td>STL</td>
<td>Rats</td>
</tr>
<tr>
<td>N-trimethyl chitosan NPs</td>
<td>Leucine – encephalin</td>
<td>443</td>
<td>+15</td>
<td>-</td>
<td>Mice</td>
</tr>
<tr>
<td>Gelatin NPs</td>
<td>Osteopontin</td>
<td>183</td>
<td>-</td>
<td>-</td>
<td>Rats</td>
</tr>
<tr>
<td>PEG-co-PCL NPs</td>
<td>NAP peptide</td>
<td>70 - 90</td>
<td>-24</td>
<td>Lf</td>
<td>Mice / Rats</td>
</tr>
</tbody>
</table>


4.3 Lipid-based nanocarriers

Lipid-based nanocarriers describe a broad category of drug delivery systems that includes liposomes, nanoemulsions and solid lipid nanoparticles. From our knowledge, Migliore et al. were the first, in 2009, to explore the use of lipid-based nanocarriers for the N-to-B delivery of protein drugs. Following this study, nanoemulsions, liposomes, cubosomes, as well as mixed systems like lipid nanoparticles with solid matrix or nanostructured lipid carriers (NLC) have been successfully employed for the N-to-B peptide delivery and analyzed below (Table 4).

In the pioneering work by Migliore et al., the authors investigated the potential of cationic liposomes with a size of 300 nm, consisting of dioleoylphosphatidylcholine (DOPC), cholesterol and stearylamine for the N-to-B transport of ovalbumin – a model protein – in rats. The i.n. administered liposomal formulation exhibited increased residence time at the olfactory mucosa and this was translated into a 10-fold protein concentration in the brain, as compared to the protein solution administered by the same route. The authors attributed these positive results to the electrostatic interactions of the positively charged carrier with the negatively charged mucus proteins. Other authors reported the fact that pegylated liposomes with a size of 110 nm and a neutral charge facilitated the direct transport of the H102 Peptide to the brain after intranasal administration to a Alzheimer’s disease rat model. In this study, the performance of the liposomal formulation
was compared with that of the free peptide administered intranasally in the presence of chitosan. The results showed that the i.n. liposomal formulation was significantly more effective than the chitosan solution in terms of enhancing the access of the H102 peptide to the olfactory bulb, cerebellum, cerebrum and hippocampus.

Cubosomes are a nanoparticulate system, consisting of amphiphilic lipids and surfactants that are organized in a cubic nanostructure. These systems contain liquid-crystal phases that may facilitate the dissolution of hydrosoluble peptides. Wu et al. explored the efficacy of pegylated cubosomes, consisting of 1-Monoolein (Glycerol Monooleate), Poloxamer 407 and maleimide–PEG–oleate, functionalized with a lectin, for the N-to-B delivery of the S14G-HN peptide, a novel peptide against Alzheimer’s disease and Cerebral ischemia. The results showed a concentration dependent enhancement of the neuroprotective effect of the S14G-HN peptide, following their intranasal administration in rats.

Although a number of reports have described the possibility to deliver small hydrophobic molecules from the nose to the brain using nanoemulsions, the only work describing a peptide-based nanoemulsion formulation was applied to Cyclosporine A (CsA). The developed nanoemulsions in this study consisted of flax-seed oil as the oil phase, known for its neuronal regulating properties, egg phosphatidylcholine, polysorbate 80 and stearylamine. Their size was 270 nm and their zeta potential was +57 mV. The i.n. administration of CsA-loaded nanoemulsion resulted in a significantly higher (6 to 18 times) drug concentration in the olfactory bulb and also in the brain compared to the levels obtained following i.n. and i.v. administration of the free peptide, as well as i.v. of the peptide loaded nanoemulsion.

Another lipid based system used for the N-to-B peptide delivery is the so called nanostructured lipid carriers (NLCs), which consist of a combination of solid and liquid lipids. Gartziandia et al. developed NLCs coated with chitosan, with a size of 114 nm and a positive zeta potential of +28 mV for the delivery of human insulin-like growth factor-I (hIGF-I). The authors reported an enhanced residence time of the nanocarriers in the nasal epithelium owing to the mucoadhesive properties of chitosan and a high brain accumulation based on the results obtained using fluorescence imaging techniques. In a later study, this system was also used by the same group for the N-to-B delivery of the glial cell-derived neurotrophic factor (GDNF), achieving a significant improvement in terms of behavior (movement recovery) and neuroprotection, in comparison to the GDNF solution, in a hemiparkinsonian rat model.

