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Brain-targeted intranasal delivery of biologics: a perspective for Alzheimer's disease treatment

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Alzheimer's disease (AD) presents significant clinical challenges due to its complex pathology and the limitations of traditional drug delivery routes, which often fail to transport therapeutic agents effectively across the blood–brain barrier (BBB). This review focuses on the potential of intranasal drug delivery to enhance therapeutic efficacy in AD treatment by providing a direct route to the central nervous system (CNS). It examines the mechanisms of intranasal administration, including the olfactory and trigeminal pathways, which facilitate rapid drug absorption and distribution to the brain. Additionally, the advantages of intranasal delivery in improving drug bioavailability, reducing systemic side effects, and enhancing patient compliance are discussed alongside innovative formulation strategies, including lipid nanoparticles and other carrier systems. Despite promising outcomes, challenges such as variability in absorption efficiency and the influence of repeated administration remain critical considerations. Furthermore, this review also surveys the current landscape of research for intranasal drug delivery in AD, integrating imaging technologies, emphasizing ongoing studies and future directions for this promising approach. By synthesizing recent findings, this review aims to provide a comprehensive exploration of the interplay of biologics, intranasal delivery, and brain disorders, offering valuable perspectives into the potential of intranasal gene therapy as a potent drug delivery system for CNS diseases.

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1. Drug development landscape in treatment of Alzheimer's disease

As the cornerstone of regulating normal physiological functions within the human body, the health and homeostasis of the central nervous system (CNS) are of paramount importance for proper operation. Unexpected pathologies within the CNS can exert significant detrimental impacts on daily life. Among the various CNS disorders, Alzheimer's disease (AD) stands out as a prevalent condition, which affects a growing number of individuals worldwide, with approximately 47 million people living with dementia, the majority of whom are afflicted with AD.[1] According to Alzheimer's Disease International, the annual global expenditure on AD therapy surpasses $1 trillion.[2] Given that AD is a CNS disorder, drugs intended to target it must successfully traverse the blood–brain barrier (BBB) and reach the site of the lesion before they can exert their therapeutic effects. While some small-molecule drugs exhibit the potential to modify the disease, with some advancing to phase III clinical trials and others currently in phases I and II, the majority of traditional small-molecule drugs can only alleviate the symptoms of AD, neglecting its fundamental aetiology,[3] and encounter substantial challenges when attempting to breach the BBB without the necessary modifications or formulations involving target ligands. Consequently, even if drugs can alleviate the symptoms of AD in the brain, they can only traverse the BBB through a targeted drug delivery system. This deficiency underscores the immense unmet clinical demand for the development of biologics and their corresponding delivery systems as promising strategies for modifying AD treatment.[1]

Monoclonal antibodies (mAbs) have exhibited tremendous promise across various disease domains, with notable examples such as Blinatumomab and Rituximab successfully employed in cancer therapy.[4,5] Researchers have also explored the potential of mAbs in the context of AD treatment.[3] However, challenges have arisen due to factors such as the large molecular size of mAbs and their difficulties in crossing the BBB. Only about 0.1% of the therapeutic antibodies administrated systemically can reach the CNS.[6] Furthermore, numerous β-amyloid (Aβ)-specific mAbs, including Solanezumab, Gantenerumab, and Crenezumab, have failed to demonstrate efficacy following intravenous or subcutaneous administration at lower doses. For instance, Solanezumab

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exhibited promising results in early clinical trials but failed to meet primary and secondary endpoints in phase III trials.[7–9] Similar setbacks have been observed in other mAb studies targeting Aβ, underscoring the complexity of this research domain.

Only a few mAbs, such as Aducanumab and Lecanemab, have demonstrated efficacy, albeit at very high doses, and have been associated with significant side effects.[10–12] These findings raise concerns about the overall benefit of this approach for AD patients. While some mAbs exhibit potential, further research and a meticulous evaluation of side effects are imperative for the development of effective and safe AD therapies.[13–15]

Concurrently, there has been a burgeoning interest in the intranasal delivery of nucleic acid-based drugs for AD treatment in recent years. Nucleic acid drugs, such as small interfering RNAs (siRNAs) and antisense oligonucleotides (ASOs), offer substantial promise as potential AD therapies due to their ability to target and modulate specific disease-related genes and proteins. Intranasal delivery represents a noninvasive and direct route to bypass the BBB, enabling the delivery of therapeutic agents directly to the brain. This approach exhibits considerable potential for enhancing the delivery efficacy of nucleic acid drugs, ushering in new prospects for the development of more effective treatments for AD and other neurological disorders.

In summary, although small molecule drugs can effectively alleviate disease symptoms, enhance memory,[16] and improve quality of life in Alzheimer's disease patients during current treatment, the majority of these drugs do not alter the course of the disease or provide a cure. Conversely, despite the promising results that mAbs have demonstrated in non-CNS diseases, they encounter substantial challenges when applied to AD treatment, primarily owing to their limited capacity to traverse the BBB. The molecular size and hydrophobic nature of mAbs impede their passive diffusion or active transport across the BBB, resulting in minimal brain exposure, with less than 1% of the administered antibody dose reaching the brain.[17,18] The remaining 99% of the dose remains in the systemic circulation, leading to persistent off-target side effects.[19] Therefore, it is imperative to explore the physiological composition and structure of the BBB and to develop appropriate drug delivery systems capable of efficiently transporting biomolecular drugs across or circumventing the BBB to target genes or proteins within related pathways in the brain.

2. Structure and low permeability of the BBB

2.1. Structure of the BBB

The BBB is a complex and multicellular structure comprising endothelial cells, pericytes, astrocytes and microglia cells. Brain microvascular endothelial cells constitute the primary component of the BBB, forming the lining of blood vessels and are interconnected through tight junctions (TJs), including claudin and occluding.[20] Pericytes and astrocytes contribute essential structural support to the barrier and are integral in regulating its metabolic functions, including maintaining the integrity of the BBB, regulating cerebral blood flow, and facilitating the appropriate localization of barrier proteins.[21,22] Although microglia are not traditional BBB constituents, they are immune cells within the brain that serve the purpose of defending against potential pathogens or toxins that might attempt to breach the BBB, as shown in Fig. 1. In addition, the basement membrane, primarily secreted by endothelial cells and astrocytes, and consists of extracellular matrix molecules such as perlecan, fibronectin and glycosaminoglycans. This structure not only provides mechanical support and anchorage for BBB cells but also facilitates intercellular communication and contributes to the polarization and organization of the surrounding cellular components.[20]

2.2. Difficulty crossing the BBB

The BBB plays a pivotal role in drug delivery and therapeutic interventions for brain disorders.[23] Within cerebral endothelial cells, arachnoid epithelium cells, choroid plexus epithelial cells, TJs and adherence junctions (Ajs) are present and are composed of junctional adhesion molecules (JAMs) and protein complexes that constitute intercellular barriers.[24,25] These structures serve as formidable defences against the penetration of toxins or biologics. Notably, certain plasma-derived proteins, such as albumin, prothrombin, and plasminogen, are present at significantly greater concentrations in the bloodstream than in the cerebrospinal fluid (CSF) due to their inability to traverse the BBB.[23] Specific biological molecules and essential nutrients vital for the CNS necessitate transport into the brain through receptors expressed on the capillary endothelium's cell membrane.[23] These include transferrin, lipoproteins, immunoglobulin G (IgG), insulin, leptin, tumour necrosis factor α (TNFα), and epidermal growth factor (EGF).

When implementing invasive treatments, such as intravenous injection of drugs and formulations into the systemic circulation, they must first breach the BBB to reach the target sites in the brain. However, the resistance posed by the BBB presents significant challenges, as more than 98% of small-molecule drugs and nearly 100% of large-molecule drugs struggle to exert therapeutic effects within the brain.[26] The BBB only permits the passage of small lipophilic molecules with a size not exceeding 400–600 Da,[27] and even these small hydrophobic molecules encounter limitations owing to the presence of p-glycoproteins expressed on the BBB.[28] On the other hand, certain small polar hydrophilic molecules can traverse the BBB through the intercellular space. However, larger molecular drugs can only access the brain through active transport mediated by transporter proteins or receptor proteins expressed on the brain endothelium's membrane.[29] Strategies, such as the use of an anti-transferring receptor (TfR) antibody fragment or cystine-dense peptide (CDP) in conjunction with mAbs or other bioactive molecules, have been explored to enhance their accumulation in the CNS.[30,31] Despite endea-

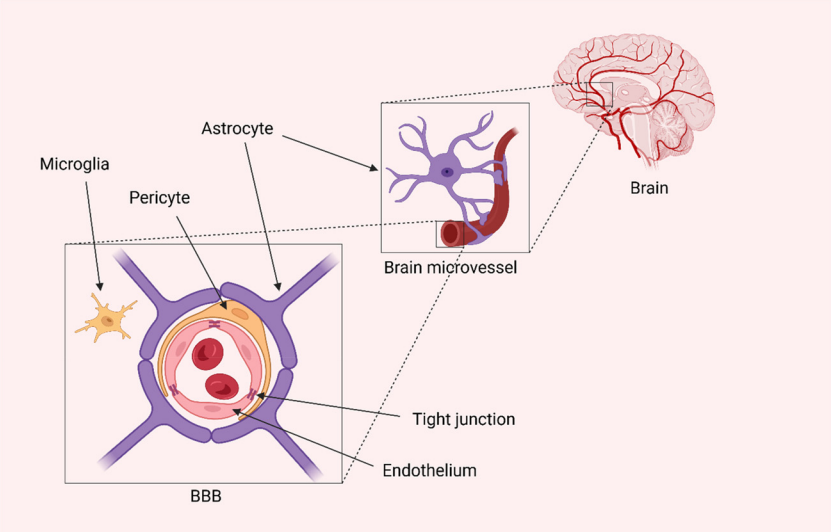

Fig. 1 Structure of the blood–brain barrier (BBB). The endothelial cells of cerebral capillaries are connected by tight junctions (TJs), enveloped by pericytes, and covered by astrocytic nerve endings, forming an intricately dense barrier. This configuration prevents substances within the blood vessels from readily undergoing passive diffusion and distributing into the brain, thereby maintaining a stable level of substances within the brain tissue.

vours in receptor-mediated transport, very few biologic drugs can successfully cross the BBB and enter the brain, with only approximately 1% of the IgG antibody conjugated with the designed anti-TfR antibody displaying the potential to traverse the BBB, and even their efficacy is influenced by factors such as molecular size.[30] Thus, while substantial development and progress have been made over the last two decades, biological therapy still confronts limitations in the treatment of brain disorders.

