

Expert Opinion

1. Introduction
2. Intranasal delivery: mechanism and pathways
3. Methodological aspects of intranasal drug deposition on the olfactory epithelium
4. How to improve intranasal delivery of proteins: pharmacological and formulation considerations
5. Different classes of intranasally delivered therapeutic proteins
6. Intranasal delivery in human clinical trials for neurodegenerative disorders
7. Future perspectives
8. Expert opinion

informa
healthcare

Intranasal delivery of therapeutic proteins for neurological diseases

Francesca Malerba, Francesca Paoletti, Simona Capsoni & Antonino Cattaneo[†]

[†]*European Brain Research Institute, Rome, Italy*

Introduction: Among the range of therapeutic protein candidates for new generation treatments of neurological diseases, neurotrophic factors and recombinant antibodies hold the greatest potential. However, major difficulties in their safe and effective delivery to the brain severely limit these applications. The BBB restricts the exchange of proteins between the plasma and the CNS. Moreover, therapeutic proteins often need to be selectively targeted to the brain, while minimizing their biodistribution to systemic compartments, to avoid peripheral side effects. The intranasal delivery of proteins has recently emerged as a non-invasive, safe and effective method to target proteins to the CNS, bypassing the BBB and minimizing systemic exposure.

Areas covered: We critically summarize the main experimental and mechanistic facts about the simple and non-invasive nasal delivery approach, which provides a promising strategy and a potential solution for the severe unmet medical need of safely and effectively delivering protein therapeutics to the brain.

Expert opinion: The intranasal route for the effective delivery of recombinant therapeutic proteins represents an emerging and promising non-invasive strategy. Future studies will achieve a detailed understanding of pharmacokinetic and mechanisms of delivery to optimize formulations and fully exploit the nose-to-brain interface in order to deliver proteins for the treatment of neurological diseases. This expanding research area will most likely produce exciting results in the near future towards new therapeutical approaches for the CNS.

Keywords: Alzheimer's disease, BBB, biologicals, intranasal delivery, neurodegenerative diseases, neurotrophins, NGF, therapeutic antibodies

Expert Opin. Drug Deliv. (2011) 8(10):1277-1296

1. Introduction

The effective treatment of neurological and neurodegenerative disorders is a severe unmet need, and the delivery of pharmacologically relevant quantities of therapeutic proteins (the so-called 'biologicals') to the brain, across the BBB, is a key aspect to be solved.

Among the wide range of biologicals (i.e., protein therapeutic candidates) for the treatment of CNS diseases, neurotrophic factors and recombinant antibodies hold, as protein classes, the greatest promises.

Indeed, different neurodegenerative diseases, such as Alzheimer's (AD), Parkinson's (PD) or Huntington's (HD) disease, or amyotrophic lateral sclerosis (ALS), affect primarily a specific class of neurons. This has led to the now well-established therapeutic concept that neurotrophic factors, specifically acting on those cells, represent promising candidates for the treatment of such diseases. Despite high enthusiasm, however, early attempts to develop clinical treatments based on such a basis were not successful [1]. Problems with the effective delivery

Article highlights.

- Numerous neurological diseases require a targeted delivery of proteins to the CNS. However, the presence of the BBB prevents an effective systemic delivery of protein therapeutics, especially as far as it concerns neurotrophic factors and antibodies/antibodies domains. This is often achieved through invasive methods, such as intracerebroventricular infusions, with severe limitations. Intranasal delivery represents a non-invasive, safe and essentially painless approach.
- The mechanisms of the intranasal delivery are regulated by the anatomy of the nasal cavity. A combination of pathways are important for the transport from the nasal cavities to the brain, depending on the chemical properties of the molecules, the type of formulation and the device used for delivery. Through the olfactory pathways, there is no systemic absorption of the delivered molecules. This feature limits the presence of undesired side effects.
- The intranasal delivery was proven to be more effective than other non-invasive methods, such as ocular delivery.
- To improve the intranasal delivery of protein drugs, the physicochemical characteristics should be considered as well as optimization of formulation.
- Different potential therapeutic proteins are being investigated for the intranasal delivery on the cure of neurological diseases. Among these, we focus in this review on neurotrophins, neuropeptides, growth factors, chemokines, hormones and antibodies, with particular emphasis on those already tested in a clinical trial.
- The future perspectives of the intranasal delivery field cover the use of the delivery of nucleic acids (both DNA and RNA), virus (especially those naturally targeted to the olfactory pathway) and stem cells.

This box summarizes key points contained in the article.

to the brain and side effects due to systemic negative actions are recognized as the hurdles and the difficulties to be overcome.

As for antibodies, human or humanized antibodies represent the largest class of therapeutic proteins in the pharmaceutical market and the clinical pipelines of the pharmaceutical industry [2,3]. Antibody-based therapies have proven efficacious in a variety of diseases, but the majority of the current antibody-based therapies are related to therapeutic areas other than the CNS [4]. One notable exception is provided by the current passive immunization clinical trials for AD targeting the A β peptide [5]. Also, in the case of antibodies, despite the huge therapeutic opportunity provided by the increasing number of target molecules potentially relevant for antibody-based therapies in the CNS, the exploitation of this potential is currently limited by the difficulty in targeting antibodies, or smaller antibody domains [6], across the BBB into the brain.

Thus, the presence of the BBB prevents an effective systemic delivery of protein therapeutics, such as

neurotrophins or antibody domains. On the other hand, the high systemic doses required to achieve pharmacologically relevant therapeutic levels in the brain can lead to adverse effects in the body. Thus, protein therapeutics has been so far directly targeted into the brain through invasive, highly impractical techniques requiring neurosurgery [1,7].

The possibility of targeting protein therapeutics in the brain by less invasive protocols remains an open question. Intranasal delivery of proteins [8] has recently emerged as a non-invasive, safe and effective method to target peptide and proteins to the CNS, bypassing the BBB and minimizing systemic exposure.

In this review, we critically discuss the evidence for the use of the intranasal delivery as a non-invasive technique to deliver peptides and proteins to the brain, the pathways and mechanism underlying their entry and distribution into the CNS, and the advantages/disadvantages for its application in a clinical context focusing mostly on neurotrophic factors, neuropeptides and antibody domains.

1.1 Bypassing the BBB: a general overview

The BBB, a physical, metabolic and immunological barrier located at the level of the brain endothelial cells [9], regulates the homeostasis of the brain, by limiting the access of plasma components, red blood cells and leukocytes into the brain, and preventing most foreign substances from entering the brain from the circulating blood [10].

The BBB limits also the distribution of systemically administered therapeutics to the brain, posing a significant challenge to drug development efforts to treat CNS disorders [11]. Despite the extensive cerebral vasculature network and the presence of several transport systems, specific for proteins and peptides [12-14], ~ 98% of small molecules and almost all larger protein molecules (with some notable exceptions, such as transferrin or IGF-1, which exploit specific transporters) fail to cross the BBB and reach the CNS following a systemic delivery, requiring high doses to achieve therapeutic levels, which can lead to adverse effects in the body [15,16]. Indeed, some proteins cannot exceed low threshold concentrations in the blood because of the onset of undesired side effects. For example, intravenous nerve growth factor (NGF) doses of 0.1 μ g/kg induce a myalgic and nociceptive reaction [17]. Thus, for such molecules, increasing the systemic dose, to increase the amount passing the BBB, is not a viable strategy.

There is also growing evidence that disruption of the BBB itself can precede, accelerate and contribute in different ways to the disease processes in neurodegenerative disorders, such as AD, PD, MS and ALS [9,18-21]. This phenomenon could modify the delivery and the clearance of the proteins into the brain in pathological conditions and should be analyzed on a case by case basis. A deeper overview on this topic is beyond the scope of this review.

Attempts to improve the capability of systemically administered biopharmaceutical drugs to cross the BBB, by

transient opening, include osmotic disruption/shrinking of the BBB by the intracarotid administration of hypertonic solutions or opening the BBB tight junctions via receptor-mediated mechanisms, [15]. However, this BBB transient opening is considered unsafe and could damage neurons due to blood components entering the brain.

Other attempts to increase BBB transport of therapeutic proteins into the brain include their coupling or engineering with specific amino-acidic domains, entire carrier proteins, antibodies or chemical groups, capable of enhancing their CNS delivery and exploiting specific BBB transporters [9,22]. However, these are not general approaches, as they require an extensive optimization on a case by case basis and could lead to immunogenic reactions [10].

In the absence of better ways to bypass the BBB, therapeutics can be introduced directly into the CNS by intracerebroventricular or intraparenchymal injections [1,7]. However, these delivery methods are invasive, risky and expensive, requiring surgical expertise and hospitalization.

The intranasal delivery route [23,24] has emerged as a viable alternative to invasive delivery methods to bypass the BBB and rapidly target protein therapeutics directly to the CNS in a safe and pharmacologically effective manner, reducing systemic exposure [25,26]. The olfactory organ is a natural port of entry to the CNS, the only part of the brain directly in contact with the external environment and provides, therefore, a unique opportunity to deliver protein therapeutics to the brain.

Intranasal delivery has received an extensive validation as a general non-invasive method for brain targeting of a large class of peptides and proteins and was also shown to work in humans, being easily administered, maximizing patient convenience, comfort and compliance [27].

2. Intranasal delivery: mechanism and pathways

2.1 The nasal cavity: a natural port of entry into the brain

The roof of the nasal cavity represents a natural port of entry in the brain. Indeed, axon bundles from the olfactory receptors neurons, located in the olfactory epithelium, cross into the brain through the manifold small holes of the cribriform plate of the ethmoid bone. These nerve bundles, with their associated cells, and these holes in the fibrous plate are exploited for the intranasal delivery of protein into the brain.

Different pathways mediate transport from the nasal cavities to the brain, depending on the chemical nature and properties of the molecules, the formulation and the delivery device.

Transport pathways include nerves (olfactory and trigeminal) connecting the nasal passages to the brain, vasculature, cerebrospinal fluid (CSF) and lymphatic system (Figure 1).

In humans, nasal cavities are subdivided into three regions: the nasal vestibule, the respiratory region and the olfactory

region. Absorption of drugs occurs in the respiratory and in the olfactory regions of the nasal cavities.

2.2 The respiratory epithelium

The respiratory region is covered by a pseudostratified epithelium formed by ciliated and non-ciliated cells, which produce an overlaid viscous mucosal layer. Materials deposited on the epithelial surface are gradually cleared from the nasal cavity by the movement of the microvilli (mucociliary clearance mechanism). Besides a defense function, microvilli increase the surface of absorption, making the respiratory epithelium the major site for systemic drug absorption from the nasal route, due also to its extensive vasculature.

2.3 The olfactory epithelium and the olfactory nerve pathway

The olfactory organ is located, in humans, in the roof of the nasal cavity. The olfactory epithelium covers a large area of the nasal cavity in rodents and carnivores (50 and 77%, respectively), but a much smaller area (~3%) in humans [28].

The olfactory epithelium comprises olfactory receptor neurons (ORNs), sustentacular cells which support ORNs and basal cells, which are able to differentiate into neuronal receptor cells and replace them every 30 – 40 days.

The olfactory receptor cells are bipolar neurons with a single dendritic process which extends to the free apical surface, where it terminates in numerous long and non-motile cilia. An unmyelinated axon penetrates the basal membrane to join other axons and form large bundles in the lamina propria. The axons bundles, ensheathed by glial cells (Schwann cells), cross into the cranial cavity through small holes in the cribriform plate of the ethmoid bone and synapse in the olfactory bulb. From the olfactory bulb, axons originate in the mitral or tufted cells and connect to the olfactory tubercle. The projections then go to the amygdala, the prepyriform cortex, the anterior olfactory nucleus and the entorhinal cortex, as well as the hippocampus, hypothalamus and thalamus.

At the nasal surface, the membranes of adjacent olfactory receptors and supporting cells are connected by classical intercellular tight junction complexes [29], creating a regulated barrier between cells. The dynamical cellular environment of the olfactory epithelium, with a rapid turnover of ORNs, plays a crucial role in the mechanism of intranasal protein delivery into the brain. Indeed, ORNs regenerate every 3 – 4 weeks from basal cells located at the basal membrane of the olfactory epithelium. As a consequence, the nasal epithelium barrier to the CNS may transiently open at the multiple sites of integration of tuning over ORNs, also by modulating the expression of junction proteins, and could become locally, but diffusely, 'leaky' to applied proteins [24].