A different type of lipid nanoparticles, is the one containing a gelatin core (GNLs) surrounded by a shell of phospholipids, cholesterol and Poloxamer 188. These nanoparticles, with a diameter of 170 nm and a surface charge of -30 mV, were tested for their capacity to enhance the N-to-B transport of the Substance P peptide, intended to treat hemiparkinsonian rats. The results
showed an enhanced response of the peptide when delivered through the nose. The same delivery vehicle was also tested for the N-to-B delivery of bFGF in order to treat cerebral ischemia. The results indicated that the intranasal administration of this formulation led to a significant increase (~1.5-fold) in the concentrations of the peptide in different brain regions (pallium, hippocampus, striatum, olfactory bulb), whereas no increase was observed after the i.v. administration of the bFGF solution or the bFGF loaded GNLS. Furthermore, the therapeutic effect of the i.n. administered bFGF GNLS was verified by the reported improvement in the neurological deficit score and locomotor activity of rats.

Overall, from these studies, it could be concluded that the efficient N-to-B peptide delivery does not depend solely on the nanocarrier size, but also on its composition. Compared to the other N-to-B lipid-based peptide delivery technologies, the highest rise of peptide concentration in the brain, relative to the i.n administration of the peptide solution, was reported for cationic liposomes (10 times higher) followed by cationic nanoemulsions (6 times higher) with diameters bigger than 200 nm (Table 4). This result could be attributed to the increased residence time achieved by these positively charged formulations in the olfactory region and more importantly to the presence of surfactants like polysorbate 80, known for its cell permeation enhancing effects. In conclusion, proper selection of lipids and surfactants can positively affect the peptide delivery through the olfactory route. Still, the limited number of studies performed so far does not allow us to draw further conclusions. Focused mechanistic studies are needed to shed light on the complex interactions of the different nano-based systems with the olfactory epithelium, the importance of the surface characteristics of the carriers and their effect on the in vivo efficacy of the systems.

Table 4. Overview of lipid-based nanocarrier systems for N-to-B peptide delivery.

<table>
<thead>
<tr>
<th>Nanocarrier</th>
<th>Drug</th>
<th>Size (nm)</th>
<th>Z-pot (mv)</th>
<th>Targeting molecule</th>
<th>Animal</th>
<th>Pharmacokinetics outcome/Therapeutic effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cationic liposomes</td>
<td>OVA</td>
<td>299</td>
<td>+19</td>
<td>-</td>
<td>Rats</td>
<td>10-fold brain levels &amp; &gt;4-fold AUC&lt;sub&gt;brain&lt;/sub&gt;/AUC&lt;sub&gt;blood&lt;/sub&gt; relative to i.n. peptide solution</td>
</tr>
<tr>
<td>Liposomes</td>
<td>H102 Peptide</td>
<td>110</td>
<td>-3</td>
<td>-</td>
<td>Rats</td>
<td>1.6-3-fold higher AUC values in specific brain areas than the i.n. solution (no drug in the brain after i.v. admin.)</td>
</tr>
<tr>
<td>Pegylated Cubosomes</td>
<td>S14G-HN (humanin derivative)</td>
<td>100 - 120</td>
<td>-14</td>
<td>OL</td>
<td>Rats</td>
<td>1.7-3.5-fold increase of the coumarin distribution in brain regions relative to the unmodified cubosomes Significant enhancement of therapeutic effect 6-fold increased brain concentration relative to i.n CsA solution</td>
</tr>
<tr>
<td>Oil-in-Water Nanoemulsion</td>
<td>CsA</td>
<td>270</td>
<td>+57</td>
<td>-</td>
<td>Rats</td>
<td>1.6-3-fold higher AUC values in specific brain areas than the i.n. solution (no drug in the brain after i.v. admin.)</td>
</tr>
</tbody>
</table>