2.3. Shortcomings of intravenous systemic administration for brain disorders

In the case of small molecule treatments for brain disorders, invasive routes of administration, such as intravenous delivery, are hindered by systemic circulation effects, exposing them to the same challenges as drugs designed for systemic diseases. Apart from the difficulties of crossing the BBB, factors such as first-pass liver metabolism and systemic off-target side effects can significantly impact the distribution and effectiveness of small-molecule drugs within the brain.[32] Over recent decades, various drug delivery systems, such as liposomes and polymeric nanoparticles, have been devised for small molecules. Nonetheless, even after intravenous administration, the liver predominantly sequesters most nanoparticle-bound drugs, with no more than 1% of these drugs reaching the brain to achieve the desired therapeutic effects in most research.[33] Besides, the ability of large molecule drugs, exemplified by mAbs, to traverse the BBB when administered intravenously or subcutaneously poses an extreme challenge. Furthermore, the repercussions of off-target effects cannot be disregarded when doses are substantially increased to elevate the drug concentrations within the brain. For instance, after intravenous or subcutaneous administration, both Aducanumab and Lecanemab exhibited amyloid-associated imaging abnormality oedema (ARIA-E) and amyloid-associated imaging abnormality haemorrhage (ARIA-H) side effects during clinical trials.[10,11,34] These issues are the primary culprits behind the failure of the majority of mAbs targeting brain diseases. Ultrasound-mediated non-invasive delivery of temporary opening of the BBB,[35] intracerebroventricular (ICV) injection[36] and intrathecal administration are limited by factors such as post-wound recovery and safety, and difficult to popularise. Hence, pioneering innovative biologic-based brain delivery strategies is imperative as an initial step in facilitating the administration of large molecule drugs for the treatment of brain disorders.

Some delivery technologies have been developed to assist in drug transport across the BBB. In early research, the enhanced permeability and retention (EPR) effect[37] was initially observed in solid tumours, including brain tumours, and subsequently used to develop various drug formulations that passively accumulate in the brain, due to the significant permeability distinction between the blood-tumour barrier (BTB) and BBB. However, the EPR effect has not yielded the anticipated outcomes and remains controversial by some researchers to be limited by drug design and tumour models.[38] Mostly importantly, it cannot contribute to drug delivery of non-tumour CNS diseases. Beyond passive targeting, active targeting strategies aimed at crossing the BBB can be categorized into several approaches, including ligand-targeting of brain endothelial cell receptors, biomimetic drug delivery systems, and externally stimulated methods temporarily disrupting the BBB. For example, TfR antibody has been widely utilized in targeting the transport across the BBB mediated by conjugating with transferrin (Tf).[39,40] The red blood cell membrane modified

with candoxin (CDX) peptide has been developed to locate them close to tumour vessels.[41] Light[42,43] and ultrasound[44,45] can also help to open the BBB temporarily and non-invasively. However, these approaches have demonstrated varying degrees of limitations in practical use, particularly regarding portability and safety in clinical applications, making intranasal delivery a more promising strategy worth development.

3. Structure of the nasal passage and drug transport pathway to the CNS

3.1. Structure of the nasal passage

The human nasal passage consists of two chambers separated by the nasal septum and is categorized into three distinct sections: the vestibule, the respiratory zone, and the olfactory zone. The total surface area of the human nasal passage is approximately 150–160 cm^2, with a volume of 20 mL, and an available volume of 200 μL for drug administration.[46–48] The nasal vestibule, located at the entrance of the nasal passage, serves as a filtration zone, where nasal hairs capture and prevent harmful substances, such as dust, from progressing deeper into the respiratory system. The respiratory zone, covering approximately 5/6 of the nasal mucosa, is the largest segment of the nasal passage.[49] This zone comprises highly vascularized mucosal membranes, providing an effective pathway for drug absorption into the human body. On the apical surface of many epithelial cells in this zone, microvilli and cilia are present. These tiny projections aid in the movement of particles, irritants, and drugs from the gel layer of mucus to the nasopharynx, where they can be swallowed, ultimately reaching the gastrointestinal tract.

The nasal mucosa is covered by several types of epithelial tissue, including compound squamous epithelium, pseudostratified columnar epithelium, and intermediate types. The primary tissue in the respiratory zone is the respiratory epithelium, which consists of columnar cells, with or without cilia, mucous-containing cupped cells, and basal cells. Below the respiratory epithelium lies the lamina propria, which is composed of fibrous elastic connective tissues that house nerves, glands, and blood vessels. The glands in this region contain plasma and mucus-secreting cells, and their secretions are released onto the surface of the respiratory epithelium.[50]

Mucociliary clearance, a combined action of cilia and the mucus layer is responsible for removing particles and irritants from the nasal cavity. The anterior non-ciliated region of the nasal cavity is cleared slowly, with foreign bodies in this area gradually transitioning from the mucus layer to the ciliated area. The ciliary clearance system propels materials backwards at a flow rate of approximately 6 mm min^{-1}. Mucociliary clearance is also accountable for rapid clearance of drugs after intranasal administration. Reversible inhibition of mucociliary clearance can prolong the residence time of the drug in the nasal cavity to enhance the absorption of intranasal drugs.

3.2. Drug transport pathway to the CNS

Following intranasal dosing, drugs can directly bypass the BBB to access the brain. Two primary pathways have been identified, namely the olfactory pathway and the trigeminal nerve pathway, both of which establish connections within the intracranial cavity.[51] Via the olfactory pathway, drugs can penetrate the olfactory bulb, access the olfactory nerve fibres, and ultimately enter the cerebral hemisphere.[52] This pathway offers the advantage of rapid drug entry into the brain. However, this approach has the drawback of potential drug clearance by secretions, necessitating higher drug doses for effective delivery.[53] Besides, the trigeminal nerve pathway provides an alternative route for drug entry into the brain through the nasal cavity. It encompasses the trigeminal nerve and the ophthalmic nerve, with their cell bodies located in the trigeminal ganglion and the ophthalmic ganglion, respectively. Drugs can access the trigeminal ganglion through this pathway and then reach the brain *via* the skull base, enabling wider distribution.[54] The advantage of this pathway is that drugs can enter the brain through numerous nerve fibres, allowing for lower drug doses to achieve effective delivery.[51]

Amongst these two pathways, two mechanisms are involved in nasal drug absorption:[55] the intracellular process and the water transport pathway, also known as the paracellular pathway. The intracellular process commences when molecules are internalized by olfactory neurons. For specific molecules such as wheat-germ agglutinin horseradish peroxidase (WGA-HRP), receptor-mediated transport may also occur.[56,57] The molecules are then transported through the axons or neurons toward the olfactory bulb or trigeminal nerves, ultimately being exocytosed to these sites. Finally, the molecules are translocated into the brain *via* trigeminal neurons or mitral and tuft cells, which project to various locations on the ventrolateral surface of the brain[58,59] or the olfactory bulb. However, neuronal translocation occurs at a slow pace before reaching the olfactory bulb or trigeminal nerves.[60] In contrast, the paracellular pathway is a passive transport process influenced by drug concentration and is initially slow. It involves navigating through TJs and traversing the paracellular cleft to cross the nasal epithelia.[61] Subsequently, drugs can migrate to the lamina propria and diffuse into the CSF through the space between olfactory nerve fibres (ONFs) and olfactory neuron-ensheathing cells.[62–65] This passage through the perineural space is considerably faster than intracellular axonal transport.[61] The same process applies to the trigeminal nerves.[66,67] Given the complex physiological environment and drug properties of actual intranasal drug delivery, intranasal administration typically involves a composite process that combines both the intracellular and paracellular pathways, as shown in Fig. 2.

The transport of molecules or nanocarriers from the nasal cavity to the brain may be driven by different forces depending on the nature of the delivery system. For unmodified or passive systems, concentration gradients serve as the primary driving force for transcellular or paracellular diffusion along

