Intranasal neprilysin rapidly eliminates amyloid-beta plaques, but causes plaque compensations: the explanation why the amyloid-beta cascade may fail?

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Abstract

Neurodegenerative brain disorders are a major burden in our society, such as Alzheimer´s disease. In order to repair or prevent such diseases, drugs are designed which enter the brain, but the blood-brain barrier limits their entry and the search for alternative pathways is important. Recently, we reported that intranasal delivery of the amyloid-beta degrading enzyme neprilysin eliminated amyloid-beta plaques in transgenic Alzheimer´s disease mice. This review describes the anatomical structure of the intranasal pathway, explains the intranasal delivery of pure neprilysin, cell-loaded neprilysin (platelets) and collagen-embedded neprilysin to destruct amyloid-beta plaques in Alzheimer´s disease in transgenic APP_SweDI mice and hypothesizes why this may cause compensation and why the amyloid-beta cascade hypothesis may fail.

Key Words: amyloid-beta; amyloid-beta degrading enzyme; clear plaque; collagen biomaterial; intranasal; neprilysin; nose-to-brain

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Introduction

Alzheimer's disease (AD) is a severe brain disorder. The major two pathologies are extracellular depositions of toxic amyloid β (Aβ) seen as plaques and the hyperphosphorylation of tau, which results in intraneuronal neurofibrillary tangles. These pathologies are accompanied by cell death of cholinergic neurons, causing loss of the neurotransmitter acetylcholine and cognitive decline. Further astrocytes are activated around the plaques and activated microglia migrate to the plaques to phagocyte and eliminate the plaques. In addition, the blood-brain barrier (BBB) is broken, vessels damaged and pro-inflammatory processes induced. In therapeutic approaches, one aims to prevent or eliminate and clear the Aβ plaques with beta-sheet breakers, secretase inhibitors, or Aβ-degrading enzymes. Definitely the infusion of plaque-degrading enzymes could be an attractive strategy to clear the plaques. Indeed, several Aβ-degrading proteases have been identified, such as e.g., neprilysin, insulin-degrading enzyme, endothelin-converting enzyme, angiotensin-converting enzyme, plasminogen activators or different matrix metalloproteinases. Neprilysin is a 90–110 kDa plasma membrane glycoprotein of the neutral zinc metalloendo-peptidase family and is a very potent Aβ-degrading enzyme.

In order to develop a target-specific brain therapy, vehicles must be designed to allow selective passage of the BBB and to target the damaged neurons or the plaque depositions without any side effects. Severe surgical interventions must be avoided, and we must find alternative ways, e.g., by circumvention of the BBB via the intranasal pathway. We have recently shown that intranasal delivery of neprilysin destructs Aβ plaques in a transgenic AD mouse model (Humpel, 2015, 2021). We have used infusion of (a) pure neprilysin, (b) neprilysin-loaded in blood cells (platelets) and (c) collagen-embedded neprilysin. Using the 3 applications via the intransal pathway we found different effects of Aβ destruction and interestingly, also compensation of the plaque load. This mini-reviews summarizes the effects found with neprilysin and hypothesizes why

the Aβ cascade-hypothesis may fail.

Retrieval Strategy

I searched the PubMed data during April–July 2021 using the search terms: amyloid-beta, amyloid-beta degrading enzyme, clear plaque, collagen biomaterial, intranasal, neprilysin, nose-to-brain.

The Intranasal Pathway

The nasal cavity is a very interesting target to infuse drugs and to allow penetration to the brain. It is easy to infuse substances into the nasal cavity and the nasal mucosa shows efficient absorption and permeability functions. A detailed and very nice review on the anatomical structure and on the transport of substances from nose-to-brain is given by Gänger and Schjindowski (2018), and a simplified scheme in **Figure 1**. Here I will only shortly summarize the most important issues. Nose-to-brain delivery is minimally invasive and can bypass the BBB. It has been shown that intranasal infusion is highly suitable for small molecule drugs, but also peptides or larger proteins, or viruses but also cells can pass from the nose to the brain. The nasal cavity is covered with olfactory epithelium and is innervated by nerves which project to the brain. Two important nerves innervate the olfactory epithelium, the olfactory nerves and the trigeminal nerves (**Figure 1**; Lee et al., 1999). The olfactory pathway targets the olfactory bulb, whereas the trigeminal pathway projects predominantly to the brain stem (**Figure 1**). In our recent study (Humpel, 2021), we investigated the frontal-middle-caudal cortex and thus delivery of neprilysin is considered to occur via the olfactory pathway, and indeed control substances were found in the olfactory bulb.