The transport/traffic of drugs (be it small molecules or proteins) to the brain, by the intranasal route, involves two main mechanistic steps, the crossing of the nasal epithelium and the subsequent, longer distance, transport/traffic to the brain.

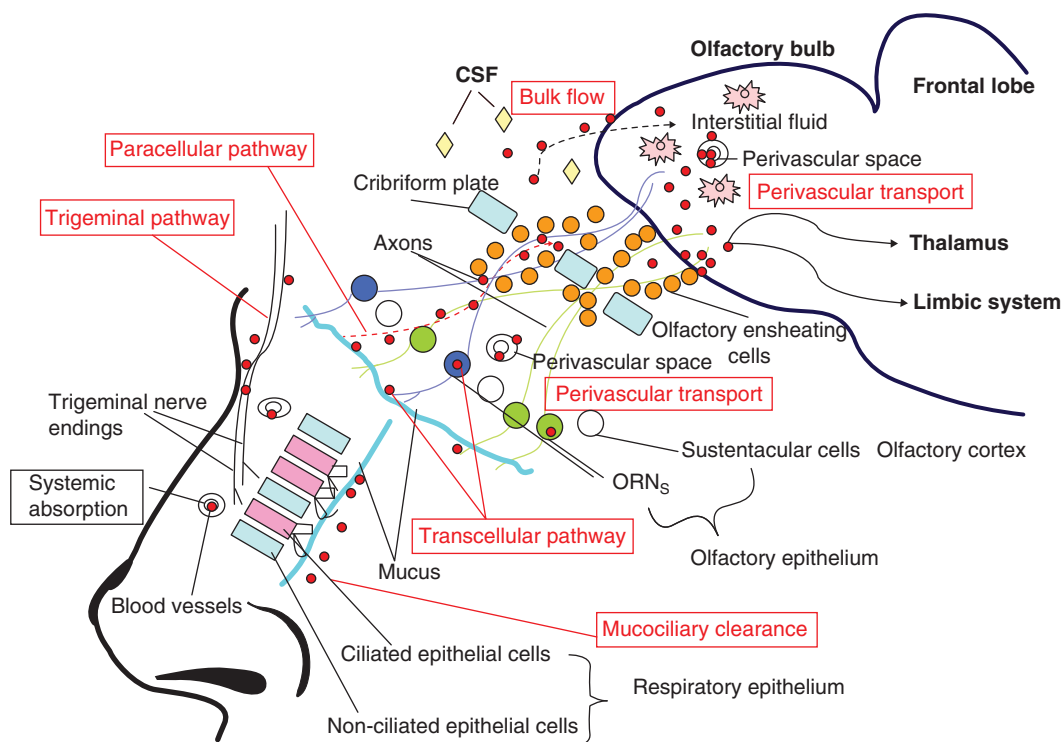


Figure 1. Schematic view of the different fate and pathways for protein/drug absorption and transport from the nasal cavity to the CNS. After intranasal administration, drugs (red circles) can enter in contact with the respiratory or the olfactory epithelium. In the respiratory region, ciliated epithelial cells and mucous secreted by non-ciliated cells contribute to the mucociliary clearance mechanism that removes substances from the nasal cavity. Drugs can penetrate the systemic circulation through passage in blood vessels found in the lamina propria underlying the respiratory epithelium region. In the olfactory epithelium region, in the roof of the nasal cavity, ORNs (blue and green circles) surrounded by sustentacular cells (white circles) contribute to the absorption of molecules through the receptor-mediated transcellular pathway, according to which proteins/drugs are endocytosed by ORNs and transported to the brain by slow axonal transport in the olfactory nerves. From the olfactory epithelium, molecules can also transit to the CNS through the rapid, nonsaturable paracellular pathway, travelling in channels formed by olfactory ensheathing cells (orange circles) along ORN axons. From these channels, drugs reach the olfactory bulb and other brain regions. Drug absorption into the CSF or into perivascular spaces provide further rapid and widespread biodistribution mechanisms. Transport via the trigeminal nerves, instead of the olfactory nerves, would transport drugs to more caudal regions of the brain.

ORN: Olfactory receptor neuron.

Drugs can cross the olfactory epithelium using two different pathways: a transcellular pathway, by receptor, carrier-mediated or vesicular transport mechanisms across the cells of the epithelium, and a paracellular pathway, via gap or tight junctions between cells and/or perineural channels. Given the unique environment of the olfactory epithelium, the same intracellular (transcellular) or extracellular (paracellular) mechanisms of transport along olfactory nerves might underlie the transport of intranasally administered proteins to the CNS.

2.3.1 Transcellular pathway

The transcellular pathway, which has been demonstrated for small lipophilic drugs or substances such as gold particles [30], aluminum salts [31] and for substances with receptors expressed on ORN surface (WGA-HRP) [23,32], requires specific receptors or adsorptive endocytosis, followed by

axonal transport and is slow: it takes from several hours to 3 – 4 days for a drug or a protein to reach the olfactory bulb and other brain areas [32]. This is a classical saturable, receptor-mediated axonal transport pathway, requiring cellular uptake and intracellular transport.

2.3.2 Paracellular pathway

The paracellular pathway is an extracellular transport mechanism involving the rapid movement of molecules between olfactory epithelium cells that can also contribute to the transport of molecules into the brain [33,34]. The extracellular transport to the olfactory bulb, via the paracellular pathway, has a fast kinetics, occurring within 30 min (or even less, in some cases) from the administration of the drug or the protein [24].

In the paracellular pathway, one mechanism through which molecules might reach the brain is via openings in gap or tight

junctions between cells of the olfactory epithelium, made leaky as ORNs turn over and regenerate [35-37], after which they get transported by bulk flow mechanisms within perineuronal channels formed by olfactory ensheathing glial cells around the olfactory axon bundles. From these perineuronal channels, intranasally delivered peptides or proteins reach the olfactory bulb and other brain regions.

As for the relative contribution of transcellular versus the paracellular transport to protein transport and delivery to the CNS, the majority of studies published so far showed a rapid kinetics of accumulation in the CNS, within 1 h of intranasal administration [33,38-39] consistently with a paracellular transport. In addition, except for a few exceptions, the transport system does not appear to be saturable, as in the case of NGF and IGF-1 [33,40], suggesting a non-receptor-mediated transport. Finally, the broad spectrum of peptides and proteins shown to be effectively delivered to the CNS following intranasal administration does not account for a receptor-mediated transcellular transport system that would involve the unlikely case of a broad range of different receptors. This evidence points to a prevailing paracellular pathway mechanism as a direct nose-to-brain route.

2.3.3 Trigeminal nerve pathway

In addition to the olfactory nerve pathway, transport to the CNS can also take place via a trigeminal nerve pathway [33], along the peripheral ophthalmic branch of trigeminal nerves, emerging from the semilunar Gasser ganglion, which innervates the respiratory and olfactory epithelium [41], and whose central processes enter the CNS, from the Gasser ganglion at the level of the pons. Collaterals from the trigeminal nerve also reach the olfactory bulb [42]. As far as the mechanism is concerned, due to these anatomical similarities, molecules might travel along the trigeminal axons through paracellular channel mechanisms analogous to those described for the olfactory nerve, although channels formed by ensheathing glial cells around trigeminal nerve fibers have not yet been described. The trigeminal pathways would allow for the transport of molecules to the posterior parts of the brain, such as cerebellum and brainstem [38,39,43]. A contribution to this form of intranasal delivery has been shown for IGF-I [33], IFN- β_{1b} [44], hypocretin-1 [45] and an antagonist of the urokinase plasminogen activator receptor [46].

This regionally divergent innervation from the nasal cavity to different brain regions offers an opportunity for experimental flexibility, provided one learns how to selectively exploit it, in terms of local administration modality, uptake or selective transport mechanisms.

2.3.4 Perivascular and cerebrospinal fluid pathways

Two additional pathways have been suggested to contribute to the biodistribution of drugs into the brain, following intranasal delivery, a perivascular and a CSF pathway [15]. The relative contribution of these additional routes with respect to the previous ones remains to be ascertained.

The intranasal administration can be a route to deliver drugs in the systemic circulation. Indeed, the respiratory and olfactory mucosae are highly vascular and receive blood supply from branches of the maxillary, ophthalmic and facial arteries, arising from the carotid artery. As the respiratory mucosa is much more vascular than the olfactory epithelium, the respiratory regions of the nasal epithelium represent the major candidate for supplying drugs to the systemic circulation after intranasal delivery. On the other hand, a selective administration to the olfactory epithelium versus the respiratory epithelium is to be preferred for the selective enrichment of CNS protein delivery while minimizing the systemic availability of the delivered protein drug. From the nasal mucosa, drugs pass into the blood circulation via continuous and fenestrated endothelia [47] or by counter-current transfer, whereby molecules enter the venous blood supply in the nasal passages, where they are transferred to the carotid arterial blood feeding the brain [48,49].

Recently, perivascular channels, formed by the outermost layer of blood vessels and the basement membrane of the surrounding tissue, have been suggested to contribute to intranasal drug delivery to the CNS [36] by acting as a lymphatic system, whereby substances produced by neurons would be cleared from the brain by a perivascular bulk flow transport in these perivascular channels [37,50]. This form of 'perivascular pump' mechanism, fuelled by the arterial pulsations, could contribute to the observed rapid distribution, throughout the brain, of exogenously applied molecules [51,52].

Substances injected into the CSF, cerebral ventricles or subarachnoid space have been shown to drain outside the olfactory bulb into channels surrounding olfactory nerves which cross the cribriform plate and reach the nasal lymphatic system [15]. For drugs applied intranasally, the same, opposite route was observed in rats [37,53] and in humans [54]. Thus, CSF drainage may reduce the efficiency of intranasal delivery.

3. Methodological aspects of intranasal drug deposition on the olfactory epithelium

From these anatomical considerations, it is clear that methodological aspects concerning the drug intranasal delivery to the brain have a great importance. This is particularly true for human subjects, where the proportion of the nasal cavity covered by the olfactory epithelium is much lower than in laboratory animals and its location is difficult to access, being on the roof of the nasal cavity. Thus, methods for delivery should be implemented to avoid, in the case of molecules that should not reach the systemic circulation, the access of drugs to the respiratory epithelium. Several different aspects and parameters for nasal delivery have been analyzed and improved, being the subject of continuing research, and include administration techniques, head position, delivery devices and drop volumes [15]. The optimization of different aspects of the delivery methods will also depend on the specific substance to be delivered and the objectives to be achieved, such as, achieving

a specific trade-off between biodistribution in the CNS versus the systemic compartment [16].

A detailed discussion of the methodological aspects of the nasal delivery technology is out of the scope of this review, and we refer to excellent comprehensive reviews that cover these aspects in detail [15].

In general, improved drop deposition on the olfactory epithelium will depend on body and head position of the subject (be it laboratory animal or human) and different nasal delivery devices (such as sprays, nose droppers, need-less syringes or cannulae) will target the drug to different regions of the nasal cavity. Drop volume will also affect targeting precision, and the number of drops delivered per injection session will, therefore, depend on liquid adsorption time and the total dose/injection required.

The required precision of drug deposition has prompted the development of *ad hoc* devices, two of which are presently under clinical evaluation [55,56].

4. How to improve intranasal delivery of proteins: pharmacological and formulation considerations

Several factors influence an optimal nasal absorption of protein therapeutics [15]. By looking into these factors, effects related to nasal physiology can be dissected from effects linked to the physicochemical characteristics of each individual protein drug and the relevant variables can be dealt with to overcome and solve the limitations [57].

Therefore, the access to CNS after nasal delivery can be improved by different strategies, from formulations for increasing solubility at the delivery site, or increasing the permeation through and within the nasal epithelium, to strategies for increasing the targeting and residence time in the olfactory epithelium and reducing the clearance of the delivered molecules. In the evaluation of the ways of optimization of the protein drug delivery, a vast knowledge comes from the experiments performed on the nasal delivery of small drugs [58].

While many of the approaches are bound to be drug-dependent, a few rather general conclusions can be derived (Figure 2).

4.1 Intrinsic characteristics of protein drugs and formulations

The degree of ionization, lipophilicity and molecular mass of an intranasally administered protein affect both its absorption across the nasal epithelium and its transport into the CSF [27,34,57]. The pK_a of the molecule, the pH of the absorption site, the solubility and the chemical and physical stability of a molecule are important factors to be considered [27,57]. In general, peptides and proteins are more prone to degradation, in comparison to low molecular-mass pharmaceuticals [27]. To improve the CNS targeting, formulations have included the addition of co-solvents or the use of alternate salt forms.