Ref. 159, 171, 143, 175
<table>
<thead>
<tr>
<th>Gelatin NCL</th>
<th>bFGF</th>
<th>170</th>
<th>-27</th>
<th>-</th>
<th>Rats</th>
<th>1.5-fold enhancement of peptide brain concentration and its therapeutic effect relative to i.n. peptide solution (no transport)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin NCL</td>
<td>SP</td>
<td>172</td>
<td>-30</td>
<td>-</td>
<td>Rats</td>
<td>Significant enhancement of therapeutic effect</td>
</tr>
<tr>
<td>Gelatin NCL</td>
<td>bFGF</td>
<td>128</td>
<td>-15</td>
<td>-</td>
<td>Rats</td>
<td>Increased peptide concentration in brain relative to i.n. peptide solution Enhanced response</td>
</tr>
<tr>
<td>Chitosan coated NCL</td>
<td>hIGF-I</td>
<td>114</td>
<td>+28</td>
<td>-</td>
<td>Mice</td>
<td>Increased residence time in the nasal epithelium and brain-accumulation Significant enhancement of neuroprotective effect</td>
</tr>
<tr>
<td>Chitosan coated NCL</td>
<td>GDNF</td>
<td>137</td>
<td>+30</td>
<td>-</td>
<td>Rat</td>
<td></td>
</tr>
</tbody>
</table>

OVA: Ovalbumin, bFGF: basic fibroblast growth factor, SP: Substance P, CsA: Cyclosporine-A, hIGF-I: Human insulin-like growthfactor-I, GDNF: glial cell-derived neurotrophic factor, NCL: nanostructured lipid carriers

5. Strategies to enhance N-to-B drug transport

5.1 Olfactory targeting strategies

As mentioned above, several studies have explored the use of covalently linked biorecognitive ligands in order to enhance the N-to-B transport of the nanosized drug delivery carriers. Table 5 summarizes the targeting moieties that have been described in the literature for targeting the olfactory region. The most commonly used targeting ligands have been proteins that have receptors in the olfactory region, i.e. lactoferrin, or glycoproteins, i.e. lectins. Lactoferrin (Lf) has received a significant attention due to the high expression of its receptor (LfR) in the brain endothelial cells and neurons. For example, Liu et al., studied the N-to-B transport of lactoferrin conjugated PEG-PCL NPs (< 90 nm), loaded with a neuroprotective octapeptide (NAP), in an Alzheimer’s disease animal model, and observed a significant neuroprotective and memory ameliorating effect. According to the in vivo biodistribution study performed with fluorescent nanoparticles in rats, Lf-functionalized NPs exhibited a rapid accumulation in various brain areas, in comparison to the unmodified NPs. Other authors showed the capacity of the same type of nanoparticles (120 nm) for the N-to-B delivery of rotigotine, a dopamine agonist used to treat Parkinson’s disease, in a mice model. The results showed a two times higher drug concentration in different brain areas, relative to the ones achieved with the unmodified NPs.

Following the study by Ferrari et al. showing the affinity of several lectins for the N-acetyl-D-glucosamine and sialic acid residues in the olfactory mucosa, a number of works has been oriented to show that the functionalization of peptide-loaded nanoparticles with specific lectins, such as wheat germ agglutinin (WGA), solanum tuberosum lectin (STL), ulex europeus agglutinin I (UEA-I), is a useful approach to N-to-B delivery. For example, the functionalization of PEG-
PLA nanoparticles with WGA was reported to double the N-to-B transport of the Vasoactive intestinal peptide associated to them, in comparison to the transport observed for the unmodified nanoparticles.\textsuperscript{131} In a different study, the authors studied the brain distribution of the labeled (with \textsuperscript{125}I and coumarin) WGA-functionalized PLGA nanoparticles after nasal administration to rats.\textsuperscript{267} The conclusion was that the WGA functionalized PEG-PLA NPs could move through the olfactory epithelium and reach the olfactory bulb in 5 minutes, suggesting their transport through the extraneuronal pathway via the olfactory epithelium. In addition, a strong radioactivity signal was found in deeper brain areas (e.g., striatum, hippocampus, medulla, cerebellum, pons) at 30 min post-administration.