The mechanism how molecules are taken up and transported by the olfactory epithelium have been well investigated and reviewed and two possible mechanisms may play a role (Gänger and Schjindowski, 2018). First, it is suggested that molecules are endocytosed in the

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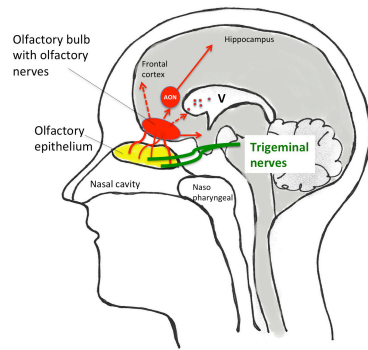https://orcid.org/0000-0001-7641-4240 (Christian Humpel)

Figure 1 | **Simplified scheme of the nose-brain pathway.**
Substances applied via the nasal cavity will reach the olfactory epithelium. There are two major nerves which innervate the olfactory epithelium, the olfactory nerves (red) and the trigeminal nerves (green). Intranasally applied substances will be taken up by olfactory nerves and reach the olfactory bulb. From there substances may be transported to the hippocampus via the anterior olfactory nucleus (AON), or to the piriform or frontal cortices. Applied substances may also diffuse and enter the ventricle (V). (Copyright by Christian Humpel).

olfactory or trigeminal nerves and transported until they reach a synaptic cleft in the olfactory bulb (termed the intracellular pathway). The molecules can travel within the nerves into the brain and are then released. In fact the olfactory nerve can endocytose molecules with a high variety in size. Second, the extracellular transport occurs along the nerves by bulk flow processes. It is suggested that the molecules cross the epithelial layer and are then included into the cleft between the axons and ensheating layers and then pass the epithelial tight junctions (Gänger and Schjindowski, 2018). The nose-to-brain delivery is quite fast and axonal transport is about 25 mm per day. The olfactory nerve in mice is about 4 mm and it was assumed that nose-to-brain delivery takes between 0.73–2.3 hours to travel from the olfactory region to the olfactory bulb. The transport along the longer (20 mm) trigeminal nerve takes longer (approximately 17–56 hours) (Gänger and Schjindowski, 2018).

Intranasal Delivery in Alzheimer´s Disease

The story of intranasal delivery is quite old, as already intranasal instillation was published in 1933 (Faber and Gebhardt, 1933). This strategy entered therapeutic interventions several decades later (see review Podolska et al., 2012). When entering the term "intranasal in Title" in PubMed (May 3rd) 7762 hits are found, when combined with "review in Title" 174 hits. The most important molecules for intranasal delivery are probably oxytocin (386 hits) and insulin (304 hits) and more than 30 clinical studies have been performed with intranasal insulin (Gänger and Schjindowski, 2018). The term "intranasal treatment in AD" gives 202 hits which are mainly focused on insulin (79 hits). Regarding AD, intranasal insulin improves memory and learning in AD models (Reger et al., 2008; Farzampour et al., 2016; Mao et al., 2016; Guo et al., 2017), it lowers Aβ in diabetes models (Subramanian and John, 2012), it modulates verbal memory (Reger et al , 2008) or it prevents tau phosphorylation in transgenic mice (Chen et al., 2014) and type II diabetes (Yang et al., 2013).

Interestingly, also other intranasal substances (e.g., naringin, erythropoietin, losartan, lactoferrin, deferoxamine) had effects in AD models, as they counteracted Aβ toxicity (Maurice et al., 2013; Kaur and Prakash, 2019), decreased Aβ and inflammation (Lin et al., 2016; Drews et al.,2019), affected amyloid-precursor protein (APP) processing (Guo et al, 2017), prevented memory deficits (Fine et al., 2012; Lou et al., 2016; Rodriguez Cruz et al., 2017), reduced aggregation of Aβ and tau in AD mice or decreased tau phosphorylation in alpha-synuclein mice (Matsuoka et al., 2007). Nerve growth factor (NGF) is very potent to protect cholinergic neurons and intranasal infusion of NGF prevented memory deficits in AD mice (Capsoni et al., 2012), or reduced Aβ deposition (Tian et al., 2012) or modulated tau phosphorylation (Lv et al., 2014). One paper showed that intranasal delivery of a single-chain variable antibody entered the brain and bound Aβ plaques and inhibited fibril formation, pointing to a strategy for intranasal vaccination (Cattepoel et al., 2011).