Co-administered peptidase/protease inhibitors or absorption enhancers (see below) may be useful [27,57]. For each of these formulation parameters, a good equilibrium between the optimal formulation and the interference with the normal nose physiology should be considered.

An optimized intranasal delivery of a protein therapeutic to the CNS through the olfactory epithelium will require overcoming the natural protective barriers in the nasal mucosa, such as the nasal mucociliary clearance mechanisms physiologically devoted to removing foreign substances. This is particularly true for these protein drugs, which are soluble into the mucus: mucociliary clearance rapidly clears inhaled protein drugs, reducing their contact with nasal epithelium and thus the delivery to the CNS after intranasal administration.

Various formulations have been described to improve the bioavailability of intranasally delivered molecules and increase nasal absorption, by affecting membrane and tight junction permeability, including bile salts, alkyl glycosides, polymers, gelatin and chitosan [27,57], tight junction modulating peptides, lipids and surfactants, cyclodextrins and chelators [15,27,57]. The transport along direct pathways to the CNS can be increased by formulations based on mucoadhesives (sodium hyaluronate, chitosan, lectins) to reduce clearance and increase residence time of the delivered formulation [15,57]. Some of these compounds can increase vascular permeability, which could be problematic for therapeutics with systemic side effects. Attempts have been made to reduce clearance into the blood from the site of delivery and increase the local availability of the therapeutic molecule on the nasal mucosa by using vasoconstrictor drugs, but contradictory findings were obtained [15]. Changes in other formulation parameters, such as osmolarity or pH, causing cells to expand or shrink, can enhance transcellular or paracellular transport mechanisms along olfactory and trigeminal nerves.

Nasal physiology can guide towards the optimization of experimental strategies and protocols for an increased delivery of protein therapeutics to the CNS, but all of these formulation substances should, however, be proven to be safe and non-toxic.

4.2 Encapsulation and coupling technologies

One emerging strategy to improve CNS uptake of peptides and proteins from the nasal route is to encapsulate or couple them in liposome carriers or particulate vectors such as microemulsions or nanoemulsions and nanoparticles [15].

Liposomes are phospholipid vesicles composed by lipid bilayers enclosing aqueous compartments wherein drugs and other substances can be included. Liposomal drug delivery systems have been found to enhance nasal absorption of peptides such as insulin and calcitonin by increasing their mucosal membrane penetration [57,59-60]. Recent studies have demonstrated the validity of the intranasal delivery of cationic liposomes for brain delivery of proteins [61].

Formulations based on microsphere technology with mucoadhesive polymers (chitosan, alginate) have been widely

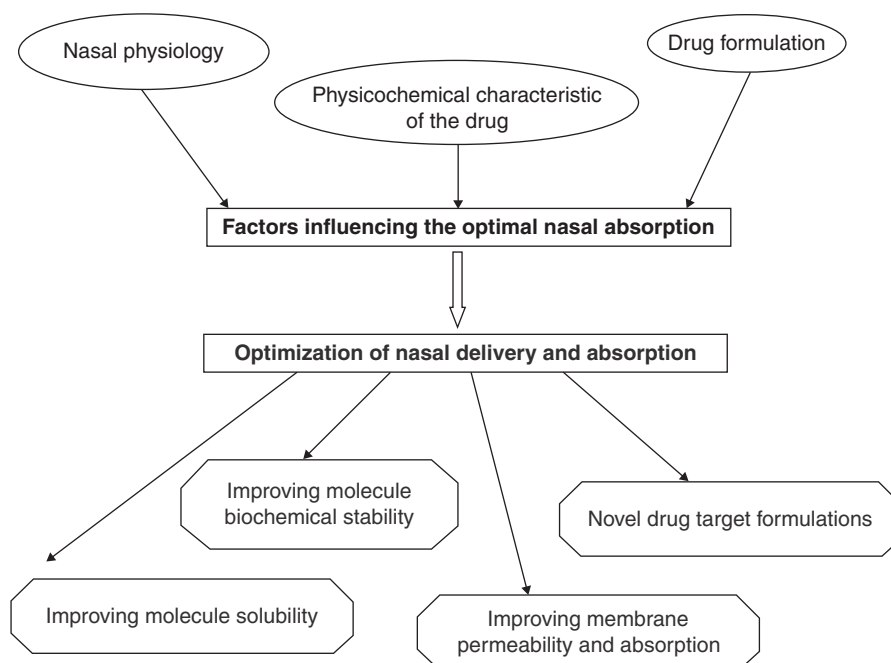


Figure 2. Schematic representation of the factors influencing the optimal nasal absorption and the possible strategies to improve it.

applied for nasal delivery of proteins, such as insulin [57,62]. Microspheres used for nasal delivery may protect the protein drug from degradation and sustain prolonged drug release, as demonstrated by several studies. However, most of the studies reported the systemic rather than CNS delivery of protein drugs [57,63]. This approach appears to be promising also for an optimization of intranasal application of CNS-targeted protein drugs.

Nanoparticle systems are being investigated for nose-to-brain drug administration, even if the precise mechanisms of the enhanced drug transport from the nasal cavity to the CNS are still unclear [15,57-58]. Nanoparticles (20 – 200 nm) are thought to be transported through the transcellular pathway in the direct nose-to-brain delivery [64]. Despite the increasing literature in the field demonstrating the efficient entering of nanoparticles through the nasal epithelium, via the transcellular mechanism [58,65], little is known about the precise endocytic pathways involved in nanoparticle absorption, via this transcellular route. Nanoparticle-mediated nose-to-brain delivery can be enhanced by several means of surface modification/engineering, including chitosan, PEG, lectin [15,58] and a new generation of poly/oligosaccharide nanoparticles composed of chitosan and cyclodextrins [65]. Nanoparticles coated with ligands preferentially binding olfactory epithelium (the lectin UEA I), with the respect to the respiratory epithelium, show an enhanced delivery along the olfactory pathway [66-68], whereas nanoparticles coated with the regionally nonspecific lectin WGA enhanced the delivery to the CNS along multiple pathways, including

vascular [66-68]. An alternative to lectins, which are toxic to mammalian cells [69] or immunogenic, would be to coat nanoparticles with ORN ‘homing peptides’, such as the phage-selected ACTTPHAWLCG peptide [70].

Filamentous bacteriophages, engineered to display peptides or proteins, including antibody fragments, can be considered living nanoparticles and have been proved to be an efficient non-toxic delivery vector for the displayed protein to the brain, when intranasally delivered [71].

5. Different classes of intranasally delivered therapeutic proteins

We briefly review some of the proteins under investigation for a therapeutic application in CNS diseases, through the intranasal pathway (Table 1), focusing on neurotrophic factors, neuropeptides and antibody domains.

5.1 Neurotrophic factors

5.1.1 Nerve growth factor for the treatment of Alzheimer's disease

Neurotrophic factors have a huge potential as protein therapeutics in the CNS that have been severely limited by problems with their delivery and systemic side effects. NGF [72] provides a paradigmatic case. In the CNS, mature basal forebrain cholinergic neurons (BFCNs) are dependent on the availability of NGF for their phenotypic maintenance and for survival after lesions or CNS insults. Beyond its neurotrophic actions on BFCNs, NGF displays a direct

Table 1. Intranasal delivery of potential protein therapeutics: a representative list.

Therapeutic protein intranasally administered	Category	Applications in CNS diseases	Tested on	
			Animal models	Clinical Trial
NGF	Neurotrophic factor	Alzheimer's disease	X	
BDNF		Alzheimer's disease	X	
		Huntington's disease		
CNTF		Alzheimer's disease	X	
	Growth factor	Huntington's disease		
NT4		Traumatic brain injury	X	
IGF-1		Alzheimer's disease	X	
		Stroke		
TGF- α	Hormone	Parkinson's disease	X	
		Stroke		
FGF		Parkinson's disease	X	
VIP		Alzheimer's disease	X	
NAP (peptide of ADNP)	Cytokine	Alzheimer's disease	X	X
		Mild cognitive impairment		
		Schizophrenia		
		Fronto-temporal dementia		
Insulin	Hormone	Alzheimer's disease	X	X
IFN- β_{1b}		MS	X	
Oxytocin		Cognitive and behavior disorders	X	X
		Autism		
	Antibody fragment	Post-traumatic stress		
		Sexual dysfunction		
		Schizophrenia		
Erythropoietin		Epilepsy	X	
	Antibody fragment	Traumatic brain injury		
TNF- α inhibitory single-chain		Parkinson's disease	X	
Fv antibody fragment		Alzheimer's disease		
		MS		

A representative summary of proteins and peptides tested as potential therapeutics via the intranasal route. Details on the pathologies where these proteins are involved are indicated.

anti-amyloidogenic and anti-neurodegenerative property [16]. Because BFCNs are the major neuronal population degenerating in the brain of AD patients, delivering NGF would provide an attractive therapeutic option, not only for preventing BFCN death and preserving cholinergic synapses, but more generally, to stop and revert AD neurodegeneration, a potentially disease-modifying therapy for AD [16]. However, NGF-based therapy is hindered by: i) the difficulty in achieving an adequate concentration in the brain areas containing target neurons, as NGF does not cross the BBB and ii) the onset of unwanted systemic side effects such as pain, due to NGF pro-nociceptive action on sensory neurons [16]. For these reasons, the strong and well-established therapeutic potential of NGF for human AD is currently being clinically explored by very invasive approaches involving neurosurgery for local intraparenchymal NGF delivery [7,73-74]. For an NGF-based therapy, the goal would be to develop a non-invasive delivery system capable of targeting NGF to the target brain areas, limiting systemic leakage and access to peripheral sensory nerve endings [75]. After the demonstration that NGF

efficiently reaches the CNS through the intranasal route [40], we demonstrated that the intranasal delivery of NGF to AD11 anti-NGF mice, a disease relevant AD mouse model, effectively targets neurodegeneration affected areas in the brain, at pharmacologically relevant concentrations, improving and reverting the memory and cognitive deficits and the neurodegeneration (Figure 3) [25,76]. After intranasal delivery, NGF does not significantly accumulate in the CSF and in the blood [77], where it is known to induce pain responses in humans. Thus, the intranasal delivery route appears to be an effective compromise to meet the required therapeutic window for NGF, leading to an effective transport into the brain, while minimizing its biodistribution to non-targeted districts where it determines unwanted side effects. The success of a non-invasive NGF-based therapy for AD will crucially depend on meeting the mentioned therapeutic window [78] and, therefore, careful dosing of the therapeutic protein in body fluids and tissues, against the background of endogenous NGF, will be crucial [78]. To this aim, a single residue tagged variant of NGF was developed, together with a specific