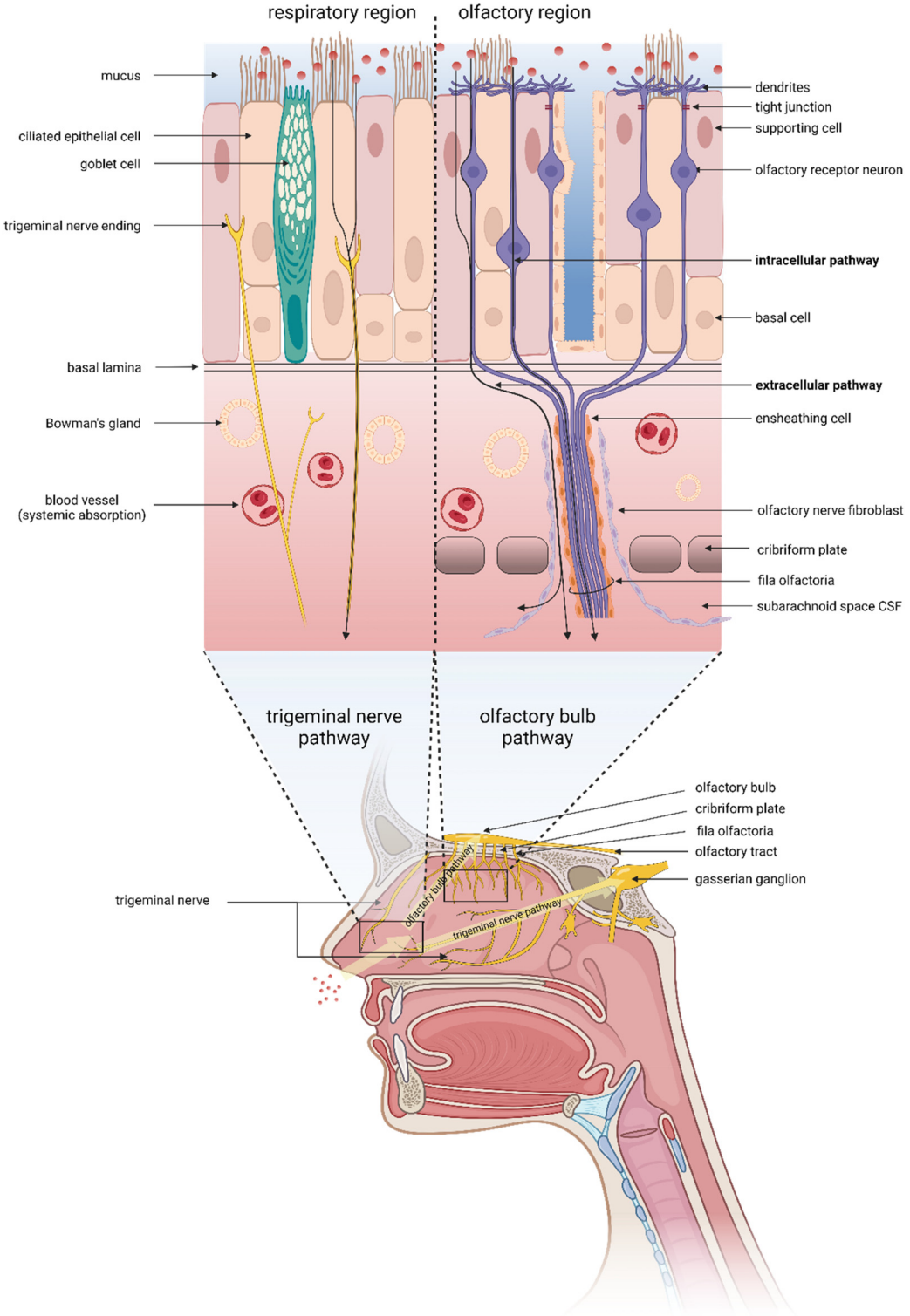

Fig. 2 Pathways of intranasal drug distribution from the nasal passage to the central nervous system (CNS). After intranasal administration, drugs move in reverse along the ciliary swing within the nasal mucus. They are subsequently captured extracellularly or intracellularly by the terminals of trigeminal neurons and olfactory receptor (sensory) neurons while traversing the respiratory and olfactory regions of the nasal cavity. Subsequently, drugs are transported to the pons or CSF in the brain through the trigeminal nerve pathway, or through the olfactory bulb pathway. While a minor portion of the drug might be absorbed into the systemic circulation by the blood vessels distributed in the lamina propria, the majority of the drug directly reaches the brain through nerve fibres and the surrounding space, effectively bypassing the blood–brain barrier (BBB).

the olfactory and trigeminal pathways. However, in systems incorporating CPPs or other active ligands, additional mechanisms such as receptor-mediated uptake, adsorptive transcytosis, or axonal internalization may be involved, facilitating more efficient and selective brain entry beyond simple passive diffusion.

Overall, a deep understanding of the intricacies of the nasal passage's structure and function is paramount when designing effective nasal drug delivery systems. Intranasal delivery has gained significant attention as an efficient and promising administration route for traditional small-molecule drugs. Additionally, the potential of intranasal delivery of biologics, such as siRNAs, ASOs and therapeutic peptides, for treating brain disorders, has been recognized, suggesting that the topic is worthy of further exploration.

4. Advantages and challenges of intranasal delivery

As detailed above, intranasal delivery involves the administration of drugs through the nasal cavity, providing several advantages over other routes, such as rapid absorption, high bioavailability, and direct delivery into the brain. Additionally, this route bypasses the harsh gastrointestinal environment of oral delivery, or the invasive procedures associated with injectable routes.

Despite the advantages of intranasal drug delivery, several challenges exist, which were also associated with concerns about the design of intranasal formulations, including limited drug volume for administration, potential nasal mucosa irritation, and potential damage with prolonged or repeated drug exposure. Nevertheless, intranasal delivery uniquely bypasses the BBB, directly reaching the brain, thereby reducing the loss of bioactivity of biologics and systemic side effects associated with systemic exposure. For example, mAbs are limited with low enrichment in the brain when administrated intravenously, but it has been reported that anti-CD3 mAb can reach into the brain of AD mice when administrated intranasally to modulate the gene expression in the hippocampus and cortex and improve cognition of AD mice.[68] Intranasal administration of a mouse mAb against the neurite growth-inhibiting and plasticity-restricting membrane protein Nogo-A has also been reported to demonstrate its repair enhancing effects on rats with large cortical strokes.[69] Despite the research in administrating Aβ-specific mAbs intranasally for AD therapy is still limited, the intranasal route could potentially offer an alternative pathway that help mAbs bypass the BBB entirely *via* olfactory and trigeminal nerve pathways, allowing more direct access to the CNS. Furthermore, compared to traditional routes such as intravenous and subcutaneous administration, the low dosage and high efficacy of biologics significantly compensate for the lower dose administered in the nasal cavity during intranasal delivery. Biologics agents also show better biocompatibility than traditional small-molecule drugs. Taken together, these findings suggest that intranasal delivery is an excellent delivery strategy for delivering biologics to the brain.

Overall, the intranasal route has demonstrated enormous potential for the treatment of CNS disorders involving biological drugs over the past two to three decades. While this strategy is not without challenges, if designed and utilized rationally, the unique advantages of intranasal delivery will significantly enhance the treatment of patients with CNS disorders.

4.1. Improved update compared to traditional administration rotes

The BBB represents the primary challenge in the treatment of brain diseases. The physiological foundation supporting the potential of intranasal drug delivery to bypass the highly selective BBB has been previously discussed. Here, we primarily focus on practical examples of research illustrating the delivery of various therapeutic agents to the CNS at increased concentrations. For instance, when administered orally, 17β-oestradiol undergoes significant degradation, with an approximately 95% loss due to first-pass metabolism. However, intranasal administration leads to a 4–9 times greater concentration of this compound in the CSF when dissolved in PBS buffer.[70] Furthermore, intranasal administration of hypocretin-1[71] or recombinant human nerve growth factor (NGF) (26.5 kDa) solutions[72] increases CNS concentrations in various brain regions by 7–13-fold and 10–45-fold, respectively. Similarly, when administered intranasally, Huperzine A,[73] insulin-like growth factor,[74] galanin-like peptide,[75] and interferon β-1b,[76] have demonstrated significantly greater concentrations in the brain than oral or intravenous formulations. Preliminary studies have often involved dissolving the drug in a buffer solution for direct nasal administration. Drug molecules can typically be detected in the olfactory bulbs and brain regions within 1 hour post-administration, which also confirms the nose-to-brain delivery pathway. However, these methods are only effective for drugs that possess certain lipophilicity and stability in nasal mucus. For more unstable drug molecules, such as biopharmaceuticals, formulation technologies are required to enhance drug stability and permeability for effective nasal delivery to the brain, which is discussed in later sections.

4.2. First-pass effect and systemic side effects

In traditional drug administration routes, such as oral administration, intravenous injection or infusion, drugs are typically absorbed into the bloodstream either through gastrointestinal absorption or direct injection. They exert their effects as they are transported throughout the body, following the hepatic first-pass effect. Small molecule drugs, in the absence of specific targeting ligands, generally adhere to the principles of pharmacokinetics and are distributed primarily in the blood or fat based on their hydrophilicity and lipophilicity, as exemplified by drugs like Cefazoline and Barbital. Conversely, large molecule drugs are prone to degradation by metabolic enzymes in the gastrointestinal tract and blood, resulting in the loss of bioactivity. Formulated drug-loaded nanoparticles,

when lacking specific targeting ligands, tend to accumulate in organs like the liver and spleen in most cases (>90%).[77–79] Even when conjugated with specific targeting ligands, after sufficient circulation time in the body, most nanoparticles are still predominantly distributed in the liver and spleen, with only a marginal increase in drug concentration in the targeted tissues.

Moreover, during the systemic circulation of drugs or drug-loaded nanoparticles in the bloodstream, all cells in the body are potentially exposed to the drug. Exposure occurs except for specific tissues protected by special barriers, such as the CNS guarded by the BBB. Regardless of whether drugs or drug-loaded nanoparticles undergo modification with specific targeting ligands, they inevitably produce off-target effects to varying degrees in other cells and tissues. This includes issues like off-target tissue toxicity associated with certain tumour-targeted drug-loaded nanoparticles.

In the case of brain-targeted drugs, one of the advantages of intranasal delivery is the avoidance of the first-pass effect of the liver and the off-target effects associated with systemic administration. Brain-targeted drugs are directly absorbed into the olfactory bulb through the nasal mucosa and subsequently transported into the brain. At this stage, only a small subsection of drug is absorbed into the systemic circulation, significantly reducing drug exposure to cells and tissues throughout the entire body. This naturally results in decreased systemic side effects. The lack of systemic circulation also implies that the drug does not undergo first-pass metabolism before it reaches the target site, which significantly enhances the bioavailability of brain-targeted drugs delivered intranasally. Even in the case of intranasally delivered drugs or formulations designed for peripheral diseases, drugs are directly absorbed into the venous circulation of the head and neck, bypassing the hepatic first-pass effect.

4.3. Limited administration volume and local nasal mucosal injury

The physiological structure and volume of the nasal cavity contribute to one of the most significant distinctions between intranasal drug delivery and other administration routes: dosage. Unlike oral administration and intravenous injection, which permit single high-dose administration, intranasal drug delivery involves smaller dosages or volumes. In the case of mice and rats, the nasal delivery volume is approximately 5 and 50 μL, respectively,[80,81] while in humans, the ideal nasal delivery volume is approximately 0.2–0.3 mL per nostril.[82] This volume is notably lower than the 10 mL typically used for general intravenous injection and the hundreds of millilitres used in intravenous infusion. Consequently, intranasal drugs targeting the CNS must exhibit high activity and efficacy at low doses, maintaining their effectiveness within the therapeutic window with lower dosages after reaching the CNS through the nasal route.

Furthermore, despite the nasal mucosa being exposed to air and various pathogens for extended periods, it remains a delicate biological membrane. Drugs administered intranasally should not damage the nasal mucosa during multiple administrations. Nasal mucosa damage during drug administration not only affects the absorption process and rate of drugs through the nasal route but also compromises an essential defence against pathogens, potentially leading to CNS lesions as pathogens invade the brain through the damaged area. Therefore, drugs intended for intranasal delivery must exhibit low toxicity toward the nasal mucosa.

Considering all the factors mentioned above, drugs delivered intranasally need to be administered at high concentrations in small volumes to increase the local drug concentration at the site of brain lesions. However, potential damage to the nasal mucosa requires that the drugs be relatively non-toxic or not prepared at excessively high dosages/concentrations. For most drugs, these two requirements are somewhat contradictory, limiting the possibility of delivering the vast majority of drugs to the brain *via* the intranasal route, particularly traditional small molecule cytotoxic drugs used in tumour treatment. It is important to note that, in comparison to traditional small molecule drugs with high doses and cytotoxicity, biologics possess characteristics such as low-dose high activity, low toxicity, and biocompatibility. For instance, biological drugs, such as siRNA, a kind of RNAi molecule, used in gene therapy can efficiently target specific mRNA at extremely low concentrations and exert therapeutic effects. Peptide and protein drugs, such as NGF, can also exert their effects on the brain after intranasal administration. Moreover, these biologics demonstrate excellent biocompatibility within the body and often do not cause irreversible damage to the nasal mucosa during intranasal administration. These findings indicate that biologics with low doses and high efficacy are well-suited for intranasal delivery, suggesting that intranasal delivery is an excellent targeted strategy for transporting them into the brain.