The question arises if it is possible to infuse Aβ via the nose and if this peptide may form aggregates in the brain. Sipos et al. (2010) intranasally applied human $Aβ_{42}$ oligomers, protofibrils and fibrils into rats and they could detect Aβ in the olfactory bulb and frontal cortex but also deeper in hippocampus, pons and cerebellum 2 hours after a single dose (Sipos et al., 2010). The intranasal application of $Aβ_{42}$ did not affect anxiety and short term memory but significantly impaired long-term spatial memory (Sipos et al., 2010). In our previous study (Humpel, 2021) we showed that intranasally applied fluorescent Aβ entered the brain and bulbus olfactorius but was captured by phagocytosing cells (probably microglia). More work is necessary, especially to show if intranasal Aβ may form plaques in the brain.

Intranasal Delivery of Neprilysin Rapidly Eliminates Plaques

Neprilysin is an Aβ-degrading enzyme and has a potent activity to degrade Aβ (Humpel, 2015, 2021). We have shown that neprilysin can degrade Aβ in organotypic brain slices taken from transgenic AD mice (Humpel, 2015, 2021). Very recently we showed that intranasal application of neprilysin cleared Aβ plaques in transgenic AD mice (Humpel, 2021). In this study, we injected 10 µL of neprilysin (100 ng) in both nostrils on 3 consecutive days. We used 9-month-old AD transgenic mice (APP_SweDI) which have a very high plaque load. At day 9 after intranasal neprilysin infusion, we found that the plaque load was reduced in the frontal cortex, but markedly increased in the middle and caudal cortex (**Figure 2A**). This suggested that infusion of pure neprilysin rapidly entered the brain, eliminated plaques quite fast, but that at later time points, this elimination caused a dramatic compensation of plaques in the cortex. The mechanism is unclear, but it seems possible that neprilysin degrades the large Aβ plaques in the brain, which then are seen as smaller Aβ plaques, or that the transgenic mice activate a compensatory process to produce more Aβ plaques.

Platelet-Loaded Neprilysin and Migration via the Intranasal Pathway

In a previous study (Kniewallner et al., 2014), we have shown that platelets-can be loaded with NGF and provide neuroprotective activity. In our recent study (Humpel, 2021), we loaded neprilysin into platelets and infused these platelets into the nose (3 × 10 µL, bilateral, each 5 million platelets, with 40 ng neprilysin each). We show that the plaque load was reduced in the frontal cortex but not in the middle and caudal cortex (**Figure 2B**). Migration of cells is a well established research topic in *Neuroscience*, especially the transmigration of blood cells across the BBB seems to be an interesting therapeutic approach (Hohsfield and Humpel, 2015b). In our hands, we have shown that monocytes can transmigrate across a BBB in mice and partly differentiate into a microglia-like cell type, which in turn phagocytoses and eliminates Aβ plaques (Hohsfield and Humpel, 2015a). Similarly, we hypothesized that platelets could be an interesting cell type to cross the BBB, however, we showed that most of the cells are trapped in the periphery, like spleen or lung and did not markedly enter the brain (Kniewallner et al., 2015). In the previous work (Humpel, 2021), we aimed to deliver platelets via the nose, and indeed we could show that there was some migration, but mainly in the frontal cortex/bulbus. This suggests a slow and delayed migration of cells via the olfactory pathway. It is also known that arteries run along the olfactory axon bundle (Gänger and Schjindowski, 2018), and we cannot exclude that platelets migrate along these vessels. Anyhow, we provide evidence that neprilysin can be released from platelets, migrate into the frontal cortex, and can partly eliminate some plaques. There was no evidence that plaques were removed in deeper brain areas. Definitely more work is necessary to investigate the use of blood cells as a therapeutic option via nasal application.