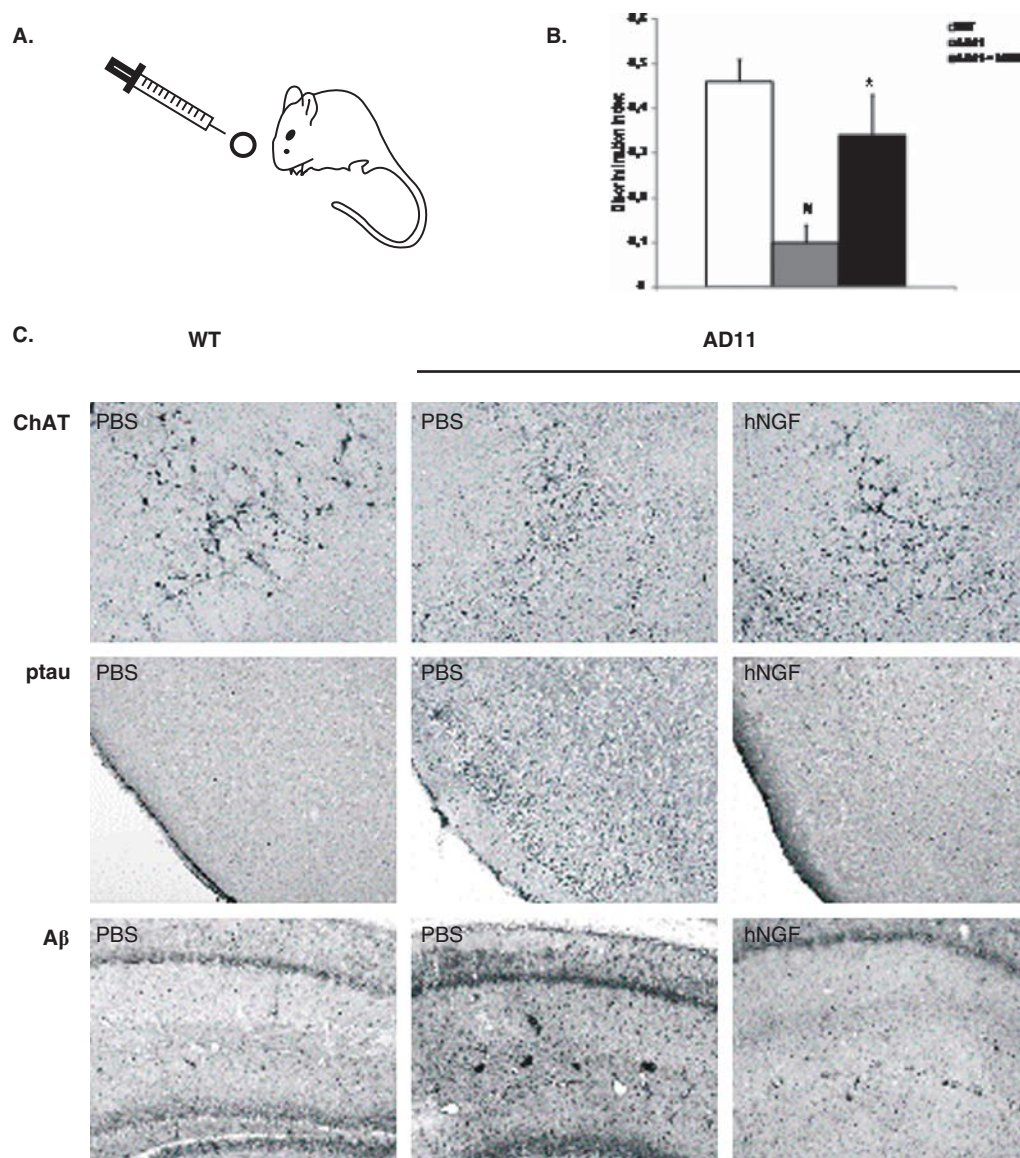


Figure 3. Effects of intranasal delivery of NGF on the neurodegenerative phenotype in AD11 anti-NGF mice. (A) hNGF (0.48 pmol) was administered intranasally, 3 μ l at a time, alternating the nostrils, with a lapse of 2 min between each administration, for a total of 14 times. The administration was repeated seven times at 2-day intervals. During these procedures, the nostrils were always kept open. As control, WT and AD11 mice were treated with PBS. (B) hNGF rescued the object recognition deficit. This rescue of memory deficit correlates well with the amelioration of the neurodegeneration, as shown in (C). hNGF administration rescues the number of ChAT-positive neurons in the nucleus basalis of Meynert (C, upper row), decreases the accumulation of phosphorylated τ (C, middle row) and the number of β -amyloid deposits (C, bottom row). hNGF: Human NGF; NGF: Nerve growth factor; PBS: Phosphate-buffered saline; WT: Wild-type.

immunoassay, in order to carry out precise pharmacokinetic and pharmacodynamic studies of the intranasally delivered therapeutic NGF [78]. Increasing the dose that can be safely delivered intranasally to increase its biodistribution in the CNS, without determining detrimental side effects, is an objective that is worthwhile pursuing. Different approaches can be taken. Zhang *et al.* [79] chemically conjugated cholera toxin B subunit to NGF (CB-NGF) to improve its

intraneuronal transport properties after intranasal delivery, with respect to NGF. The bioactivity of this CB-NGF molecule needs, however, to be better characterized. In another approach, the CNS bioavailability of NGF was increased [80] by counteracting the barrier properties of the olfactory epithelium with the permeation enhancer chitosan, a biodegradable polycationic linear polysaccharide that is bioadhesive and able to interact strongly with the nasal mucosa [81,82].

NGF-chitosan nanoparticles were shown to increase by ~ 14-fold the CNS bioavailability of NGF [80], with respect to that of naked NGF.

As a complementary approach to meet the therapeutic window for NGF, in addition to increasing its biodistribution in the CNS, while limiting detrimental systemic side effects, one could engineer NGF variants with reduced pain inducing activity [83]. Thus, 'painless' NGF single residue variants have been engineered and characterized [83,84] that would allow higher doses to be safely delivered, without inducing adverse painful responses. This 'painless' NGF has identical neurotrophic properties than the wild-type form [84]. The combination of these improved molecules with formulations that allow increased CNS biodistribution will probably provide the best candidate for the development of a non-invasive disease-modifying therapy for AD [16].

In a recent report, intranasal NGF was found to enhance striatal neurogenesis in adult rats with focal cerebral ischemia [85].

5.1.2 The intranasal route to deliver NGF to the brain is more effective than the ocular route

Recently, the topical eye application of NGF was proposed as an alternative option for non-invasive delivery of NGF to the brain on the basis of results obtained in a rat model in which cholinergic deficit was caused by natural aging [86] or by ibotenic acid [87]. The authors show an enhanced expression of NGF receptors and of ChAT immunoreactivity in rat BFCNs after ocular delivery of NGF. A side by side comparison of the ocular and intranasal routes, in the AD11 neurodegeneration model, showed the latter to be far more effective and specific than the former in reverting neurodegeneration, and safer in terms of NGF-related adverse effects [88]. Indeed, after ocular NGF delivery, no rescue of BFCNs or -related neurodegeneration was found, even with a 10-fold higher dose than the fully effective intranasal NGF dose [88]. Ocular NGF distributes widely in the systemic compartment and in peripheral tissues [86,88-89], most likely via the naso-lacrimal ducts, at concentrations well above the threshold for the adverse nociceptive response to occur [17,90].

We conclude that the intranasal administration provides a novel, non-invasive, safe and effective route for the delivery of NGF, which is superior to the ocular route, in terms of pharmacological effectiveness and brain targeting versus systemic exposure (Table 2).

5.1.3 Other neurotrophins and growth factors

Other members of the neurotrophins family, besides NGF, show an increasing therapeutic interest [1] due to the broad spectrum of their biological actions, including neuroprotective effects in animal models of neurodegenerative diseases or after traumatic and ischemic lesions of the nervous system.

The neurotrophin BDNF has been considered a candidate therapeutic protein for different neurological

diseases, including HD, ALS, AD and stroke. A major loss of BDNF protein in the striatum of HD patients and the well-established functional interaction between mutated huntingtin protein and BDNF expression show that BDNF is likely to play a role in the etiology of the disease [91]. BDNF is, therefore, considered a promising therapeutic candidate for HD, notwithstanding the potential liabilities deriving from its potentially pro-epileptogenic activities [92]. BDNF has also potent neurotrophic survival actions on motoneurons, both in physiological situations and in pathology models, which raised hopes that BDNF could be used for the treatment of neurodegenerative disorders specifically affecting motoneurons, such as ALS [1]. These cogent observations prompted the first BDNF clinical trials with a large number of ALS patients in the early 1990s, and because of the difficulty of delivering BDNF to the brain, the neurotrophin was delivered systemically. The failure of systemically administered BDNF (and of CNTF, see below) to reach the motoneurons in sufficient quantities is considered the reason why those large multi-center trials with these factors failed [1]. BDNF is produced in the entorhinal cortex, a brain region profoundly and early affected in AD [93] and undergoes anterograde transport in the hippocampus, where it regulates synaptic plasticity. Moreover, levels of BDNF and its mRNA are consistently reduced in AD patients and correlate to pathological changes in AD brains [94]. For these reasons, BDNF is considered a therapeutic candidate for AD, although its poor pharmacokinetic profile and poor delivery limit its therapeutic application. The neuroprotective effects of BDNF in different rodent and primate AD models were achieved using the same invasive approaches of gene delivery as for NGF [95]. A recent proof of concept study demonstrated that BDNF can be intranasally delivered to the brain, where it exerts dose-dependent neuroprotective effects, to contrast neurodegeneration in AD11 anti-NGF mice [96].

Ciliary neurotrophic factor (CNTF) and neurotrophin NT-4 were also shown to have clinically promising properties. CNTF effectively rescues learning and memory impairments in AD and HD models [97,98], and was even tested in human clinical trials for ALS, with a systemic or an intraventricular delivery [1]. NT-4 reduces hippocampal neuronal loss after traumatic brain injury [99]. Both CNTF and NT-4 can be efficiently delivered through the intranasal route in adult rats, resulting in a substantial concentration of the neurotrophic factor throughout the brain and cervical spinal cord [98]. The delivery efficiency was determined by analysis of the induced downstream signaling in CNS target neurons and was shown not to depend on the molecular mass of the considered neurotrophins [98]. On the basis of these results, a clinical testing of these neurotrophic factors was suggested for the non-invasive treatment of patients with traumatic or ischemic CNS injuries or degenerative situations of a different sort [98].

Delivery of IGF-I to the CNS may be beneficial for the treatment of AD or stroke [33], potently promoting neuronal

Table 2. Comparison between two non-invasive NGF delivery routes, intranasal and ocular.

Phenotype	Route and doses of hNGFP61S		
	Intranasal (0.48 pmol)	Ocular (0.48 pmol)	Ocular (4.8 pmol)
Rescue of vORT deficit	Yes	Yes	Yes
Rescue of cholinergic deficit	Yes	No	No
Decreased τ phosphorylation	Yes	No	Yes
Decreased A β deposition	Yes	Yes	Yes
Leakage in peripheral blood circulation*	No	Yes	Yes

NGF was administered to AD11 mice via the intranasal or ocular routes. In the case of ocular administration, two concentrations of the neurotrophin were tested. In the case of the ocular administration, even at the 10-fold higher concentration, the rescue of AD11 phenotype was partial when compared to the complete rescue obtained by intranasal administration. Moreover, when the same rescue level was observed, as for the Object Recognition Test or the A β end point, the doses used for the ocular administration were 10 times higher than those for the intranasal administration (from [88]).

*Measured by evaluating the activity on sympathetic ganglia as surrogate markers.

A β : β -Amyloid; NGF: Nerve growth factor.

survival, rescuing hippocampal neurons from A β -induced neurotoxicity, reducing τ phosphorylation, protecting cortical neurons against NO-mediated neurotoxicity, rescuing neurons from glucose deprivation and stimulating neurogenesis and synaptogenesis [33]. Despite the fact that IGF-1, unlike most other growth factors, can cross the BBB on systemic administration [100], a brain targeted intranasal delivery of IGF-1 would be useful to increase specifically targeted IGF-1 dosage. IGF-1 was shown to be successfully delivered to the brain and spinal cord by intranasal administration [33] and activate signaling pathways in brain regions expressing IGF-1 receptor [33].

TGF- α is a mitogenic polypeptide with pleiotropic functions, expressed in the developing and adult CNS, which regulates the proliferation of progenitor cells in the subventricular zone of the adult brain and neuronal differentiation *in vitro*. TGF- α stimulates neurogenesis and behavioral deficits in neurodegeneration models. TGF- α was investigated as a therapeutic candidate via the intranasal delivery route [101]. To improve TGF- α stability and the CNS delivery efficacy, TGF- α was covalently coupled to PEG. Intranasal administration of PEG-TGF- α induced a significant cell proliferation and improvement of behavioral deficits in a chronic stroke model [101].

The intranasal delivery of fibroblast growth factor (FGF) family members (acidic and basic FGF; aFGF or FGF-1 and bFGF or FGF-2, respectively) was explored in an MPTP-induced mouse model of PD [34]. Although the striatal concentrations of dopamine and its metabolites did not return to pre-lesion levels, this study demonstrated that intranasal aFGF and bFGF can rescue behavioral and biochemical deficits in a PD model [34].

Altogether, these animal model studies demonstrate that the intranasal route can be exploited to target a wide spectrum of different neurotrophic factors to the brain, in pharmacologically relevant quantities, leading to measurable and therapeutically relevant end points.

5.1.4 Other protein therapeutics: insulin, cytokines, neuropeptides

Insulin, the protein hormone involved in glucose metabolism in muscle, influences several aspects of neuronal physiology, participating in neuronal maintenance, energy metabolism, neurogenesis and neurotransmitter regulation and also influencing neuronal firing and synaptic plasticity. AD patients show insulin-related dysmetabolism and reduced levels of insulin in CSF and brain; hence, insulin is currently under investigation for the treatment of AD in clinical trials [102]. Obviously, peripherally administered insulin is not a viable option due to risks associated with hypoglycemia and direct targeting of insulin to the human brain, via intranasal delivery, with little peripheral build-up, has been demonstrated [54].