PEG-PLGA nanoparticles have also been functionalized with STL.\textsuperscript{122,266,268} The resulting nanoparticles showed an almost 2.5 times higher brain targeting efficiency (AUC\textsubscript{Brain}/AUC\textsubscript{Blood}) for the STL functionalized PEG-PLGA NPs relative to the values attained for the unmodified NPs after their i.n. administration in rats.\textsuperscript{266}

Some authors have argued about the potential immunotoxicity of lectins and suggested the use of smaller peptides with lectin-like function, like Odorranalectin (OL), a small peptide (1.7 KDa) from frog skin secretions.\textsuperscript{269} This lectin recognizes and specifically binds to L-fucose, which is highly expressed in the olfactory mucosa. In vivo fluorescence imaging studies showed that the interaction of fluorescent PEG-PLGA nanoparticles (95 nm), as well as that of pegylated cubosomes (100-200 nm) functionalized with OL, with the olfactory epithelium was significantly improved (up to a 3.5-fold increase) as compared to the one observed for the unmodified NPs.\textsuperscript{143,167}

Table 5. Examples of targeting ligands and nanocarriers used for Nose-to-Brain drug delivery

<table>
<thead>
<tr>
<th>Nanosystem</th>
<th>Targeting ligands</th>
<th>Animal model</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-PCL NPs</td>
<td>Lactoferrin</td>
<td>Rats/Mice</td>
<td>129</td>
</tr>
<tr>
<td>PEG-PLGA NPs</td>
<td>Lactoferrin</td>
<td>Mice</td>
<td>264</td>
</tr>
<tr>
<td>PEG-PLGA NPs</td>
<td>STL</td>
<td>Rats</td>
<td>122,266,268</td>
</tr>
<tr>
<td>PEG-PLA NPs</td>
<td>WGA</td>
<td>Rats/Mice</td>
<td>131,251,267,270</td>
</tr>
<tr>
<td>PEG-PLA NPs</td>
<td>UEA I</td>
<td>Rats</td>
<td>271</td>
</tr>
<tr>
<td>PEG-PLGA NPs</td>
<td>OL</td>
<td>Rats/Mice</td>
<td>167</td>
</tr>
<tr>
<td>Pegylated Cubosomes</td>
<td>OL</td>
<td>Rats</td>
<td>143</td>
</tr>
</tbody>
</table>


5.2 Cell-penetrating peptides (CPPs)
CPPs are traditionally positively charged oligopeptides, containing arginine and lysine residues, that have been long recognized for their ability to interact with biological membranes and enable the efficient cellular uptake of biomacromolecules, via different endocytosis pathways or direct cytoplasmic translocation. Recent research on N-to-B drug delivery has proven the ability of these peptides to significantly increase the permeation of several nanosystems through the olfactory epithelium (Table 6). Among them, the human immunodeficiency virus Tat peptide (GRKKRRQRRRPQ) is the most commonly used so far. Tat peptide’s cell penetrating properties have been directly associated to the presence of the guanidinium groups of arginine, which can induce both electrostatic and hydrogen bonding with the cell-surface. In a recent study, the authors described a nanostructured lipid carrier (particle size of 206 nm and surface of +22 mV) coated with Tat-conjugated chitosan, for the intranasal delivery of glial cell-derived neurotrophic factor (GDNF). According to the results, the surface modification of the system with the Tat peptide led to a significant enhancement of the therapeutic effect of the administered protein, in terms of behavior, histological evaluation and microgliosis reduction in a mouse model of Parkinson’s disease. On the other hand, insulin-loaded PLGA NPs, surface modified with the cationic Tat peptide (~200 nm, +11 mV), were able to accumulate nearly 6.5 times more in the olfactory bulb in a matter of a few hours, in contrast to the non-modified NPs, that exhibited poor transport from the nose to the brain.