5. Intranasal formulations of biologics for AD treatment

The advantages and potential of intranasal delivery route for the treatment of brain disorders have been described above. The drug molecules are encapsulated into intranasal formulations for brain delivery according to their hydrophilicity and lipophilicity, or electrostatic interaction, and other intermolecular interactions. In the context of AD treatment, intranasal delivery of biologics has assumed a significant role, resulting in numerous research breakthroughs, albeit with the majority concentrating on the intranasal administration of insulin. In the context of intranasal delivery to the brain, the site of drug release from nanoparticles plays a critical role in determining therapeutic efficiency. Ideally, the nanocarrier should remain intact while traversing the nasal mucosa, minimizing premature drug leakage and avoiding systemic absorption or mucociliary clearance. Controlled or stimuli-responsive release mechanisms, triggered by pH changes, enzymatic activity, or intracellular environments, are often employed to

ensure that drug release occurs after the nanoparticles have crossed the epithelial barrier, preferably within the olfactory bulb, trigeminal nerve region, or brain parenchyma. Such spatially selective release enhances the concentration of the therapeutic agent at the target site, improving central nervous system specificity while reducing off-target exposure. Here, the formulations commonly used for brain-targeted intranasal delivery are described as follows.

5.1. Nano formulations

5.1.1. Polymer-based nanoparticles. Polymeric nanoparticles (PNPs) have been developed as promising drug delivery systems for intranasal delivery due to their ability to encapsulate a wide range of drugs, improve drug stability, and achieve targeted delivery to the brain. PNPs are often formulated by the self-assembly of hydrophobic block copolymers using methods including solvent evaporation, nanoprecipitation, homogenisation, and supercritical fluid technology. Commonly used polymer molecules include PLA, PLGA, and some natural polymers, such as chitosan.

However, in addition to amphiphilic block co-polymer self-assembly, polymer nanoparticles can be formed by a number of other routes. For delivery of biologics, like nucleic acid molecules, amine-rich polymer molecules are frequently used to prepare cationic PNPs, which assemble with nucleic acids through electrostatic interactions. For instance, the natural polymer chitosan has been employed to deliver plasmid DNA into the brain, ultimately enhancing VGF expression *via* intranasal delivery.[83] However, some cationic PNPs have been demonstrated to show the cytotoxicity to cells,[84] and it has been noted that the charge density of polycationic nanoparticles is a key factor to be optimised in PNP design to ensure the correct balance of nucleic acid condensation, membrane interaction and cytocompatibility. Payload release from PNPs occurs *via* a variety of mechanisms, and with careful polymer design, controlled release kinetics can be achieved. Polymer delivery systems can be prepared with chemical bonds that degrade under tissue or cell-specific conditions to release drugs, for example, some PNPs have been encoded with chemistries enabling breakdown in the acidic microenvironment of local tumours and/or in the reduced pH of late endosomal compartments.[85] In addition, the design flexibility inherence in PNPs enables functionalization with fluorescent labels, MRI agents and targeting ligands. In the context of this review, PNPs have been utilised for intranasal brain delivery of an antibody mimicking Aβ to clear Aβ and rescue memory deficits in AD mice.[86]

In this respect, there are many ongoing research efforts to develop innovative nanoparticle formulations and explore their applications in drug delivery and disease therapy. The versatility, chemical design space, and tunability of PNPs make them promising candidates for advancing intranasal drug delivery technologies.

5.1.2. Lipid-based nanoparticles. Lipid-based nanoparticles are widely used in drug and nucleic acid delivery and can be prepared from a range of physiological and synthetic lipids. They have excellent biocompatibility and stability and have advantages in drug delivery for CNS diseases with its biodegradability, controlled and modified drug release pattern. Typical particle sizes are in the range of 50–1000 nm, and these can be varied by preparation method. Lipid nanoparticles can be divided into several diverse classes with a variety of properties.

5.1.2.1. Liposomes. Liposomes are nanoscopic vesicles composed of lipid bilayers, mimicking cell membranes, which can encapsulate drugs for targeted delivery. These structures have an aqueous interior and hydrophobic membrane, making them advantageous carriers for hydrophobic and hydrophilic drugs. Due to their good biocompatibility and ability to encapsulate a wide range of therapeutic agents, including mRNA, siRNA and ASO, liposomes are extensively used in drug delivery systems, as illustrated in Fig. 3. For instance, cationic liposomes have been utilized to transport c-myc siRNA due to their electrostatic interactions with negatively charged nucleic acids, offering a method for glioblastoma treatment through intranasal administration.[87] They can be utilized to enhance drug solubility, prolong circulation time, and target specific tissues or cells. Additionally, liposomes can be modified with ligands or surface coatings, such as RGD peptide modification,[88] to achieve targeted delivery to specific sites within the body, including tumours, inflamed tissues, or the brain. This versatility makes liposomes a valuable tool in pharmaceutical research and clinical practice for improving drug efficiency and minimizing side effects. In the context of intranasal delivery to target the brain, liposomes can improve drug utilization and minimize systemic side effects, thus increasing drug efficacy.

5.1.2.2. Lipid nanoparticles. Lipid nanoparticles (LNPs) have many advantages in comparison to other particle systems, including biocompatibility and biodegradability,[90] low toxicity, potential for controlled and modified drug release,[91] and large-scale production.[92] Based on their preparation methods, they can be classified into solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC). These two types on LNP vary in drug loading efficiency, storage stability, and controlled release properties.[93,94] However, both are manufactured from mixtures of solid lipids or combinations of solid and liquid lipids along with emulsifiers.[95] Similar to liposomes, their surfaces can be modified with various ligands to target specific organs. Additionally, cationic lipid nanoparticles can be utilized for drug loading and delivery of nucleic acid molecules through electrostatic interactions.

Regarding AD therapy, intranasal LNPs have been widely trialled to enhance the delivery of drugs to the brain. The brain concentration and AUC value of resveratrol were higher than oral formulation when encapsulated into intranasal NLC, with a T_{max} at 30 min, compared to 2 h for oral formulation.[96] Pioglitazone[97] NLC also demonstrated a similar ability for brain targeting after intranasal administration. For other brain disorders, intranasal delivery of siRNA LNP (Fig. 4) ameliorated depression-like behaviours in mouse depression model.[98]

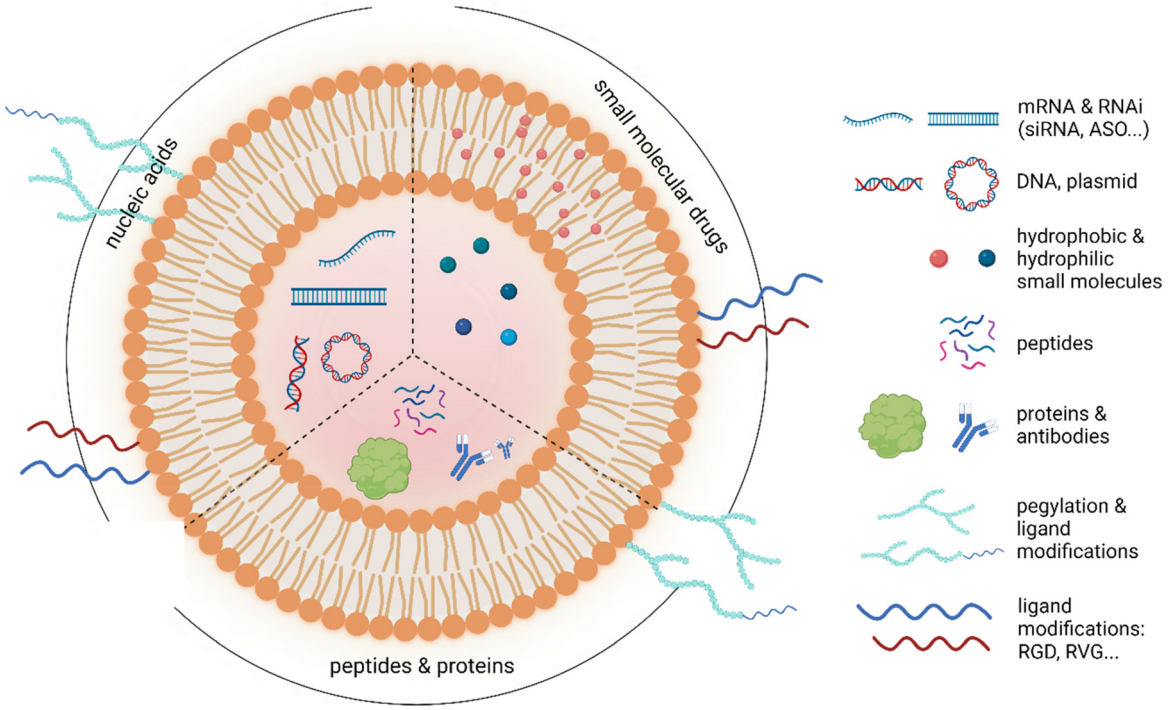

Fig. 3 Liposome application for intranasal therapeutics. Taking liposomes as an example, strategies such as modifications with ligands like polyethylene glycol (PEG) can be employed to enhance the *in vivo* stability of liposomes, and enhance mucosal penetration for intranasal administration.[89] Alternatively, specific targeting ligands can be incorporated to augment their ability to target brain tissue, such as the rabies virus glycoprotein (RVG) peptide. Hydrophilic drugs are encapsulated within the aqueous phase enclosed by the bilayer, while lipophilic molecules tend to distribute within the hydrophobic regions of the bilayer. Biomacromolecular drugs, such as nucleic acids and protein/peptide-based therapeutics, can be encapsulated into liposomes to protect them from metabolic degradation within the circulatory system. Subsequently, these vesicles can be transported by liposomes to target regions, facilitating their therapeutic effects. The depicted structures in the diagram are not drawn to scale and are presented solely for a schematic representation.