Type 1 IFNs are cytokine factors with antiviral, antiproliferative and immunomodulatory actions that have been approved for clinical treatment of hepatitis (IFN- α), cancer (IFN- α) and MS (IFN- β). Application of IFN- β in MS may benefit from a method of administration that targets the CNS yet also allows a sufficient systemic exposure to elicit peripheral effects. This would increase patient's compliance to the treatment with respect to the current repeated injection scheme. To this aim, the intranasal delivery of IFN- β in monkeys was explored [44], demonstrating a significant IFN- β concentration build-up across many CNS regions and regional lymph nodes. This result prospects the use of intranasally-delivered IFN- β in MS as an alternative to systemic injections.

The vasoactive intestinal peptide exerts a potent neuroprotective action in AD animal models by inducing the secretion of astroglial-derived substances such as the potent activity-dependent neurotrophic factor, ADNP [103]. ADNP provides neuroprotection at very low concentrations, but its size is too large (124 kDa) to cross the BBB. Peptide activity scanning identified NAP (NAPVSIPQ, molecular mass = 824.9), an active fragment of ADNP, endowed with the same neuroprotective properties as the entire protein [104].

NAP demonstrated neuroprotective activity against A β peptide toxicity in cell cultures and neurodegeneration animal models. NAP was successfully delivered through the intranasal route, providing neuroprotection against loss of cholinergic functions and memory impairments [103].

Other neuropeptides effectively delivered to the brain by the nasal route include hypocretin I (orexin A) [45], for narcolepsy treatment, glucagon-like peptide-I antagonist exendin (9 – 39) [38] and leptin [15].

Erythropoietin (EPO) is a hormone regulating erythropoiesis, but recently its potent neuroprotective function has been demonstrated in neuron loss induced by experimental epilepsy or traumatic brain injury and cisplatin-induced neuronal toxicity [98]. A small amount of systemically administered EPO can cross the BBB, but a more effective CNS delivery, limiting systemic exposure, would be advantageous. The intranasal delivery of EPO has been explored in adult rats, showing the potential of intranasal EPO as a neuroprotective agent [98].

5.1.5 Oxytocin: a neuropeptide and a hormone for social binding

Oxytocin (OT), a nonapeptide synthesized by specialized cells of the hypothalamus, exerts a dual role in mammals [105], acting peripherally as a hormone, regulating lactation and parturition, but acting also centrally as neuropeptide, controlling a wide range of social cognitive and emotional functions [106]. The properties of OT have been extensively studied exploiting intranasal delivery in animals and humans. Various studies in healthy humans have provided evidence that OT is implicated in facilitating human bonding, trust and generosity toward others and decreasing fear and diffidence [105]. Moreover, OT has empathogenic properties, improving recognition of complex mental states and emotions, and memory for faces [107]. Other studies indicate that OT facilitates stress regulation, both inhibiting amygdala activity and buffering cortisol response [108,109].

Due to its chemical characteristics, OT does not cross the BBB and, administered orally or intravenously, is rapidly degraded by peptidases in the plasma and gut. Furthermore, its peripheral administration could interfere with OT role as a hormone, resulting in undesired side effects, especially in females at various reproductive phases. Intranasal delivery is the only reliable current means to obtain demonstrable effects only in the CNS and exploit the enormous therapeutic potential of the peptide [54]. For this reason, intranasally delivered OT has been extensively studied in humans.

OT has shown an important therapeutic potential in treating mental disorders or psychopathological states in which empathy and human social relationships are affected. The existing literature on intranasally delivered OT suggests several therapeutic areas in which OT could be beneficial: anxiety and stress disorders (e.g., social phobia, post-traumatic stress disorders, separation anxiety disorders), psychopathologies with a prominent social dysfunction (autism, Asperger's syndrome,

schizophrenia), mood disorders (various forms of depression), sexual disorders and borderline personality disorders [105,110].

Currently, intranasal administration of OT in clinical populations has been reported to improve emotion recognition in autism or Asperger's syndrome, and ameliorate symptoms in schizophrenia, post-traumatic stress disorders and sexual dysfunction [105,111-114].

5.2 Recombinant antibodies

Antibodies represent the largest class of biologicals (therapeutic recombinant proteins) [2,3,115]. Antibody-based therapies have shown high efficacy in a variety of diseases and, due to their high target specificity, are considered safer than small chemical molecules. Due to delivery difficulties, the overwhelming majority of current therapeutic antibodies relate to diseases not affecting the CNS, as autoimmune disorders, cancer and cardiovascular diseases, even if the number of targets for CNS antibody-based therapies is greatly increasing and antibody biologicals are expanding into neural disorders [116].

The high molecular mass of mAbs (~ 150 kDa) prevents them from efficient penetration across tissue barriers following systemic administration. To overcome the problems of delivery into the brain related to the antibodies size and also to avoid the inflammatory properties potentially elicited by the antibody Fc domains, smaller antibody fragments can be engineered, endowed with the same binding properties as their whole immunoglobulin counterpart (Figure 4).

Intranasal administration of antibodies or antibody fragments could be an alternative route for immunotherapies, opening the CNS therapeutic arena to large class of recombinant antibody biologicals.

A TNF- α inhibitory single-chain Fv antibody fragment (scFv) (ESBA105) was administered intranasally. CNS local bioavailability of ESBA105 was considerably higher following repeated intranasal administration, while systemic exposure was significantly lower, when compared to a bolus intravenous injection. Thus, intranasal administration of ESBA105, and of recombinant scFv fragments in general, might be an attractive approach for the treatment of neurological disorders [117].

Another future major application of intranasal delivery of antibodies, or antibody fragments, is likely to be represented by the passive A β (β -amyloid) immunization strategy, presently under clinical testing for the treatment of AD [5]. The A β peptide, generated following proteolytic cleavage of the amyloid precursor protein, has become a major therapeutic target in AD, with various anti-A β strategies being pursued. One major approach involves A β immunotherapy, following A β vaccination or passive immunization with anti-A β antibodies passively administered by systemic route [5].

Passive A β immunotherapies are evolving to include second-generation conformation-specific anti A β antibodies or antibody fragments that recognize more toxic, soluble A β oligomeric forms [118]. The absence of the Fc portion in small

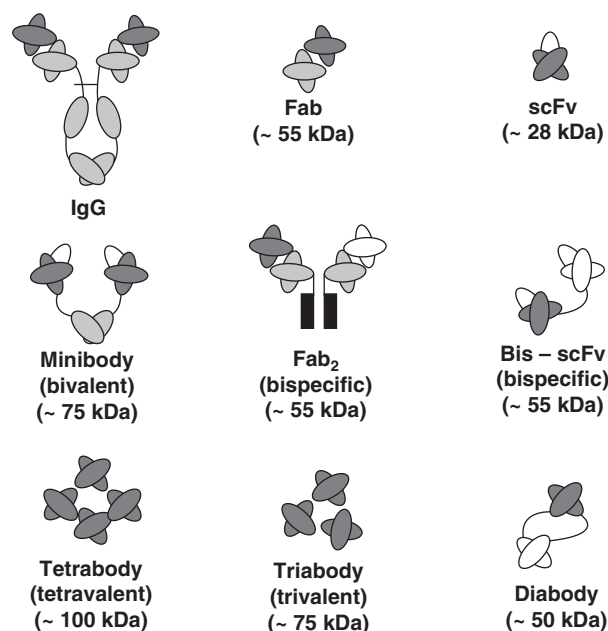


Figure 4. Schematic representation of different antibodies formats. The intact IgG molecule is shown together with a variety of antibody fragments, including Fab, scFv and multimeric formats such as minibodies and diabodies. The most widely used type of therapeutic antibodies presently utilized is represented by the IgG whole immunoglobulin format. To overcome the problems connected with the size of the whole molecule and avoid the inflammatory effect of Fc portion, small fragments are increasingly being used in numerous application. scFvs are a format in which the VH and VL domains are joined with a flexible polypeptide linker preventing dissociation. Antibody Fab and scFv fragments usually retain the specific, monovalent, antigen-binding affinity of the parent IgG, while showing improved pharmacokinetics for tissue penetration (reviewed in [6]). Even smaller fragments have been described (single domain antibodies), constituted by an isolated VH domain. Small antibody fragments or domains are likely to become the antibody format of choice for intranasal delivery to the CNS.

Adapted from [6].

scFv: Single-chain variable Fragment.

antibody fragment could improve the safety of the immunotherapies, without diminishing their efficacy for immunotherapy-induced A β clearance [119,120]. Intranasal delivery of anti-A β antibody fragments could represent an innovative approach for the treatment of AD, avoiding the need for systemic injections, with improved safety aspects. Indeed, intranasal delivery of anti-A β antibody fragments, by their direct targeting to the brain, could be advantageous to avoid the onset of an inflammatory response and the generation of auto antibodies. Intranasally delivered anti-A β scFv antibody fragments have been shown to reach the brain and dissolve A β plaques in animal models, when displayed on the surface of filamentous phage vectors [71,121].

6. Intranasal delivery in human clinical trials for neurodegenerative disorders

Evidence presented above demonstrated the efficacy of intranasal delivery of proteins in animal models. Can intranasal delivery be efficiently applied to clinical settings? First of all, the anatomy of rodent nose is quite different from the human one. The nasal cavity is much larger in humans than in rodents. The olfactory epithelium is located on the roof of the nasal cavity and covers a much smaller area. This might imply difficulties in the methods of delivery of drugs in human patients due to the position of the olfactory epithelium and the greater dilution of the drug in the nasal cavity. Second, the volume of CSF and its turnover is quite different in humans with respect to rats. CSF volume is 1000-fold higher in humans but its turnover is slower (every 5 h in humans; every hour in rodents). Because CSF is implicated in the diffusion of molecules from the nose-to-brain parenchyma, this implies the necessity of re-evaluating dosage and frequency of administration. Despite these issues, numerous reports have shown that therapeutic proteins delivered to human subjects by the intranasal route effectively reach the CNS, without major systemic leakage, and have the potential to treat neurological disorders. Most of the studies evaluating nose-to-brain delivery of neuropeptides in humans do not directly measure their transport into the CNS, but rather the indirect pharmacological effects on functional end points [122,123]. Although measuring pharmacodynamic effects rather than neuropeptide CNS concentration, these studies provide conclusive evidence that the intranasal administration of peptides results in fast onset effects on the CNS that are not observed after intravenous administration [122,123]. Direct evidence of nose-to-brain transport in humans was reported for the intranasal delivery of insulin, melanocortin and vasopressin, [54] in a placebo-controlled study (that did not include, however, a comparison to a systemic delivery route). It was demonstrated that CSF insulin levels significantly increased after intranasal treatment with no change in blood levels. The intranasal delivery of melanocortin and vasopressin to healthy humans further demonstrated a direct nose-to-brain intranasal route for an efficient delivery of neuropeptides to the brain, without systemic build-up [54].

Although a group of studies [15] concluded that there was no evidence for a direct nose to CSF in humans, for the drugs tested (including vitamin B₁₂, melatonin and estradiol), these conclusions were questioned based on methodological criticisms of the measurements performed [15,122].

A number of ongoing or completed clinical trials, based on intranasal delivery of therapeutic proteins to patients affected by specific neurological diseases, are briefly described.

6.1 Insulin

In humans, intravenous insulin administration was observed to determine amelioration in verbal memory; AD has been considered by some a 'brain diabetes' or

'type 3 diabetes' [15]. Obviously, peripherally administered insulin is not a viable treatment option for CNS disorders due to hypoglycemia risks. It was necessary to find alternative delivery routes that would not influence the systemic levels [15]. The first trial of intranasal insulin administration was developed as an alternative to subcutaneous insulin injections in diabetic patients. This trial was, however, not successful, with respect to the planned end points because insulin did not reach an adequate concentration in blood. Years later, the intranasal delivery method was proposed for a direct delivery of insulin to the brain along olfactory pathways for the treatment of AD and other brain disorders [15]. Indeed, energy metabolism in the CNS is dependent on glucose uptake and is regulated by insulin, while glucose uptake and utilization are deficient in AD patients, providing a rationale for delivering insulin to the CNS without altering blood glucose, as demonstrated in a proof of concept study in healthy human subjects [54]. Using intranasal delivery, it was also found that insulin significantly improved memory and mood in normal individuals [124] without altering blood insulin or glucose levels. Intranasal administration of Insulin Aspart, an insulin analog more rapidly absorbed was more effective than regular insulin to improve memory in normal subjects [125].