Other studies have been oriented to analyze the potential of the Tat peptide to improve the N-to-B transport of surface-conjugated MPEG-PCL nanomicelles with a size <100 nm and a slightly positive surface charge. The same group employed this carrier for the nose-to-brain delivery of an anti-tumor drug (camptothecin) and a small interfering ribonucleic acid. In a follow up study, a similar system (50-80 nm, 10-15 mV) was employed, where MPEG-PCL micelles, conjugated with a different arginine-based CPP, were tested for their ability to improve the N-to-B delivery of macromolecules, using dextran as a model drug. A comparison was also made with a system containing a hydrophobic derivative of the CPP (stearyl modification, STR) (100 nm, 20 mV), proving the possibility of targeting different brain areas, depending on the hydrophilicity of the CPP. Using in vivo fluorescence imaging, it was found that both systems reached the olfactory bulb in the short period of 15 minutes, in contrast to the labeled dextran control for which almost no fluorescence was observed in the brain. More interestingly, the more hydrophobic complex accumulated mostly in the forebrain, whereas the regular CPP complex could be found throughout the brain. Still, the mechanisms involved throughout this brain translocation remain unknown.

Another CPP type that has attracted attention for nose-to-brain delivery are short peptide fragments of the well-known CPP protamine, produced by enzymatic degradation, called low molecular weight protamine (LMWP, CVSRRRRRRGGRRRR). The advantage of these chain-shortened peptides is their significant lower toxicity and immunogenicity than their parent protein. For example, it has
been reported that the attachment of LMWP onto the surface of PEG-PLGA nanoparticles led to a significant enhancement of the N-to-B delivery of coumarin. Taking all these results into account, we can conclude that the CPPs hold a potential in terms of facilitating the nanoparticle-mediated transport of macromolecules from the nose directly to the brain.

Table 6. Examples of nanocarriers surface-modified with CPPs for Nose-to-Brain drug delivery

<table>
<thead>
<tr>
<th>Nanosystem</th>
<th>Surface modification</th>
<th>Animal model</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan coated NCL</td>
<td>Tat</td>
<td>Mice</td>
<td>147</td>
</tr>
<tr>
<td>PLGA NPs</td>
<td>Tat</td>
<td>Mice</td>
<td>276</td>
</tr>
<tr>
<td>Nanomicelles MPEG-PCL</td>
<td>Tat</td>
<td>Rats</td>
<td>273-275,277</td>
</tr>
<tr>
<td>Nanomicelles</td>
<td>CH2R4H2C</td>
<td>Rats</td>
<td>279</td>
</tr>
<tr>
<td>PEG-PLA NPs</td>
<td>LMWP</td>
<td>Rats</td>
<td>280</td>
</tr>
</tbody>
</table>

NCL: nanostructured lipid carriers, PLGA: poly(lactic-glycolic acid), PEG-PCL: PEG-poly (ε-caprolactone), PLA: polylactic acid, LMWP: low molecular weight protamine

6. Comparative analysis of in vivo studies

In a review by Merkus and van den Berg, the authors defined the principles for a reliable animal study aiming to prove the direct N-to-B transport. These principles are: (i) the selection of realistic administration volumes and concentrations (ii) the comparison of the data with those obtained upon intravenous administration of the same formulation, (iii) the pharmacokinetic analysis in plasma and CNS and (iv) studying the brain biodistribution of the administered formulations. Other authors defined specific parameters to express the efficacy of different N-to-B delivery approaches, i.e. the drug targeting efficiency % (DTE %), which represents the efficiency of the drug to reach the brain compared to the blood, following intranasal versus parenteral administration, and the direct transport percentage % (DTP %), which represents the percentage of the drug that reaches the brain due to direct N-to-B transport, with respect to the total amount of drug found in the brain %), as described by the following equations:

- **Drug targeting efficiency % (DTE%)**

  \[
  DTE\% = \left( \frac{AUC_{\text{brain}}}{AUC_{\text{blood}}} \right)_{\text{intranasal}} \cdot 100\%,
  \]

  where \(AUC_{\text{brain}}\) and \(AUC_{\text{blood}}\) are the area under the curve of the drug in the brain or blood respectively, estimated each time for the corresponded route and

- **Direct transport percentage % (DTP%)**

  \[
  DTP\% = \left( \frac{B_{\text{in}} - B_x}{B_{\text{in}}} \right) \cdot 100\%,
  \]
where \( B_{\text{in}} \) is the brain AUC after the intranasal administration, \( B_x \) is the brain AUC fraction from the blood after intranasal administration and equals to \( B_x = (B_{\text{in}}/P_{\text{par}})P_{\text{in}} \), where \( P_{\text{par}} \) is the blood AUC following parenteral administration, and \( P_{\text{in}} \) is the blood AUC following intranasal administration.