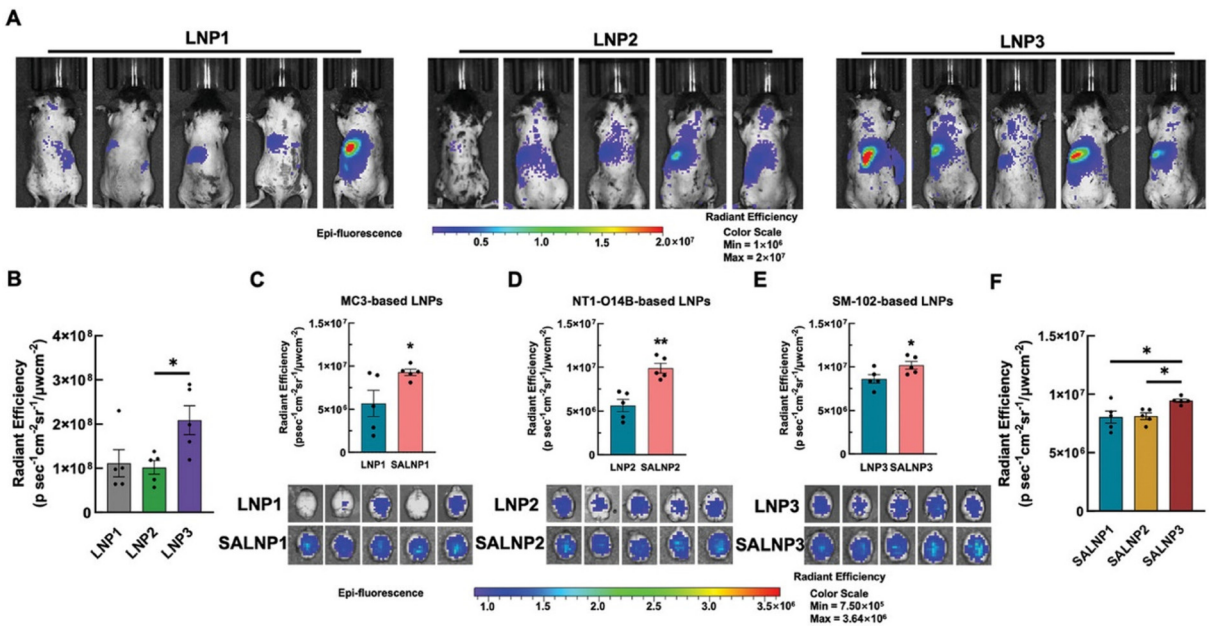

Fig. 4 (A) Intranasal delivery of circATF7IP siRNA *via* lipid nanoparticles; (B–F) Alleviation of LPS–induced depressive–like behaviors,[98] showing permeability improvement of si-circATF7IP by LNPs.

These studies have demonstrated the potential of LNPs for intranasal brain delivery of drug molecules. Compared with other administration routes, including intranasal solutions or suspensions of naked drugs, intranasal LNPs can effectively increase the transport rate and accumulation of drugs into the brain, and reduce drug loss during nasal absorption, which results from mucociliary clearance and enzymatic degradation.

5.1.2.3. Nanoemulsions. Nanoemulsions are stable colloidal systems consisting of an aqueous phase, an oil phase and surfactants with or without co-surfactant, which are prepared by phase inversion, ultrasonication, microfluidics, or high-pressure homogenization.[99,100] Multiple studies have demonstrated that nanoemulsions can effectively achieve brain-targeted drug delivery *via* nasal administration. In addition to the physiological state of the nasal mucosa, this process is influenced by several factors, including the size and zeta potential of the nanoemulsions. The droplet size primarily affects the penetration of particles through the nasal cavity and their clearance time within the nasal cavity.[100] Particles smaller than 200 nm are less likely to be cleared from the nasal cavity, thereby retaining a longer residence time and higher drug absorption.[101] The zeta potential impacts the stability of the colloidal system and its adhesion to the nasal mucosa. For instance, a zeta potential exceeding ±30 mV can form a stable electrostatic system,[102] and positively charged nanoemulsions exhibit better adhesion to the negatively charged nasal mucosa.[103]

For AD therapy, intranasal administration of nanoemulsions containing curcumin,[104,105] huperzine A,[106] nimodipine,[107] and resveratrol[108] has been employed to reduce the production, aggregation and deposition of Aβ in mouse model. These small molecule nanoemulsions exhibit high mucosal permeability in both *in vivo* and *in vitro* studies, with drug concentrations in the brain after nasal administration surpassing those achieved through intravenous administration. Additionally, these nanoemulsions possess similar surface modifiability. Mucosal adhesive polymers, such as chitosan, can significantly enhance flux and permeation across the nasal mucosa. Although the application of nanoemulsions for macromolecular drugs is relatively rare, there has been a study utilizing intranasal cationic nanoemulsions to encapsulate siRNA for the knockdown of TNFα mRNA. This approach aimed at anti-inflammatory therapy in neurodegenerative disease-affected brains, achieving higher siRNA uptake in the brain than pure siRNA administration.[109]

5.1.2.4. Exosomes. Exosomes, naturally occurring lipid bilayer nanovesicles, are generated within the endosomal compartments of most eukaryotic cells and subsequently secreted upon fusion with the cell membrane. They are widely recognized to participate in intercellular communication as well as various physiological and pathological processes. Exosomes often contain a repertoire of bioactive molecules of cellular origin, such as proteins, nucleic acids, and lipids, endowing them with significant advantages in clinical diagnostics. While the diverse biomolecular cargoes can serve as biomarkers to indicate pathophysiological states, this is outside the focus of this review. However, the innate biocompatibility, stability, low immunogenicity, and membrane-penetrating capacity of exosomes underscore their outstanding potential as natural drug nanocarriers.[110] Although similar to liposomes, exosomes are more complex. Their lipid composition originates from many cellular components, and these can impart higher biocompatibility and circulation time. Moreover, their lipid bilayers are highly asymmetrical and enriched with non-lamellar forming lipids,[111] which enhances their interaction with target cells and facilitates drug delivery. Additionally, the presence of various integral and peripheral membrane proteins in exosomes provides further functionality.

Research on exosomes is mainly constrained by challenges in isolation, purification, and drug-loading processes. As a biologically derived-nanovesicle, the extraction and purification of exosomes relies on purification techniques such as ultracentrifugation, ultrafiltration, immunoaffinity, polymer precipitation, microfluidics, and size-exclusion chromatography. These techniques pose challenges for the widespread industrial production of exosomes. Moreover, the drug-loading process of exosomes is also relatively complex. Pre-isolation cargo loading involves pre-transfecting drugs into the origin cells of exosomes to induce the secretion of drug-loaded exosomes. This method is only applicable to loading nucleic acids and protein drugs, but the loading efficiency has often been shown to be unpredictable, posing additional difficulties for the end-application. Post-isolation loading methods include co-incubation, electroporation, extrusion, sonication, and freeze–thaw cycles.[112,113] However, each of these methods has its advantages and disadvantages, and the specific choice depends on practical use. Co-incubation and freeze–thaw cycles are simple operations but are limited by low drug loading efficiency or difficulty in control and may lead to extracellular vesicle aggregation. Although sonication and extrusion methods have relatively high drug loading efficiency, they may affect the structural integrity of extracellular vesicles. Electroporation, while performing well in terms of drug loading efficiency, may still cause aggregation of drugs such as charged therapeutic nucleic acids. However, this can be mitigated by adding protective agents such as citrate and EDTA.[114–116] Exosomes have also shown the high potentials of being utilized in intranasal delivery for brain disorders (Fig. 5)[117,118] and personalised medicine approaches.[119,120]

5.1.3. Surface modification of intranasal formulations. Surface modification of nanoparticles can improve targeting specificity, biocompatibility, formulation stability, and control of drug release. Over the past decade, various modifications have been developed, with polyethylene glycol (PEG) modification being the most commonly used. The PEGylation of nanoparticle surfaces helps to increase the hydrophilicity of nanoparticles, add steric hindrance, and enhance circulation time.[121,122] It protects particles from degradation,[123] and enhances nanoparticle brain uptake moderately by prolonging the blood-circulation time and slightly improving permeability.[89,124] Although drug circulation time may not be a major concern in brain-targeted intranasal delivery of nano-

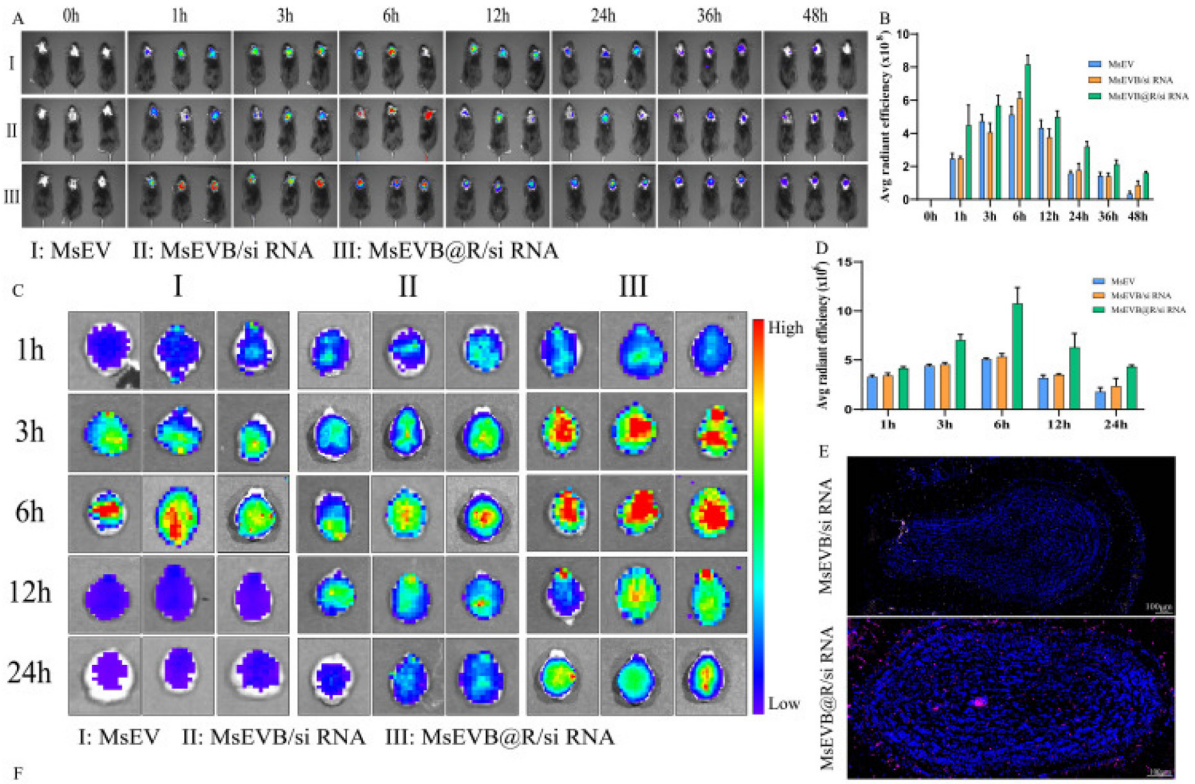

Fig. 5 (A–E) Intranasal delivery of BACE1 siRNA *via* engineered stem cell exosomes and distribution post-transport,[118] showing permeability improvement of BACE1 siRNA by engineered exosomes.

particle formulations, PEGylation remains common in CNS drug delivery. However, repeated administration of PEG has been shown to induce the production of PEG antibodies in the body, necessitating careful consideration of PEG's use in drug delivery systems. Additionally, other polymeric modifications have demonstrated similar functions, such as the surfactant polyoxymethylene sorbitan monooleate (Tween 80).