In memory-impaired old subjects, a single intranasal insulin treatment determined a significant verbal memory improvement, an effect seen primarily in patients without the AD susceptibility ApoE ϵ 4 allele [126]. A 3-week long placebo-controlled clinical trial with intranasal insulin in either early stage AD or mild-cognitive impairment showed enhanced memory and attention compared to placebo subjects, as well as a dose-dependent modulation of A β [102,127].

Beyond these promising clinical perspectives, for the improvement of memory disorders linked to AD, more generally, these studies provide a proof of concept in humans that the intranasal administration indeed allows the delivery to the CNS of a protein therapeutic while limiting its systemic accumulation and ensuing side effects.

6.2 NAP and oxytocin

In addition to insulin, other neuropeptides administered by the intranasal route are being clinically evaluated in humans.

The ADNP derived NAP peptide, following results obtained in animal models, is currently in Phase II clinical trials, being intranasally delivered to patients affected by mild cognitive impairment, schizophrenia-related cognitive impairment, AD and frontotemporal dementia [15,104,128].

Intranasally delivered OT has been extensively studied in healthy and sick human subjects. Central positive effects of OT in clinical populations have been reported in mental disorders related to psychopathological states in which empathy and human social relationships are affected. In all, 25 clinical trials based on intranasal administration of OT are presently registered on US National Institutes of Health. In these clinical trials, intranasal OT is administered to treat cognitive

and behavior disorders, including schizophrenia, headache, autism spectrum disorders, anxiety and post traumatic stress disorders, addiction, depression and fronto temporal dementia (<http://www.clinicaltrials.gov/>).

7. Future perspectives

The direct nose-to-brain route demonstrated to be a general strategy for the non-invasive delivery of numerous therapeutic protein candidates to the CNS of animal models and offers an exciting opportunity to deliver biologicals to the human brain for the treatment of neurological and neurodegenerative disorders. Based on what this strategy has proved so far, several aspects require more research and development to improve and exploit knowledge about the mechanisms of protein biodistribution from the nasal cavity and obtain a more sustained release of the proteins into the brain. Clearly, the possibility to quantitatively image and follow the biodistribution of the delivered protein in the living (animal or) human brain represents a primary objective. In any event, it can be anticipated that neurotrophic factors, neuropeptides and small antibody domains will be the classes of biologicals that will enjoy the most exciting developments with this delivery route. Moreover, as the research into the intranasal delivery of protein therapeutics to the CNS progresses, new experimental avenues can be conceived to exploit the intranasal route to the brain for therapeutic and regenerative purposes.

7.1 Towards intranasal gene- and cell-therapy for neurological diseases

The intranasal delivery of nucleic acids (DNA or RNA) to the brain would provide a new gene therapy approach. A study using 7 – 14 kb DNA expression plasmids encoding for the β -galactosidase gene showed that brain levels of DNA plasmids can reach a threefold higher concentration with intranasal than endovenous delivery. DNA plasmids integrated mainly in endothelial cells of different brain regions, including striatum, septal area and hippocampus [129]. However, safety issues were not addressed in this study. This first study opens a promising avenue, and it will be important to study how naked, or suitably modified, nucleic acids are transported to the brain via this route and evaluate their biodistribution in terms of transport as well as cell internalization and reporter gene expression.

One significant class of nucleic acid molecules of emerging interest that should be considered for delivery through this route are small-interfering RNA (siRNA) molecules to modulate gene expression for functional studies or therapeutic purposes. Current pilot approaches to target siRNA to the brain across the BBB, from the systemic compartment, involve complexing siRNA to a short peptide derived from the rabies virus glycoprotein that binds specifically to acetylcholine receptors on neuronal cells [130]. Efficient peptide-mediated delivery of siRNA to the brain

after intravenous injection relies on protecting the complex from nuclease and protease degradation en route, which is performed by complexing or encapsulating siRNA with liposomes [131]. While the direct intranasal administration of siRNA was initially targeted to cells in the lungs and the respiratory pathways [132], an effective target gene knock-down in different brain regions by nasally administered siRNAs was recently demonstrated in a first preliminary study [133]. The generality of these findings remains to be ascertained, but the rewards, if the approach will turn out successful, will be high.

Several studies have evaluated the intranasal delivery of proteins to the brain using replication-defective adenoviral vectors. Conflicting and controversial data have been obtained so far. In early studies, the use of the replication incompetent adenovirus to deliver β -galactosidase to the rodent brain showed a biodistribution confined to mouse olfactory nerve axons, as well as their terminal glomeruli, in the olfactory bulbs [134,135] with a persistence of the expression that varied from 8 to 21 days. Subsequent studies showed that the expression can be prolonged until 90 days after injection [136]. Only one study reported the expression in brain regions different than the olfactory bulbs [137].

An alternative to adenovirus-based vectors is represented by vectors based on viruses with a natural tropism for neurons [138]. Herpes simplex virus was the more studied, even if safety concerns remain [139,140]. A growth defective herpes simplex virus type 2 mutant RR vector was engineered which, when delivered intranasally to mice and rats, did not induce epileptic seizures nor neuronal loss, providing a first evidence of the feasibility of this approach [141].

A more recent, potentially promising, approach is represented by the intranasal delivery of stem cells to repair damaged tissues in the brain. Most of the few studies performed so far addressed the access and distribution of cells after intranasal delivery.

In one study [142], fluorescently labeled rat mesenchymal stem cells or human glioma cells were intranasally administered to naive mice and rats and the transnasal delivery of cells to the brain was followed. After cells crossed the cribriform plate, two migration routes were identified: i) migration into the olfactory bulb and to other parts of the brain and ii) entry into the CSF with movement along the surface of the cortex followed by entrance into the brain parenchyma.

While brain repair paradigms based on nasally delivered stem cells may still be in its infancy, a more feasible and sustainable approach in the near future would be represented by the use of stem cells to deliver therapeutic proteins, not necessarily requiring transport or integration of the donor cell in the nervous tissue. Towards this objective, our group explored the capacity of mesoangioblasts (MABs), engineered to secrete neurotrophins NGF and BDNF, to localize selectively in brain lesion areas, when intranasally or peripherally administered. MABs can be isolated from the

perivascular human adult tissue and, having a high adhesion-dependent migratory capacity, can reach perivascular targets, particularly in damaged areas [143]. After both peripheral and intranasal administration *in vivo*, GFP-positive MABs selectively reached the lesioned brain areas in a mouse model of AD [144].

8. Expert opinion

The effective treatment of neurological and neurodegenerative disorders is a severe unmet medical need. New disease modifying therapies may come from the promising class of the so-called biologicals (recombinant therapeutic proteins), provided the limitations of their effective delivery to the brain, across the BBB, are solved. Among the wide range of candidate biologicals for the treatment of CNS diseases, neurotrophic factors and recombinant antibodies hold, as protein classes, the greatest promises. The nasal cavity, through the olfactory epithelium and the cribriform plate, represents a natural port of entry to the brain. A large body of research has now provided evidence, in animal models and in humans, that the intranasal route constitutes a safe, non-invasive and effective strategy to deliver peptides and proteins to the brain, reducing systemic exposure and thus unwanted side effects. The intranasal delivery was proven to be more effective than other non-invasive methods, such as ocular delivery. Already at the present stage, the intranasal route greatly expands the range of biologicals that could be clinically tested as potential treatments for human neurological diseases (including the neurotrophins NGF and BDNF, as well as antibody domains for passive immunization approaches). However, despite the increasing knowledge that has been collected since the introduction of this method of delivery, further studies are required to achieve a better and more detailed understanding of pharmacokinetic and of the mechanisms of drug delivery, to optimize formulations and fully exploit the nose-to-brain interface to deliver proteins for the treatment of human neurological and psychiatric diseases. Research in this area will aim at improving the delivery effectiveness (increasing the percentage of the applied protein drug to reach the brain) and the delivery selectivity (brain vs systemic exposure), also exploiting coated nanoparticle formulations. A further refinement of the delivery selectivity, in terms of targeting to selected brain regions, will depend on our ability to exploit different neural pathways (trigeminal vs olfactory) or distinct transport modalities (intraneuronal vs paracellular pathways). These improvements will require carefully controlled, quantitative pharmacokinetic and pharmacodynamic studies to obtain conclusive and general results. The possibility of non-invasively imaging the therapeutic proteins, at first in animal models, as they are transported and biodistributed within the brain, would provide a breakthrough goal. Besides protein delivery, the nasal route is being explored

for the delivery of nucleic acids (DNA or small-interfering RNAs) to the brain. In any event, the development of viral- or cell-based intranasal delivery systems, exploiting the nose-to-brain interface, is likely to represent a research area that will produce exciting results in the near future towards new therapeutical approaches for the CNS.

Declaration of interest

The authors declare no conflict of interest. *Grant Sponsors:* Italian Institute of Technology (IIT); PNR MIUR Project RBP063ANC001; UE MEMORIES Project (No. 037831); Italian MIUR FIRB RBLA03FLJC.

Bibliography

1. Thoenen H, Sendtner M. Neurotrophins: from enthusiastic expectations through sobering experiences to rational therapeutic approaches. *Nat Neurosci* 2002;5:(Suppl):1046-50
2. Leader B, Baca QJ, Golan DE. Protein therapeutics: a summary and pharmacological classification. *Nat Rev Drug Discov* 2008;7(1):21-39
3. Reichert JM. Monoclonal antibodies as innovative therapeutics. *Curr Pharm Biotechnol* 2008;8(6):423-30
4. Reichert JM, Rosensweig CJ, Faden LB, et al. Monoclonal antibody successes in the clinic. *Nat Biotechnol* 2005;23(9):1073-8
5. Lemere CA, Masliah E. Can Alzheimer disease be prevented by amyloid-beta immunotherapy? *Nat Rev Neurol* 2010;6(2):108-19
6. Holliger P, Hudson PJ. Engineered antibody fragments and the rise of single domains. *Nat Biotechnol* 2005;23(9):1126-36
7. Tuszynski MH, Thal L, Pay M, et al. A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease. *Nat Med* 2005;11(5):551-5
8. Thorne RG, Emory CR, Ala TA, et al. Quantitative analysis of the olfactory pathway for drug delivery to the brain. *Brain Res* 1995;692(1-2):278-82
9. Zlokovic BV. The blood-brain barrier in health and chronic neurodegenerative disorders. *Neuron* 2008;57(2):178-201
10. de Boer AG, Gaillard PJ. Strategies to improve drug delivery across the blood-brain barrier. *Clin Pharmacokinet* 2007;46(7):553-76
11. Pardridge WM. Molecular biology of the blood-brain barrier. *Mol Biotechnol* 2005;30(1):57-70
12. Zlokovic BV. Cerebrovascular permeability to peptides: manipulations of transport systems at the blood-brain barrier. *Pharm Res* 1995;12(10):1395-406
13. Zlokovic BV, Segal MB, Davson H, et al. Circulating neuroactive peptides and the blood-brain and blood-cerebrospinal fluid barriers. *Endocrinol Exp* 1990;24(1-2):9-17
14. Banks WA. The CNS as a target for peptides and peptide-based drugs. *Expert Opin Drug Deliv* 2006;3(6):707-12
15. Dhuria SV, Hanson LR, Frey WH II. Intranasal delivery to the central nervous system: mechanisms and experimental considerations. *J Pharm Sci* 2010;99(4):1654-73
16. Cattaneo A, Capsoni S, Paoletti F. Towards non invasive nerve growth factor therapies for Alzheimer's disease. *J Alzheimers Dis* 2008;15(2):255-83
17. Petty BG, Cornblath DR, Adornato BT, et al. The effect of systemically administered recombinant human nerve growth factor in healthy human subjects. *Ann Neurol* 1994;36(2):244-6
18. Zlokovic BV, Martel CL, Matsubara E, et al. Glycoprotein 330/megalin: probable role in receptor-mediated transport of apolipoprotein J alone and in a complex with Alzheimer disease amyloid beta at the blood-brain and blood-cerebrospinal fluid barriers. *Proc Natl Acad Sci USA* 1996;93(9):4229-34
19. Zlokovic BV, Yamada S, Holtzman D, et al. Clearance of amyloid beta-peptide from brain: transport or metabolism? *Nat Med* 2000;6(7):718-19
20. Engelhardt JI, Appel SH. IgG reactivity in the spinal cord and motor cortex in amyotrophic lateral sclerosis. *Arch Neurol* 1990;47(11):1210-16
21. Brettschneider J, Petzold A, Sussmuth SD, et al. Axonal damage markers in cerebrospinal fluid are increased in ALS. *Neurology* 2006;66(6):852-6
22. Pardridge WM. Blood-brain barrier delivery. *Drug Discov Today* 2007;12(1-2):54-61
23. Shipley MT. Transport of molecules from nose to brain: transneuronal anterograde and retrograde labeling in the rat olfactory system by wheat germ agglutinin-horseradish peroxidase applied to the nasal epithelium. *Brain Res Bull* 1985;15(2):129-42
24. Balin BJ, Broadwell RD, Salzman M, et al. Avenues for entry of peripherally administered protein to the central nervous system in mouse, rat, and squirrel monkey. *J Comp Neurol* 1986;251(2):260-80
25. Capsoni S, Giannotta S, Cattaneo A. Nerve growth factor and galantamine ameliorate early signs of neurodegeneration in anti-nerve growth factor mice. *Proc Natl Acad Sci USA* 2002;99(19):12432-7
26. Capsoni S, Giannotta S, Cattaneo A. Beta-amyloid plaques in a model for sporadic Alzheimer's disease based on transgenic anti-nerve growth factor antibodies. *Mol Cell Neurosci* 2002;21(1):15-28
27. Costantino HR, Illum L, Brandt G, et al. Intranasal delivery: physicochemical and therapeutic aspects. *Int J Pharm* 2007;337(1-2):1-24
28. Morrison EE, Costanzo RM. Morphology of olfactory epithelium in humans and other vertebrates. *Microsc Res Tech* 1992;23(1):49-61
29. Engstrom B, Ekblom A, Hansson P. The olfactory and respiratory epithelium in Rhesus and Squirrel monkeys studied with freeze fracture technique. *Acta Otolaryngol* 1989;108:259-67
30. Gopinath P, Gopinath G, Kumar TCA. Target site of intranasally sprayed substances and their transport across the nasal mucosa: a new insight into the intranasal route of drug delivery. *Curr Ther Res* 1978;23:596-607