Collagen-Loaded Delivery of Neprilysin

Direct nose-to-brain delivery has some limitations (Gänger and Schjindowski, 2018): rapid elimination by mucociliary clearance, anatomy of the individual nasal cavities, administration techniques, dosage and volume, mucosal toxicity, allergic reactions, frequency of adminstration, and different molecular weights of the drugs. In order to overcome these problems different agents are used to increase uptake and transport of small peptides or proteins from nose-to-brain. The most important are mucoadhesive excipients (e.g., chitosan), adsorption enhancer (e.g., cyclodextrins) or preservatives (e.g., chlorobutol), semisolid formulations (e.g., hydrogels) and particulate

formulations (e.g., nanoparticles) or also lipid-based formulations (e.g., liposomes). Sodium hyaluronate is a mucoadhesive component and increased the transport of the 4 kDa peptide dextran (same size as Aβ) from the nasal cavity to the brain (Horvat et al., 2009). Chitosan nanoparticles with bromocriptine showed beneficial effects in a mouse model of Parkinson´s disease (Gänger and Schjindowski, 2018). In our hands, we have extensive experience with collagen to deliver drugs (Ucar and Humpel, 2018). Collagen is classified as a hydrogel and has drawn interest for brain repair, as it is highly biocompatible, biodegradable and non-toxic. In addition to its natural advantages, collagen already has very diverse applications in medicine, such as bone and cartilage reconstruction, wound dressing, tissue engineering applications, drug/gene delivery and cell encapsulation systems. We have extensive experience using collagen hydrogels and loaded different growth factors, such as NGF (Foidl et al., 2018), GDNF (Ucar et al., 2021) or FGF-2 (Ucar et al., 2020) into hydrogels and showed potent activity onto cholinergic, dopaminergic neurons or brain vessels, respectively.

In the previous study (Humpel, 2021), we loaded neprilysin into collagen hydrogels, showed potent activity *in vitro* in brain slices and loaded the collagen hydrogels into the nose of APP_SweDI mice (3x, each 30 ng, bilateral). Our data show that neprilysin is slowly released from the collagen, is stable and causes a decrease of the plaque load in the frontal cortex but markedly stronger effects are seen in middle and caudal cortex (**Figure 2C**). This type of intranasal application was the most powerful one and it was surprising to see that the majority of the plaques was eliminated. We suggest that neprilysin is slowly released from the collagen in the nose and then rapidly diffuses into the caudal areas. As we injected collagen-loaded neprilysin on 3 consecutive days, this guarantees a continous slow and stable release over several days, which probably gave the optimal dose during the 9 days of experimentation. As we did not test the plaque load after 10 days, we cannot definitely say if the plaques are again compensated in the transgenic AD mouse model and increase after > 10 days. This needs to be proven in further experiments. Anyhow, independent of the outcome, we think that collagen-loaded neprilysin provides the optimal strategy to deliver an Aβ-degrading enzyme deep into the brain to clear plaques.

Outlook on the Amyloid-Beta Hypothesis

The Aβ-cascade hypothesis is the most prominent hypothesis in AD (Selkoe, 2002). This hypothesis suggests that a toxic Aβ species is an initial pathological driver in AD, leading to the formation of extracellular Aβ plaques and to intraneuronal tau neurofibrillary tangles, and subsequent down-events, such as neurodegeneration, inflammation and vascular damage resulting finally in memory loss. However, this hypothesis is now surrounded by more and more controversy (Armstrong, 2014; Morris et al., 2014; Nisbet et al., 2015). The reasons for questioning the lonely Aβ cascade hypothesis are: (1) Why does vaccination against Aβ not eliminate plaques and partly improve symptoms in AD in any clinical human trials. (2) What is the role of tau in the Aβ cascade, does it modulate or potentiate Aβ or is even tau the lonely driver in the AD cascade, formulating the "tau-cascade hypothesis". Indications for a tau-induced dementia are the tauopathies and frontal lobe dementia, which do not need Aβ. (3) PET scans show that also younger individuals > 30 years have Aβ depositions. Why do these depositions not clearly correlate with the cognitive decline? (4) What is the role of native, oligomeric and fibrillar species of Aβ in the cascade, and when and how does the cascade start? What is the first event in targeting APP processing and affecting the secretase enzymes? (5) What is the role of microglia in the progression of AD, why does elimination of toxic Aβ at early stages not stop the disease, indicating also parallel events. (6) What is the role of vascular Aβ dositions resulting in cerebral amyloid angiopathy and how do platelets, expressing high amounts of APP contribute to the progression of AD? This all formulates the "vascular hypothesis of AD" (Humpel and Marksteiner, 2005; Humpel, 2017). (7) And finally, all the different complex heterogenities and comorbidities associated with AD clearly point to different subtypes of AD, which are not Aβ only dependent.