In non-intranasal brain-targeted formulations, the influence of the BBB cannot be ignored. Various ligands, peptides, small molecules, and antibodies are often conjugated to the surface of nanoparticles to enhance the uptake and targeting specificity of drug-loaded nanoparticles. These include Tf,[125] TfR antibodies, ApoE, RGD peptides, glutathione, transmembrane peptides, and glucose derivatives, among others. For intranasal delivery, although the use of ligand modifications to facilitate BBB crossing is unnecessary, cell-penetrating peptides (CPPs) can still enhance the transport of nanoparticles across cell membranes.[126] Some CPPs also possess additional brain-targeting properties, promoting the accumulation of modified nanoparticles in brain regions besides facilitating absorption, such as RVG peptides.[125,127–129]

In summary, although numerous nanoparticle systems have been developed for intranasal drug delivery, including polymeric nanoparticles, solid lipid nanoparticles, liposomes, and dendrimers, there is no single platform that can be considered categorically superior. This is because nanoparticle properties such as size, surface charge, mucoadhesion, and mucopenetration can be extensively tuned through material composition and surface modification. As a result, the optimal choice of nanoparticle depends on the characteristics of the therapeutic cargo, the target brain region, and the specific design goals of the delivery system.

5.2. Non-NP based formulations/approaches

5.2.1. Viral vectors and gene therapies. Gene therapy is based on the delivery of therapeutic nucleic acids into target cells or tissues. Delivery vectors can be broadly classified into viral and non-viral vectors, such as LNPs or exosomes with their respective advantages and challenges. Non-viral vectors generally elicit a lower immune response and can carry larger genetic loads, while viral vectors have higher transport efficiency and specificity.[130] Recombinant adeno-associated viruses (rAAVs) are currently the main viral vehicle used for gene delivery to the CNS due to their safety and neurotropism.[131,132] AAV is regarded as a non-pathogenic DNA virus for humans.[133] Thirteen different primate serotypes of AAV (AAV1–13) have been identified, along with numerous AAV pseudotypes that combine different serotype capsids and genomes, displaying varied cell tropisms.[134] Among these, AAV1, AAV2, AAV5, AAV8, AAV9, and the rhesus monkey isolate AAVrh.10 have been investigated and shown efficacy in transducing neurons.[135,136]

AAV has been widely used in the therapy of brain disorders through various administration routes, including intravenous administration, stereotaxic intraparenchymal injection, ICV injection, intracisternal and intralumbar injection, and intranasal administration.[137] These administration routes exhibit different shortcomings, such as the difficulty of repeated administration in ICV and the challenges and trauma associated with brain injections. In contrast, intranasal delivery stands out due to its non-invasive drug delivery advantages. Intranasal administration of AAV encoding the Aβ gene to transgenic mice overexpressing a mutant APP led to the accumulation of anti-Aβ immunoglobulins, resulting in reduced Aβ deposits and improved behavioural performance in the mice.[138] In another study addressing dysregulated cerebral calcium balance in AD treatment, intranasal administration of an AAV2 encoding the calcium channel-binding domain 3 reduced plaque accumulation and hippocampal cell apoptosis, leading to improved cognitive function in AD mice.[139] Similarly, for other brain disorders, such as mucopolysaccharidosis type I (MPS I),[140] α-L-iduronidase (IDUA)-deficient[140,141] and brain-derived neurotrophic factor (BNDF) application in depression prevention,[142] AAV-mediated intranasal administration has also shown significant therapeutic potential.

5.2.2. Hydrogels. Hydrogels are three-dimensional, hydrophilic polymer networks capable of retaining a large amount of water and storing biological fluids. Their swelling behaviour and unique structure contribute to their water-holding capacity and biological properties.[143] Hydrogels can be categorized based on their origins, composition,[144] configuration, and crosslinking.[145] For intranasal brain delivery, hydrogels can be divided according to their drug-loading, including hydrogels that are directly loaded with free drugs and hydrogels that are loaded with drugs *via* nanocarriers.[146] Drug molecules are typically loaded into hydrogels to extend mucosal retention time, enhance the effectiveness of intranasal uptake, and protect against chemical and enzymatic degradation in the nasal passages.[147] For instance, D-penicillamine was loaded into natural hydrogel consisting of chitosan with β-glycerophosphate for *in vivo* studies in AD mice.[148] Similarly, hydrogels prepared with gelatin and hydroxypropyl methylcellulose (HPMC) have been utilized to encapsulate rivastigmine tartrate for AD treatment.[149]

Considering the co-application of hydrogels and nanocarriers, drug-loaded nanoparticles can be encapsulated into hydrogels to combine the advantages of these two delivery systems. Hydrogels can increase the mucociliary retention time and improve nanoparticle absorption through the nasal epithelium,[146] and retain the advantage of cell or tissue targeting of nanoparticles. For instance, curcumin was encapsulated into silica nanoparticles and then loaded into poloxamer 407 and HPMC hydrogel for intranasal delivery of AD.[150] In another research, a ^{32}P-siRNA was loaded in poly-amidoamine dendrimers and then loaded into hydrogel consisting of chitosan in poloxamer for the treatment of AD and brain tumours.[151]

Overall, hydrogels are promising mucosal drug delivery systems, as they can significantly enhance drug adsorption and penetration at the mucosal site and help protect drugs from chemical and enzymatic metabolism. Nasal mucosal hydrogels can be designed as thermosensitive, pH-sensitive, and ion-responsive hydrogels based on the nasal cavity's low pH (5.5–6.5), temperature (32 °C), and ion environment (sodium, calcium, and potassium ions).[152,153] These features indicate that hydrogels are promising for further development in nasal drug delivery.

5.3. Excipients for intranasal formulations

The physicochemical properties of drugs and their distribution within the nasal cavity largely determine their efficiency of nasal absorption. Therefore, besides modifying drug structure and physicochemical properties, strategies aimed at enhancing the bioavailability of drugs in the nasal mucosa mainly focus on improving drug stability and increasing nasal residence time and nasal absorption. These strategies include the use of absorption enhancers, mucoadhesive agents, nasal enzyme inhibitors, solubilisers and buffers, amongst others.[154]

5.3.1. Enzymatic inhibitors. Nasal enzyme inhibitors are primarily used for drugs metabolized nasally to reduce drug loss during nasal delivery, which can be pre-administrated or co-administrated with drugs.[155] Enzyme inhibitors for protein and peptide drugs often include pancreatin and aminopeptidase. Some common pharmacological inhibitors of P-glycoprotein (P-gp) can also be utilized to enhance the absorption of drug molecules, such as amiodarone[156] and clarithromycin.[157]

5.3.2. Absorption enhancers. Absorption enhancers are primarily used to enhance the absorption of therapy agents.[158] Ideally, absorption enhancers can reversibly reduce mucin viscosity or elasticity without causing nasal mucosal damage or permanent alteration.[159] They also can reduce mucociliary clearance, widen tight junctions,[160] or dissolve and stabilize drug molecules when enhancing absorption is necessary. Typical chemical agents that enhance permeation include surfactants, bile salts, chelating agents, and fatty acid salts.[161]

5.3.3. Mucoadhesive agents. Mucoadhesive agents can be used to increase the residence time of drugs in the nasal cavity, thereby enhancing drug absorption. They enhance drug retention in the nasal cavity by generating adhesive forces between the formulation and nasal mucosa, thereby reducing the rate of mucociliary clearance for the formulation.

Chitosan is a commonly used excipient in intranasal formulations and exhibits highly functional properties in the design of intranasal delivery nanoparticles. It can interact electrostatically with the negatively charged surface of nasal epithelial cells,[162] prolonging the nasal clearance time of modified nanoparticles while increasing the uptake of drug-loaded nanoparticles.[163] For example, chitosan hydrogels have been used for Alzheimer's disease drug delivery.[164] Furthermore, the high hydrophilicity of chitosan can lead to its absorption of moisture from nasal mucosa upon contact, causing swelling and providing a larger surface area for drug-loaded nanoparticles to traverse.[165,166] Overall, chitosan, as a polymer, can be used alone for synthesizing nanoparticles, but it is more commonly

used in combination with other functionalized nanoparticles to achieve more efficient drug delivery to the brain.

The remaining components that are not specific for intranasal delivery such as solvents, preservatives, antioxidants, buffers, and moisturizers, among others, will not be elaborated on here.

6. Efficacy evaluation of intranasal formulations targeting brain

Various approaches have been developed to investigate the intranasal administration efficacy of intranasal formulations. The two significant aspects of *in vitro* and *in vivo* to determine the diffusion of drug-encapsulated formulations are discussed below.

6.1. *In vitro* diffusion studies

Concerning research on intranasal delivery to the brain, the efficiency of trans-mucosal delivery needs to be investigated and optimized. Due to ethical and material constraints, researchers have developed various nasal mucosa models as alternatives to human nasal mucosa. These include *in vitro* cell models such as RPMI 2650 cells, *ex vivo* animal nasal mucosa such as *ex vivo* porcine nasal mucosa, and *in vivo* animal models such as mice or rats.

6.1.1. *In vitro* nasal cell model. Building upon the physiological principles discussed above, *in vitro* nasal mucosa models are used to investigate the interaction between drugs and the nasal mucosa. These models are instrumental in assessing drug permeability across the nasal mucosa and drug cytotoxicity toward this vital barrier. They are designed to replicate the intricate structure and functions of the nasal mucosa and offer unique advantages in pharmaceutical research and development. They are straightforward to establish and maintain, providing a platform for the investigation of drug absorption, permeability, metabolism, and nasal cavity toxicity. Moreover, they offer a solution to ethical concerns related to animal testing when compared to *in vivo* nasal mucosa tests.