31. Perl DP, Good PF. Uptake of aluminium into central nervous system along nasal-olfactory pathways. *Lancet* 1987;1(8540):1028
32. Baker H, Spencer RF. Transneuronal transport of peroxidase-conjugated wheat germ agglutinin (WGA-HRP) from the olfactory epithelium to the brain of the adult rat. *Exp Brain Res* 1986;63(3):461-73
33. Thorne RG, Pronk GJ, Padmanabhan V, et al. Delivery of insulin-like growth factor-I to the rat brain and spinal cord along olfactory and trigeminal pathways following intranasal administration. *Neuroscience* 2004;127(2):481-96
34. Thorne RG, Frey WH II. Delivery of neurotrophic factors to the central nervous system: pharmacokinetic considerations. *Clin Pharmacokinet* 2001;40(12):907-46
35. Mackay-Sim A. Neurogenesis in the adult olfactory neuroepithelium. Marcel Dekker, New York; 2003
36. Pollock H, Hutchings M, Weller RO, et al. Perivascular spaces in the basal ganglia of the human brain: their relationship to lacunes. *J Anat* 1997;191(Pt 3):337-46
37. Bradbury MW, Cserr HF, Westrop RJ. Drainage of cerebral interstitial fluid into deep cervical lymph of the rabbit. *Am J Physiol* 1981;240(4):F329-36
38. Banks WA, During MJ, Niehoff ML. Brain uptake of the glucagon-like peptide-1 antagonist exendin(9-39) after intranasal administration. *J Pharmacol Exp Ther* 2004;309(2):469-75
39. Charlton ST, Whetstone J, Fayinka ST, et al. Evaluation of direct transport pathways of glycine receptor antagonists and an angiotensin antagonist from the nasal cavity to the central nervous system in the rat model. *Pharm Res* 2008;25(7):1531-43
40. Chen XQ, Fawcett JR, Rahman YE, et al. Delivery of nerve growth factor to the brain via the olfactory pathway. *J Alzheimers Dis* 1998;1(1):35-44
41. Clerico DM, To W, Lanza DC. Anatomy of the human nasal passages. Marcel Dekker, Inc., New York; 2003
42. Schaefer ML, Bottger B, Silver WL, et al. Trigeminal collaterals in the nasal epithelium and olfactory bulb: a potential route for direct modulation of olfactory information by trigeminal stimuli. *J Comp Neurol* 2002;444(3):221-6
43. Dufes C, Olivier JC, Gaillard F, et al. Brain delivery of vasoactive intestinal peptide (VIP) following nasal administration to rats. *Int J Pharm* 2003;255(1-2):87-97
44. Thorne RG, Hanson LR, Ross TM, et al. Delivery of interferon-beta to the monkey nervous system following intranasal administration. *Neuroscience* 2008;152(3):785-97
45. Hanson LR, Martinez PM, Taheri S, et al. Intranasal administration of hypocretin 1 (orexin A) bypasses the blood-brain barrier & targets the brain: A new strategy for the treatment of narcolepsy. *Drug Del Tech* 2004;4:66-70
46. Ross TM, Zuckermann RN, Reinhard C, et al. Intranasal administration delivers peptoids to the rat central nervous system. *Neurosci Lett* 2008;439(1):30-3
47. Grevers G, Herrmann U. Fenestrated endothelia in vessels of the nasal mucosa. An electron-microscopic study in the rabbit. *Arch Otorhinolaryngol* 1987;244(1):55-60
48. Einer-Jensen N, Larsen L. Transfer of tritiated water, tyrosine, and propanol from the nasal cavity to cranial arterial blood in rats. *Exp Brain Res* 2000;130(2):216-20
49. Skipor J, Grzegorzewski W, Einer-Jensen N, et al. Local vascular pathway for progesterone transfer to the brain after nasal administration in gilts. *Reprod Biol* 2003;3(2):143-59
50. Zhang ET, Richards HK, Kida S, et al. Directional and compartmentalised drainage of interstitial fluid and cerebrospinal fluid from the rat brain. *Acta Neuropathol* 1992;83(3):233-9
51. Rennels ML, Gregory TF, Blaumanis OR, et al. Evidence for a 'paravascular' fluid circulation in the mammalian central nervous system, provided by the rapid distribution of tracer protein throughout the brain from the subarachnoid space. *Brain Res* 1985;326(1):47-63
52. Hadaczek P, Yamashita Y, Mirek H, et al. The "perivascular pump" driven by arterial pulsation is a powerful mechanism for the distribution of therapeutic molecules within the brain. *Mol Ther* 2006;14(1):69-78
53. Boulton M, Flessner M, Armstrong D, et al. Contribution of extracranial lymphatics and arachnoid villi to the clearance of a CSF tracer in the rat. *Am J Physiol* 1999;276(3 Pt 2):R818-23
54. Born J, Lange T, Kern W, et al. Sniffing neuropeptides: a transnasal approach to the human brain. *Nat Neurosci* 2002;5(6):514-16
55. Djupesland PG, Docekal P. Intranasal sumatriptan powder delivered by a novel breath-actuated bi-directional device for the acute treatment of migraine: a randomised, placebo-controlled study. *Cephalalgia* 2010;30(8):933-42
56. Giroux M. Controlled particle dispersion: applying vortical flow to optimize nasal drug delivery. *Drug Del Tech* 2007;5(3):44
57. Pires A, Fortuna A, Alves G, et al. Intranasal drug delivery: how, why and what for? *J Pharm Pharm Sci* 2009;12(3):288-311
58. Mistry A, Stolnik S, Illum L. Nanoparticles for direct nose-to-brain delivery of drugs. *Int J Pharm* 2009;379(1):146-57
59. Jain AK, Chalasani KB, Khar RK, et al. Muco-adhesive multivesicular liposomes as an effective carrier for transmucosal insulin delivery. *J Drug Target* 2007;15(6):417-27
60. Law SL, Huang KJ, Chou VH, et al. Enhancement of nasal absorption of calcitonin loaded in liposomes. *J Liposome Res* 2001;11(2-3):165-74
61. Migliore MM, Vyas TK, Campbell RB, et al. Brain delivery of proteins by the intranasal route of administration: a comparison of cationic liposomes versus aqueous solution formulations. *J Pharm Sci* 2010;99(4):1745-61
62. Ozsoy Y, Gungor S, Cevher E. Nasal delivery of high molecular weight drugs. *Molecules* 2009;14(9):3754-79
63. Wang J, Tabata Y, Morimoto K. Aminated gelatin microspheres as a nasal delivery system for peptide drugs: evaluation of in vitro release and in vivo insulin absorption in rats. *J Control Release* 2006;113(1):31-7
64. Miyamoto M, Natsume H, Satoh I, et al. Effect of poly-L-arginine on the nasal absorption of FITC-dextran of different molecular weights and recombinant human granulocyte

- colony-stimulating factor (rhG-CSF) in rats. *Int J Pharm* 2001;226(1-2):127-38
65. Teijeiro-Osorio D, Remunan-Lopez C, Alonso MJ. New generation of hybrid poly/oligosaccharide nanoparticles as carriers for the nasal delivery of macromolecules. *Biomacromolecules* 2009;10(2):243-9
66. Gao X, Chen J, Tao W, et al. UEA I-bearing nanoparticles for brain delivery following intranasal administration. *Int J Pharm* 2007;340(1-2):207-15
67. Gao X, Tao W, Lu W, et al. Lectin-conjugated PEG-PLA nanoparticles: preparation and brain delivery after intranasal administration. *Biomaterials* 2006;27(18):3482-90
68. Gao X, Wu B, Zhang Q, et al. Brain delivery of vasoactive intestinal peptide enhanced with the nanoparticles conjugated with wheat germ agglutinin following intranasal administration. *J Control Release* 2007;121(3):156-67
69. Reynoso-Camacho R, Gonzalez de Mejia E, Loarca-Pina G. Purification and acute toxicity of a lectin extracted from tepary bean (*Phaseolus acutifolius*). *Food Chem Toxicol* 2003;41(1):21-7
70. Wan XM, Chen YP, Xu WR, et al. Identification of nose-to-brain homing peptide through phage display. *Peptides* 2009;30(2):343-50
71. Solomon B. Filamentous bacteriophage as a novel therapeutic tool for Alzheimer's disease treatment. *J Alzheimers Dis* 2008;15(2):193-8
72. Levi-Montalcini R. The nerve growth factor 35 years later. *Science* 1987;237(4819):1154-62
73. Tuszynski MH. Nerve growth factor gene therapy in Alzheimer disease. *Alzheimer Dis Assoc Disord* 2007;21(2):179-89
74. Mandel RJ. CERE-110, an adeno-associated virus-based gene delivery vector expressing human nerve growth factor for the treatment of Alzheimer's disease. *Curr Opin Mol Ther* 2010;12(2):240-7
75. Cattaneo A, Capsoni S, Paoletti F. A new generation of noninvasive NGF-based therapies for Alzheimer's Disease: RSC Publishing; 2010
76. De Rosa R, Garcia AA, Braschi C, et al. Intranasal administration of nerve growth factor (NGF) rescues recognition memory deficits in AD11 anti-NGF transgenic mice. *Proc Natl Acad Sci USA* 2005;102(10):3811-16
77. Frey IW, Liu J, Chen XQ, et al. Delivery of 125I-NGF to the brain via the olfactory route. *Drug Deliv* 1997;4:87-92
78. Covaceuszach S, Capsoni S, Ugolini G, et al. Development of a non invasive NGF-based therapy for Alzheimer's disease. *Curr Alzheimer Res* 2009;6(2):158-70
79. Zhang Q, Liu Y, Yang N, et al. Nasal administration of cholera toxin B subunit-nerve growth factor improves the space learning and memory abilities in beta-amyloid protein(25-35)-induced amnesic mice. *Neuroscience* 2008;155(1):234-40
80. Vaka SR, Sammeta SM, Day LB, et al. Delivery of nerve growth factor to brain via intranasal administration and enhancement of brain uptake. *J Pharm Sci* 2009;98(10):3640-6
81. Illum L, Farraj NF, Davis SS. Chitosan as a novel nasal delivery system for peptide drugs. *Pharm Res* 1994;11(8):1186-9
82. Illum L. Nasal drug delivery—possibilities, problems and solutions. *J Control Release* 2003;87(1-3):187-98
83. Covaceuszach S, Capsoni S, Marinelli S, et al. In vitro receptor binding properties of a "painless" NGF mutein, linked to hereditary sensory autonomic neuropathy type V. *Biochem Biophys Res Commun* 2010;391(1):824-9
84. Capsoni S, Covaceuszach S, Marinelli S, et al. Taking pain out of NGF: a "painless" NGF mutant, linked to hereditary sensory autonomic neuropathy type V, with full neurotrophic activity. *PLoS One* 2011;6(2):e17321
85. Zhu W, Cheng S, Xu G, et al. Intranasal nerve growth factor enhances striatal neurogenesis in adult rats with focal cerebral ischemia. *Drug Deliv* 2011 [Epub ahead of print] doi:10.3109/10717544.2011.557785
86. Lambiasi A, Tirassa P, Micera A, et al. Pharmacokinetics of conjunctivally applied nerve growth factor in the retina and optic nerve of adult rats. *Invest Ophthalmol Vis Sci* 2005;46(10):3800-6
87. Lambiasi A, Pagani L, Di Fausto V, et al. Nerve growth factor eye drop administrated on the ocular surface of rodents affects the nucleus basalis and septum: biochemical and structural evidence. *Brain Res* 2007;1127(1):45-51
88. Capsoni S, Covaceuszach S, Ugolini G, et al. Delivery of NGF to the brain: intranasal versus ocular administration in anti-NGF transgenic mice. *J Alzheimers Dis* 2009;16(2):371-88
89. Di Fausto V, Fiore M, Tirassa P, et al. Eye drop NGF administration promotes the recovery of chemically injured cholinergic neurons of adult mouse forebrain. *Eur J Neurosci* 2007;26(9):2473-80
90. Apfel SC. Neurotrophic factors in the therapy of diabetic neuropathy. *Am J Med* 1999;107(2B):34S-42S
91. Zuccato C, Cattaneo E. Role of brain-derived neurotrophic factor in Huntington's disease. *Prog Neurobiol* 2007;81(5-6):294-330
92. Simonato M, Tongiorgi E, Kokaia M. Angels and demons: neurotrophic factors and epilepsy. *Trends Pharmacol Sci* 2006;27(12):631-8
93. Price JL, Ko AI, Wade MJ, et al. Neuron number in the entorhinal cortex and CA1 in preclinical Alzheimer disease. *Arch Neurol* 2001;58(9):1395-402
94. Tapia-Arancibia L, Aliaga E, Silhol M, et al. New insights into brain BDNF function in normal aging and Alzheimer disease. *Brain Res Rev* 2008;59(1):201-20
95. Nagahara AH, Merrill DA, Coppola G, et al. Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease. *Nat Med* 2009;15(3):331-7
96. Berardi N, Braschi C, Capsoni S, et al. BDNF intranasal administration rescues visual memory deficits in a mouse model of Alzheimer disease (AD), the AD11 mouse. *FENS Forum* 2008. Geneva, CH: 2008
97. Garcia P, Youssef I, Utvik JK, et al. Ciliary neurotrophic factor cell-based delivery prevents synaptic impairment and improves memory in mouse models of Alzheimer's disease. *J Neurosci* 2010;30(22):7516-27