In our study, we show that intranasal delivery is very potent to infuse neprilysin deep and fast into the brain. Biocompatible biomaterials like collagen are a suitable tool to protect neprilysin and to allow a slow release. Our data also show that a fast elimination of Aβ plaques
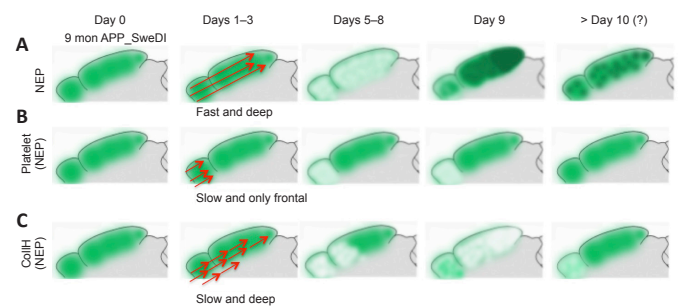

Figure 2 │ **Hypothetical model of the effects of the three different intranasal neprilysin applications into transgenic Alzheimer mice.**
Neprilysin was applied into the nose of 9-month-old transgenic mice overexpressing amyloid-precursor protein (APP) with the Swedish-Dutch-Iowa mutations (APP_SweDI), and these mice have a very high amyloid-beta plaque load in the frontal, middle and caudal cortex (day 0, green). Neprilysin was applied through the nose (bilateral) via 3 routes on 3 consecutive days (days 1–3). First, neprilysin was applied as a pure solution (3 × 10 µL, each 100 ng neprilysin, bilateral), which rapidly diffuses into the frontal and caudal cortex (A). Second, neprilysin was loaded into platelets (5 million platelets, 40 ng neprilysin loaded, 10 µL bilateral) and as this application is limited by migration of the cells it rather enters only the frontal cortex (B). Third, neprilysin was loaded into collagen hydrogels and they were placed into the nose (30 ng neprilysin, bilateral), and as collagen hydrogels only slowly degrade, it is assumed that neprilysin is released slowly and slowly diffuses deep into the caudal cortex (C). Infusion of pure neprilysin caused a slight decrease of plaque load in the frontal cortex, but markedly enhanced plaques in middle and caudal cortex at day 9. It is assumed that neprilysin eliminates plaques very rapidly between days 5–8 but causes a compensation of the plaque load after 10 days (A). Infusion of platelet-loaded neprilysin may eliminate plaques only in the frontal but not middle and caudal cortex on day 9, as the platelets will not migrate deeper into the brain (B). Infusion of collagen-loaded neprilysin causes a slight reduction of plaques in frontal cortex, but a dramatic reduction of the plaque load in the middle and caudal cortex on day 9 (C). It is assumed that collagen releases neprilysin very slowly, and that released neprilysin is stable and diffuses delayed also in deeper brain areas. It is hypothesized that in all cases, the neprilysin application causes a compensation of the plaque load after 10 days, suggesting the need for a continous and repeated neprilysin treatment in a human AD therapy. (Copyright by Christian Humpel).

results in a compensation and rapid new production of Aβ plaques. While on one hand neprilysin degrades plaques on the other hand new plaques are produced. This clearly shows that a single delivery of an Aβ-degrading enzyme is not efficient to eliminate all plaques in the brain. Thinking of a therapeutic drug, neprilysin must be delivered at an early time point before many plaques develop and also continuously for the whole life span. Our data provide clear evidence that neprilysin can degrade and eliminate Aβ plaques in a transgenic AD mouse model, but the plaque load is compensated over time. This may explain why the clinical (vaccination) strategies to destruct plaques may have failed and why the Aβ-cascade hypothesis needs to be re-adjusted. Finally, AD is a very complex disorder and the effects of an intranasal drug must be tested not only on the Aβ pathology but also on the tau pathology, on inflammatory cells or neurodegeneration, in order to consider an intranasal human therapeutic strategy.

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