The variety of pharmacokinetics/pharmacodynamics parameters analyzed in the different literature reports (Table 3&4) renders the comparison of the in vivo performance of nanocarriers very difficult. After a thorough screening of the selected publications, it is apparent that not all of them provide adequate information to substantiate the direct N-to-B peptide transport, according to the criteria set by Merkus and van den Berg. In fact, most of the reports do not disclose accurate pK/biodistribution data, but rather indirect efficacy or semiquantitative biodistribution results (Fig. 6-7).

![Figure 6](image-url)

**Figure 6.** (A) Mean concentration of cyclosporine-A (CsA) in the brain and (B) Brain-to-blood concentration ratio of cyclosporine-A (CsA) after intranasal (IN) or intravenous (IV) administration of CsA loaded nanoemulsion (CsA-NE) or CsA-solution (CsA-S) to rats. (*p < 0.05 or **p < 0.01 vs control groups). Adapted with permission from [175].
In addition, some studies lack of proper controls, such as a systemic comparison or biodistribution data, a fact that makes it inappropriate to conclude about the direct N-to-B transport mechanism.\textsuperscript{152,155,160} It is, however, important to highlight that a significant number of studies have reported direct evidence of the ability of nanocarriers to travel through the olfactory pathway and deliver their payload to the different brain areas, employing fluorescence and radioactivity-based imaging techniques (Fig. 7).\textsuperscript{122,129,131,136,140,142,143,151,159,160,167,276} Nevertheless, quantitative information on the N-to-B transport of the nanocarriers is still missing in the literature.

![Figure 7](image-url)

**Figure 7.** Brain distribution study of PEG-PLGA NPs (Odorranalectin-functionalized (OL-NP) and non-functionalized (NP)) in mice by in vivo imaging system. (A) Fluorescence images of animal following i.n. administration at different time points; (B) Fluorescence intensity in the brain versus time. (*p<0.05) Adapted with permission from [167].

Among the studies analyzed throughout this review, high nose-to-brain delivery efficiency was reported for both, lipid based and polymer-based systems. Comparatively, the highest performance of a nanocarrier in terms of enhanced transport of the associated peptide was reported for cationic liposomes of 300 nm, which resulted in 10 times enhanced bioavailability of OVA in the brain.\textsuperscript{159} Similarly, PLA nanoparticles coated with chitosan (<150 nm) were found to lead to a 9.5-fold enhanced concentration of Neurotoxin-I, in the brain.\textsuperscript{142} Another example, describes a 7-fold peptide (vasoactive intestinal peptide) transport increase following the i.n. administration of WGA
functionalized PEG-PLGA nanoparticles as compared to the i.n. administered free peptide solution. Similar transport efficiency was observed for the CsA administered in the form of a cationic oil-in-water nanoemulsion (~270 nm).

Among the discussed studies, the one from Zhang et al. is considered to be particularly rigorous as it complies with all the criteria mentioned above. The group employed PEG-PLGA NPs surface modified with STL, with a particle size of 120 nm and a negative surface charge of -32 mV, for the N-to-B delivery of bFGF. Radioisotopic tracing method enabled the tracking of the radiolabeled protein molecule after its i.v. and i.n. administration with or without drug delivery system, proving the superiority of the i.n. administered nanoformulation. DTE% and DTP% values provided additional evidence of the ability of the carrier to enhance the nose to brain peptide delivery (DTE% = 1050% and DTP%=90.44% for the olfactory bulb). The enhanced therapeutic effect was later verified in rats after the i.n. administration of the peptide loaded nanocarriers.

A general conclusion from these studies is that despite the recognized importance of the particle size, the composition of the carrier plays a critical role in the fate of the intranasally administered nanocarriers. Overall, the tendency is to observe that cationic or functionalized nanocarriers perform better than plain negatively charged nanocarriers. Nevertheless, the variability in the parameters and criteria, as well as the different animal models employed, do not allow an in-depth comparison among the different systems. Therefore, more exhaustive comparative studies are required in order to distinguish among the different nano-based N-to-B peptide delivery systems.