Typically, these models comprise cultured nasal epithelial cells that form a barrier closely resembling the *in vivo* nasal mucosa, as described in Fig. 6. Various cell lines are employed, including 16HBE14o cells, Calu-3 cells and RPMI 2650 cells,[167] which have demonstrated relevance in the nasal mucosa research related to drug transport and permeability.[168–174] The creation of *in vitro* nasal mucosa models involves the cultivation of the chosen cell line using specialized growth and differentiation medium on permeable support, such as a Transwell insert. This setup establishes an air–liquid interface, allowing the cells to differentiate into a pseudostratified epithelium, such as RPMI 2650 cells, that closely mimics the nasal mucosa structure. RPMI 2650 cells cultured at the air–liquid interface have been reported to exhibit sufficient barrier properties, such as permeation coefficients and high transepithelial electrical resistance (TEER) values.[168,171,175] Compared to the liquid–liquid interface, a cell model established under an air–liquid interface system can provide a more representative study environment that closely resembles *in vivo* conditions.[176] In addition, to enhance the physiological relevance of these models regarding the nasal mucosa, researchers often incorporate factors such as mucus production, ciliary movement, and the presence of TJs between cells. Mucus production can be stimulated by the addition of substances such as mucin or cytokines, while ciliary movement can be induced through culture conditions and appropriate culture media supplementation.

In vitro nasal mucosa models furnish a controlled environment for the investigation of several factors related to intranasal drug delivery, encompassing drug absorption mechanisms, formulation optimization, drug metabolism, and toxicity assessment. They enable researchers to assess the efficacy, safety, and bioavailability of nasal drug formulations before progressing to *in vivo* studies. Moreover, these models contribute to an enhanced understanding of nasal mucosa biology, barrier function, and drug interactions, ultimately leading to improved nasal drug delivery strategies and therapeutic outcomes.

6.1.2. *Ex vivo* nasal tissue model. *Ex vivo* tissue models show several advantages over *in vitro* animal models, providing more details, such as the toxicity of drugs on the nasal mucosa, on interaction between drug-encapsulated formulations and nasal mucosa membrane. Besides, *ex vivo* models are readily accessible as they can be obtained from abattoirs and have been previously validated their suitability and potential for clinical applications.[177] One subject can also offer numerous tissue samples,[178] which presents time and cost gains over other models. However, additional and careful attention should be given to the reproducible collection and preservation of animal tissues.

Various kinds of animal nasal mucosa tissues are used in nose-to-brain delivery research. Bovine nasal tissue was collected to evaluate the permeability and tissue toxicity of the self-emulsifying drug delivery systems.[179] Permeation and ciliotoxicity studies of thiolated chitosan complexes have also been conducted with caprine nasal tissue.[180] Nasal mucosa from porcine,[181] leporine[182] and ovine[183,184] tissues are used to evaluate the permeation and toxicity of the drug-loaded formulations.

In conclusion, as illustrated in Fig. 6, nasal mucosa models —both *in vitro* and *ex vivo*—are essential tools for intranasal delivery studies, as they provide accessible and controllable platforms representative of human nasal physiology. While these models cannot fully replicate the complex dynamics of the *in vivo* environment, such as blood flow, mucociliary clearance, neural connectivity, and systemic absorption, they offer critical insights into mucosal permeability, formulation–tissue interactions, and potential transport mechanisms. Specifically, *in vitro* epithelial models allow for quantification of passive diffusion (*e.g.*, Papp), TEER, and TJ modulation, while *ex vivo* nasal tissues preserve native architecture and mucus layers for more physiologically relevant screening. Coupling these data with *in vivo* pharmacokinetic and biodistribution studies

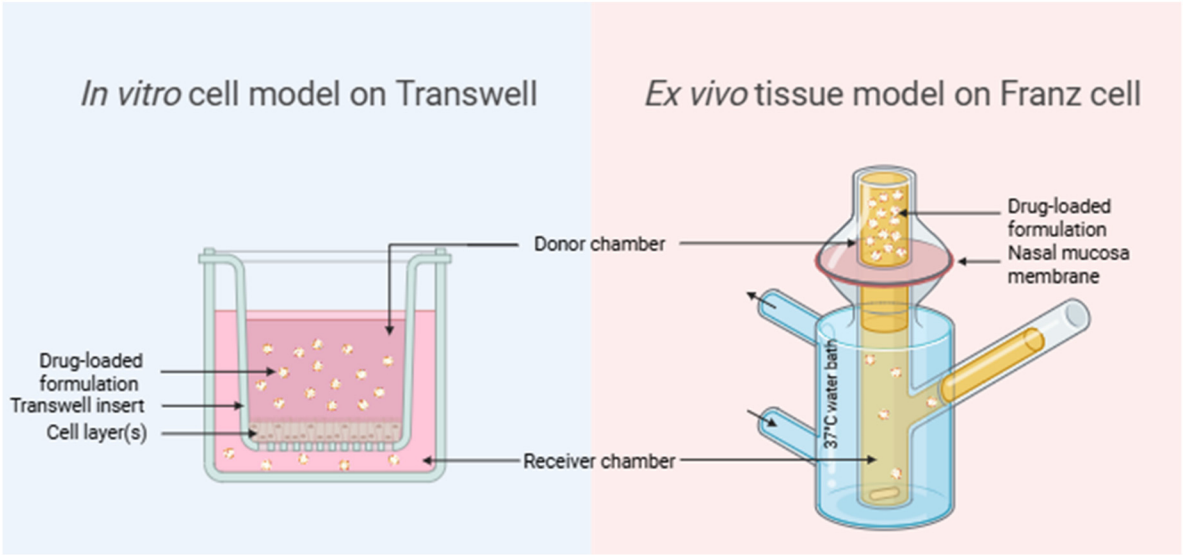

Fig. 6 *In vitro* or *ex vivo* permeability study of drug-loaded formulations utilizing Transwell inserts with epithelial cells or Franz diffusion cells (left) with *ex vivo* nasal mucosa membrane (right).

enables researchers to improve the translational relevance of their delivery systems. Therefore, these models remain pivotal in the preclinical development and optimization of intranasal-to-brain targeting platforms and should not be overlooked in this field.

6.2. *In vivo* intranasal absorption studies

Compared to *in vitro* cell models and *ex vivo* tissue models, *in vivo* animal models provide a more realistic representation of the complex physiological environment. These methods offer insights into the local metabolism, clearance mechanisms, and toxicity of formulations, aiding in the understanding of how substances are processed within the nasal mucosa.[167] However, in comparison to cell and tissue models, they require additional ethical considerations related to animal use and are influenced by interindividual animal differences such as age, diet, and pathology.[185]

Regarding the *in vivo* animal models for brain targeting research, rats and mice are widely used for intranasal administration studies. For instance, mice are used in research on insulin delivery to the brain to evaluate transport efficiency,[186] and rats are involved in studies of morphine administration.[187] There are also applications of other mammals, such as monkeys[188] and sheep.[189]

The efficacy of brain-targeted intranasal delivery is typically investigated *in vivo* through classical pharmacokinetic studies, which involve the isolation of brain tissues, olfactory bulbs, and other relevant tissues after a certain administration period. Subsequently, appropriate analytical techniques are employed to quantitatively assess the targeting efficiency of the drug-encapsulated formulations. In addition to conventional pharmacokinetic methods, there are also supplementary techniques available for evaluating the delivery efficiency (Table 1).

6.3. Quantitative and imaging analysis

The evaluation of intranasal delivery systems relies heavily on advanced quantitative and imaging techniques to assess the biodistribution, absorption, and efficacy of biologics. These methods provide critical insights into the behaviour of drug formulations within biological systems, enabling researchers to track the delivery pathway, measure drug concentrations, and visualize tissue-specific localization. Among these techniques, fluorescence imaging and radiological labelling have emerged as powerful tools to quantify and visualise biologics' distribution in real time and at high resolution *in vivo* or *in vitro*.

6.3.1. Fluorescent labelling. Fluorescent labelling has been widely developed in drug delivery research, commonly used to monitor the biodistribution of drug-encapsulated formulations, bio-imaging, and transport pathway research. It can also be used to investigate the intranasal administration efficacy for brain-targeted intranasal formulations. Fluorescent molecules can be linked to the drug-encapsulated formulations through covalent conjugation, noncovalent bonds, or co-encapsulation, then transported into the brain with the formulations after intranasal administration, which provides opportunities for *in vivo* imaging and fluorescent analysis of relevant tissues.

Fluorescent molecules can often be covalently linked to nucleic acid molecules such as siRNAs and ASOs. This reaction can occur at the 5′-end, 3′-end or in the middle of the sequence, with fluorescent tags such as FAM, FITC, CY3 and Cy5. For siRNA and non-sterically hindered ASO, however, this modification mostly occurs at the ends of the sequence as it offers a simple and easily adapted alternative.[196] A study has labelled siRNA with DyLight 647 fluorescent dye (DY647) and then administered it through the nasal cavity to investigate the

Table 1 Recent advances in intranasal delivery of drugs for central nervous system (CNS) disorders: models used and key therapeutic outcomes

Payload	Formulation	Intranasal model	Source	Outcome	Ref.
Theophylline, antipyrine, antipyrine, *etc.*	Solution	*In vitro* nasal cell model	RPMI 2650 cell line	RPMI 2650 cell model grown at A-L interface has shown superior differentiation between high permeability model drugs and low permeability model drugs.	190
Clonazepam	*In situ* gel		Caco-2 cell line	Reduced drug cytotoxicity and improved clonazepam permeability.	191
HLS-3	HBSS		Calu-3 cell line	Intranasally delivered HLS-3 exhibited significant higher central cholinergic mediated responses without obvious peripheral side effect.	192
Centella	Thiolated chitosan	*Ex vivo* tissue model	Caprine excised nasal tissue	The affinity of thiolated chitosan for receptors associated with BBB confirms its potential to cross the BBB and attain therapeutic concentration in the brain.	180
Dimenhydrinate	Self-emulsifying drug delivery system		Bovine excised nasal tissue	Improved drug solubility and an enhanced *ex vivo* permeation compared to the control.	179
Nile Red	PLGA nanoparticle			The smaller diameter nanoparticles were transferred more compared with the larger nanoparticles.	193
Insulin	Solution		Rabbit excised nasal tissue	Didecanoyl-L-α-phosphatidylcholine significantly enhanced the paracellular transport of insulin across rabbit nasal mucosa *in vitro*.	194
IgG	Solution (medium w/o FBS)		Porcine excised nasal tissue	A role for Fc receptor (FcRn) and Fc-gamma receptor2 (FCGR2) in modulating IgG transport and degradation in nasal mucosa.	195
Insulin	CPP conjugated liposome			CPP functionalized liposome improved insulin permeability transport through nasal mucosa.	181
Morphine	Solution	*In vivo* animal model	Mice and rats	Morphine was transferred along the olfactory pathway to the CNS.	187
Opioid	Solution		Monkey	A trend for opioids to have a faster onset of action when given intranasally.	188
Scrapie strain	Solution		Sheep	Well-established sheep scrapie model.	189

route of siRNA transported into the brain through the nose in mice. It revealed that the fluorescently labelled siRNA was transported and observed from the nasal cavity to the olfactory bulb through the olfactory nerve pathway 30 minutes after administration.[62]