98. Alcala-Barraza SR, Lee MS, Hanson LR, et al. Intranasal delivery of neurotrophic factors BDNF, CNTF, EPO, and NT-4 to the CNS. *J Drug Target* 2010;18(3):179-90
99. Royo NC, Conte V, Saatman KE, et al. Hippocampal vulnerability following traumatic brain injury: a potential role for neurotrophin-4/5 in pyramidal cell neuroprotection. *Eur J Neurosci* 2006;23(5):1089-102
100. Nishijima T, Piriz J, Dufloot S, et al. Neuronal activity drives localized blood-brain-barrier transport of serum insulin-like growth factor-I into the CNS. *Neuron* 2010;67(5):834-46
101. Guerra-Crespo M, Sistos A, Gleason D, et al. Intranasal administration of PEGylated transforming growth factor-alpha improves behavioral deficits in a chronic stroke model. *J Stroke Cerebrovasc Dis* 2010;19(1):3-9
102. Reger MA, Watson GS, Green PS, et al. Intranasal insulin improves cognition and modulates beta-amyloid in early AD. *Neurology* 2008;70(6):440-8
103. Gozes I, Giladi E, Pinhasov A, et al. Activity-dependent neurotrophic factor: intranasal administration of femtomolar-acting peptides improve performance in a water maze. *J Pharmacol Exp Ther* 2000;293(3):1091-8
104. Gozes I, Morimoto BH, Tiong J, et al. NAP: research and development of a peptide derived from activity-dependent neuroprotective protein (ADNP). *CNS Drug Rev* 2005;11(4):353-68
105. Macdonald K, Macdonald TM. The peptide that binds: a systematic review of oxytocin and its prosocial effects in humans. *Harv Rev Psychiatry* 2010;18(1):1-21
106. Lee HJ, Macbeth AH, Pagani JH, et al. Oxytocin: the great facilitator of life. *Prog Neurobiol* 2009;88(2):127-51
107. Kirsch P, Esslinger C, Chen Q, et al. Oxytocin modulates neural circuitry for social cognition and fear in humans. *J Neurosci* 2005;25(49):11489-93
108. Hurlemann R, Patin A, Onur OA, et al. Oxytocin enhances amygdala-dependent, socially reinforced learning and emotional empathy in humans. *J Neurosci* 2010;30(14):4999-5007
109. Quirin M, Kuhl J, Dusing R. Oxytocin buffers cortisol responses to stress in individuals with impaired emotion regulation abilities. *Psychoneuroendocrinology* 2011 [Epub ahead of print]. doi:10.1016/j.psyneuen.2010.12.005
110. Heinrichs M, Domes G. Neuropeptides and social behaviour: effects of oxytocin and vasopressin in humans. *Prog Brain Res* 2008;170:337-50
111. Feifel D, Macdonald K, Nguyen A, et al. Adjunctive intranasal oxytocin reduces symptoms in schizophrenia patients. *Biol Psychiatry* 2010;68(7):678-80
112. Guastella AJ, Einfeld SL, Gray KM, et al. Intranasal oxytocin improves emotion recognition for youth with autism spectrum disorders. *Biol Psychiatry* 2010;67(7):692-4
113. Hollander E, Novotny S, Hanratty M, et al. Oxytocin infusion reduces repetitive behaviors in adults with autistic and Asperger's disorders. *Neuropsychopharmacology* 2003;28(1):193-8
114. Ishak WW, Berman DS, Peters A. Male anorgasmia treated with oxytocin. *J Sex Med* 2008;5(4):1022-4
115. Pavlou AK, Reichert JM. Recombinant protein therapeutics—success rates, market trends and values to 2010. *Nat Biotechnol* 2004;22(12):1513-19
116. Vastag B. Monoclonals expand into neural disorders. *Nat Biotechnol* 2006;24(6):595-6
117. Furrer E, Hulmann V, Urech DM. Intranasal delivery of ESBA105, a TNF-alpha-inhibitory scFv antibody fragment to the brain. *J Neuroimmunol* 2009;215(1-2):65-72
118. Meli G, Visintin M, Cannistraci I, et al. Direct in vivo intracellular selection of conformation-sensitive antibody domains targeting Alzheimer's amyloid-beta oligomers. *J Mol Biol* 2009;387(3):584-606
119. Bacskaï BJ, Kajdasz ST, McLellan ME, et al. Non-Fc-mediated mechanisms are involved in clearance of amyloid-beta in vivo by immunotherapy. *J Neurosci* 2002;22(18):7873-8
120. Das P, Howard V, Loosbrock N, et al. Amyloid-beta immunization effectively reduces amyloid deposition in FcRgamma-/- knock-out mice. *J Neurosci* 2003;23(24):8532-8
121. Frenkel D, Solomon B. Filamentous phage as vector-mediated antibody delivery to the brain. *Proc Natl Acad Sci USA* 2002;99(8):5675-9
122. Illum L. Is nose-to-brain transport of drugs in man a reality? *J Pharm Pharmacol* 2004;56(1):3-17
123. Fehm HL, Perras B, Smolnik R, et al. Manipulating neuropeptidergic pathways in humans: a novel approach to neuropharmacology? *Eur J Pharmacol* 2000;405(1-3):43-54
124. Benedict C, Hallschmid M, Hatke A, et al. Intranasal insulin improves memory in humans. *Psychoneuroendocrinology* 2004;29(10):1326-34
125. Benedict C, Hallschmid M, Schultes B, et al. Intranasal insulin to improve memory function in humans. *Neuroendocrinology* 2007;86(2):136-42
126. Reger MA, Watson GS, Frey WH II, et al. Effects of intranasal insulin on cognition in memory-impaired older adults: modulation by APOE genotype. *Neurobiol Aging* 2006;27(3):451-8
127. Reger MA, Watson GS, Green PS, et al. Intranasal insulin administration dose-dependently modulates verbal memory and plasma amyloid-beta in memory-impaired older adults. *J Alzheimers Dis* 2008;13(3):323-31
128. Merenlender-Wagner A, Pikman R, Giladi E, et al. NAP (davunetide) enhances cognitive behavior in the STOP heterozygous mouse—a microtubule-deficient model of schizophrenia. *Peptides* 2010;31(7):1368-73
129. Han IK, Kim MY, Byun HM, et al. Enhanced brain targeting efficiency of intranasally administered plasmid DNA: an alternative route for brain gene therapy. *J Mol Med* 2007;85(1):75-83
130. Kumar P, Wu H, McBride JL, et al. Transvascular delivery of small interfering RNA to the central nervous system. *Nature* 2007;448(7149):39-43
131. Pulford B, Reim N, Bell A, et al. Liposome-siRNA-peptide complexes cross the blood-brain barrier and significantly decrease PrP on neuronal cells and PrP in infected cell cultures. *PLoS One* 2010;5(6):e11085
132. Bitko V, Barik S. Nasal delivery of siRNA. *Methods Mol Biol* 2008;442:75-82
133. Kim I, Kim SW, Lee JK. Gene Knockdown in the Olfactory Bulb, Amygdala, and Hypothalamus by

- Intranasal siRNA Administration.
Appl Microbiol Biotechnol
2009;42(4):285-92
134. Zhao H, Otaki JM, Firestein S. Adenovirus-mediated gene transfer in olfactory neurons in vivo. *J Neurobiol* 1996;30(4):521-30
 135. Holtmaat AJ, Hermens WT, Oestreicher AB, et al. Efficient adenoviral vector-directed expression of a foreign gene to neurons and sustentacular cells in the mouse olfactory neuroepithelium. *Brain Res Mol Brain Res* 1996;41(1-2):148-56
 136. Doi K, Nibu K, Ishida H, et al. Adenovirus-mediated gene transfer in olfactory epithelium and olfactory bulb: a long-term study. *Ann Otol Rhinol Laryngol* 2005;114(8):629-33
 137. Draghia R, Caillaud C, Manicom R, et al. Gene delivery into the central nervous system by nasal instillation in rats. *Gene Ther* 1995;2(6):418-23
 138. Graff CL, Pollack GM. Nasal drug administration: potential for targeted central nervous system delivery. *J Pharm Sci* 2005;94(6):1187-95
 139. Wu HM, Huang CC, Chen SH, et al. Herpes simplex virus type 1 inoculation enhances hippocampal excitability and seizure susceptibility in mice. *Eur J Neurosci* 2003;18(12):3294-304
 140. Najioullah F, Bosshard S, Thouvenot D, et al. Diagnosis and surveillance of herpes simplex virus infection of the central nervous system. *J Med Virol* 2000;61(4):468-73
 141. Laing JM, Gober MD, Golembewski EK, et al. Intranasal administration of the growth-compromised HSV-2 vector DeltaRR prevents kainate-induced seizures and neuronal loss in rats and mice. *Mol Ther* 2006;13(5):870-81
 142. Danielyan L, Schafer R, von Ameln-Mayerhofer A, et al. Intranasal delivery of cells to the brain. *Eur J Cell Biol* 2009;88(6):315-24
 143. Cossu G, Bianco P. Mesoangioblasts—vascular progenitors for extravascular mesodermal tissues. *Curr Opin Genet Dev* 2003;13(5):537-42
 144. Binaschi A, Su T, Paradiso B, et al. Mesoangioblast-based supplementation of neurotrophic factors for the treatment of neuronal damage. Society for Neuroscience. 40th Annual Meeting 2010. San Diego, California; 2010

Affiliation

Francesca Malerba^{1,2}, Francesca Paoletti²,
Simona Capsoni^{1,2} & Antonino Cattaneo^{†1,2}
[†]Author for correspondence
¹Scuola Normale Superiore,
Piazza dei Cavalieri 7,
56126 Pisa, Italy
²European Brain Research Institute,
Via del Fosso di Fiorano 64,
00143 Rome, Italy
E-mail: a.cattaneo@ebri.it