**Comparative analysis of the in vivo efficacy of the nano-based DDS vs. permeation enhancers**

As mentioned above, various authors have proven the ability of non-covalent permeation enhancers to facilitate the N-to-B delivery of the co-administered peptide drugs through the olfactory epithelium. One of the most popular permeation enhancers used for the N-to-B peptide delivery is chitosan, followed by various cell permeating peptides (i.e. penetratin, low molecular weight protamine, Pz-peptide). In terms of the peptide bioavailability in the brain, the most promising results so far have been reported by Morishita’s group who presented up to 20 times increased levels of insulin in different brain areas after its co-administration with L-penetratin (0.5 mM) as compared to the i.n. administered free protein solution. Following these studies, Vaka and co-workers showed a 13−14-fold increase in the brain bioavailability of NGF and BDNF respectively, when i.n. co-administered with chitosan, in the form of a solution. The same authors showed the ability of peppermint oil to increase around 8 times the brain concentration of NGF, after their i.n. co-administration. Peppermint oil is also believed to have a tight junctions’ opening effect, promoting thus the paracellular uptake in the olfactory epithelium.

Unfortunately, we have identified only one report showing the performance of penetration enhancers vs. nanocarriers using the same peptide drug. In particular, in 2015, Zheng et al. compared the
efficiency of the permeation enhancer chitosan, and a formulation of PEGylated liposomes, with a particle diameter of 110 nm, for the N-to-B delivery of the H102 peptide. The results showed significantly higher (1.6–3-fold) values of the drug concentration over the time in different brain areas for the liposomal formulation, as compared to the AUC values observed for the chitosan solution. According to the authors, this significant difference in performance was attributed to the ability of the nanoformulation to provide adequate protection to the peptide from enzymatic degradation, something that a simple co-administration with a permeation enhancer failed to provide.\textsuperscript{171} In another study, the authors explored the use of chitosan as a permeation enhancer for the i.n. administration of bFGF, achieving up to 1.95 times increased concentrations in different brain areas as compared to the i.n. administered free peptide in rats.\textsuperscript{74} Similar results though were reported by later studies, using gelatin based nanocarriers for the N-to-B transport of the same peptide.\textsuperscript{123,124}

Overall, it is clear that, more comparative studies based on the same peptide drugs are required in order to make a clear conclusion on the comparison between nano-based drug delivery systems and permeation enhancers.

### 7. Conclusions and Future Perspectives

The increasing number of the in vivo studies published over the last years on the direct nose-to-brain transport of protein and peptide drugs indicates the growing interest in this route. This modality of administration is particularly attractive for peptides and proteins as they have the chance to reach the CNS directly, avoiding the disadvantages and limitations of the systemic route. Several studies have already proven the ability of peptide and protein drugs to be transported through this route to different brain areas in animal models, while several of them are already undergoing clinical studies. However, the bioavailability of these peptides remains quite low. The contribution of nanotechnology in this field is becoming more and more evident. Nanosized drug delivery carriers provide a versatile platform with great potential in overcoming the underlying challenges of this route. In general, the particle size and the composition of the carrier are considered to be important factors that may influence the fate of the nanocarriers after their intranasal administration. Specifically, a particle size in the range of 100-200 nm has been explored with the conclusion that the importance of this size range is also connected with the composition of the nanocarrier. Regarding the composition, both muco-permeating agents (i.e. PEG and pegylated surfactants) and cell permeation enhancing agents (i.e. chitosan) were reported to lead to some positive effects. However, the mechanism underlying this positive outcome has not been elucidated. In addition, a tendency is observed towards the surface functionalization of the nanocarriers with biorecognitive ligands. Still, no nanosystem has reached the clinical development phase up to now. The limited number of in vivo studies in non-primate animals and the differences in nasal anatomy between
human and animals, do not allow us to draw clear conclusions. In conclusion, the field of nano-based N-to-B peptide delivery is still at an early development stage and its potential is not fully unveiled. To translate this research from the bench to the clinic, more conclusive studies involving pharmacokinetic and pharmacodynamic data are required to shed light on the brain targeting efficiency of the nanosystems and the underlying mechanisms.

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