In addition to covalent conjugation, fluorescent molecules are typically combined with drugs or formulations through non-covalent methods, including adsorption, electrostatic interactions, hydrophobic interactions, host–guest interactions[197] and co-encapsulation. These combinations might be reversible and would be affected by water solubility and some intermolecular interactions among drugs, formulations, and the body's internal environment. For example, two lipophilic fluorescent molecules, 6-coumarin and rhodamine B, was encapsulated into the lipid layers of lipid nanoparticles for labelling but showed different biodistribution in rats after intranasal administration. Most of 6-coumarin was distributed and accumulated in the brain due to its water insolubility and retention in the lipid nanoparticles, while rhodamine B is partially soluble in water and was promptly released in the nasal mucosa.[198] Compared to radiolabelling or other bioimaging techniques, fluorescent labelling offers advantages such as low toxicity, minimal equipment requirements, simple operation, and ease of observation. One study has developed a series of near-infrared fluorescent diaza-indacene (BODIPY) and aza-BODIPY dyes as fluorescent labels to investigate the nose-to-brain pathway in SD rats, detailing the transport routes *via* the trigeminal nerve and olfactory bulb pathways into the brain, as well as the impact of particle size on clearance and transport.[199]

In summary, fluorescent labelling of intranasal formulations is an extremely sensitive and specific technique, which allows multi-tag labelling and visualized real-time monitoring. However, it presents challenges for long-term imaging and demands delicate design, as the ideal coupling between fluorescent molecules and formulations must be stable without compromising the functionality of the drug-encapsulated formulations.[197] Fluorescent imaging is also hampered by the limited tissue depth penetration, restricting applications to near surface-level and/or *ex vivo* imaging.

6.3.2. Radioisotope labelling and PET imaging. Nuclear imaging refers to a technology that uses radioactive isotopes as label signals to study biological processes in the body. Similar to fluorescent labelling, by tracking the changes and movements of radioactive signals, the transport pathways and metabolic processes of nanomaterials in organisms can be investigated. Unlike fluorescent imaging, however, the ionizing gamma radiation involved in nuclear imaging can penetrate through tissue, allowing full imaging throughout the body. Radioisotope labelling can be divided into self-labelling and marker labelling. Self-labelling means that the chemical components of the nanomaterials themselves carry radioactive labels when they are initially synthesized, which can produce stable radioactive nanoparticles simply and directly, but it is mostly used in inorganic nanomaterials since the radioactive

isotopes need to be embedded in the lattice of nanocrystals stably in the process of radioactive synthesis, such as ^{14}C-labelled graphene[200,201] and ^{64}Cu-labelled copper sulfidic nanoparticles.[202] Instead, marker labelling uses radioactive label carriers to label the target nanoparticles by covalent binding, chelating adsorption and chemical adsorption, such as ^{64}Cu and ^{89}Zr, which can be easily attached to the surface of nanoparticles through chelating agents.[203]

To evaluate the biodistribution and kinetics of therapeutics delivered intranasally to the brain, positron emission tomography (PET) has emerged as a powerful nuclear imaging tool, offering sensitive and quantitative insights into *in vivo* transport mechanisms. PET is a nuclear imaging technique that utilizes positron-emitting radioisotopes to visualize and quantify biological processes *in vivo* with high sensitivity and deep tissue penetration. The underlying mechanism involves the annihilation of positrons, emitted from the radiotracer, with electrons in the tissue, producing two 511 keV gamma photons emitted in opposite directions. These photons are detected in coincidence by the PET scanner, enabling precise three-dimensional localization of tracer accumulation.[204] PET imaging has been applied in preclinical studies of nose-to-brain delivery.[205,206]

PET can also be used to indirectly measure drug delivery, such as by monitoring drug-induced regional metabolic changes. The most commonly used radiotracer for this approach is ^{18}F-fluorodeoxyglucose (^{18}F-FDG), a clinically approved glucose analogue that enables real-time mapping of metabolic activity in the brain and other organs. For example, the uptake and distribution of ^{18}F-FDG in the nasal passages and brain regions can be visualized using PET alone or in combination with anatomical imaging parietal regions.[205,207,208] Furthermore, PET has been used to evaluate cerebral glucose metabolism after intranasal administration of insulin, offering insights into therapeutic responses in Alzheimer's disease models.[209,210]

6.3.3. MRI imaging. Magnetic resonance imaging (MRI) complements PET by providing high-resolution anatomical information, making it a valuable modality for assessing the spatial localization of intranasally administered therapeutics within brain structures. MRI is a non-ionizing imaging modality that leverages strong magnetic fields and radiofrequency pulses to generate high-resolution anatomical images. It is particularly valuable for brain imaging due to its superior spatial resolution and soft-tissue contrast. Unlike PET, MRI does not require radioactive tracers. Instead, it typically relies on the relaxation properties of hydrogen protons in water molecules, which are influenced by tissue composition and, in some cases, by contrast agents. Upon the application of a radiofrequency pulse, these spins are deflected and subsequently relax back to the original state,[211] resulting in the generation of two relaxation parameters known as T_1 and T_2 relaxation times. MRI contrast agents are capable of shortening these relaxation times separately or simultaneously, thus, resulting in contrast in the corresponding weighted images. However, contrast-free MRI remains the standard in many clinical and preclinical applications.

In intranasal delivery to the brain, MRI serves to precisely localize therapeutic nanoparticles and cellular therapies. For instance, in an animal model of glioblastoma, mesenchymal stem cells encapsulated with micron-sized paramagnetic iron oxides (MPIOs; a common T_2 contrast agent) were tracked using MRI 24 hours after intranasal administration.[212] Similarly, in a mouse study, Gd^{3+} (clinically used in T_1 contrast agents) was employed to label a type of cholera toxin B subunit-derived nanoparticle, which was subsequently observed in the hippocampus 1 hour after intranasal administration.[213] In another study focusing on intranasal insulin for memory effect for AD treatment, subregional brain MRI volumes of some brain regions were higher in patients who demonstrated memory improvement when compared to normal patients, including the hippocampus, superior frontal, cuneus, middle cingulum, and parietal regions.[207] While used in 1/3 of clinical MR scans, for tracking delivery MRI contrast agents can suffer from a lack of sensitive, with orders of magnitude differences in detection limits when compared to PET tracers (Fig. 7).

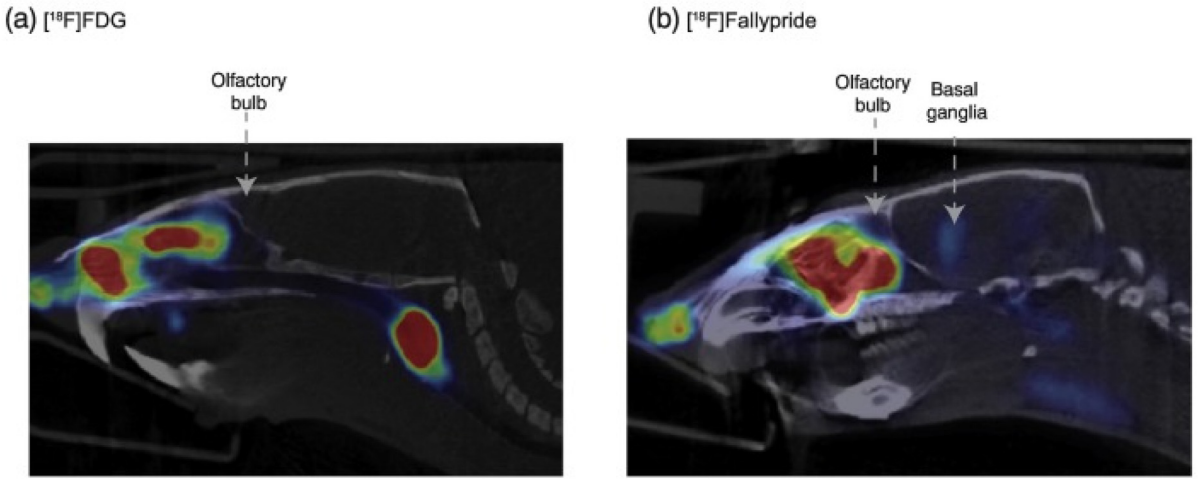

Fig. 7 PET/MRI scanning of lateral view of the brain of mice after intranasal administration of (a) [^{18}F] FDG and (b) [^{18}F] Fallypride.[205]

The integration of PET and MRI into a single hybrid imaging platform can overcome these issues and enables simultaneous acquisition of functional and anatomical data. In PET/MRI systems, PET provides sensitive, quantitative tracking of radiolabelled compounds, while MRI provides high-resolution anatomical context for co-registration and precise spatial without additional radiation exposure. This combination allows for more precise interpretation of tracer distribution and therapeutic targeting in the brain is particularly useful for evaluating complex delivery routes like the nose-to-brain pathway.[205]

In summary, the combined use of PET and MRI holds significant promise for evaluating the nose-to-brain drug delivery pathway as it merges the precise quantification and sensitivity of molecular imaging with the excellent tissue contrast and spatial resolution of MRI. Despite its potential, this dual-modality approach has been utilized in only a limited number of studies so far, which aim to enhance our fundamental understanding of *in vivo* biological processes and underscore the importance and potential in biomedical research and clinical applications.

7. Summary and prospects

In contrast to traditional administration routes, intranasal delivery is characterized by its requirement for a smaller dosage and capacity to bypass the BBB, allowing direct targeting of the brain. The feature eliminates first-pass metabolism and reduces systemic side effects. The need for lower doses aligns with the characteristics of gene therapy, which often demonstrates high activity and efficacy at low doses. Owing to the low-dose, high-efficiency nature of gene therapy, this approach is a promising candidate for intranasal drug delivery, further benefiting from bypassing systemic circulation and the BBB. This administration route safeguards biologics from enzymatic degradation in the bloodstream and significantly enhances their concentration in the CNS.

For brain targeting delivery, intranasal drug delivery stands apart from conventional administration routes, such as intravenous and subcutaneous delivery. While numerous studies have explored intranasal delivery for brain disorders or systemic diseases, yielding general principles, its full potential for AD treatment and the intricate relationship between them remain incompletely understood. AD, as one of the most intricate brain disorders, presents a range of therapeutic targets throughout its progression. The dynamic interplay among gene therapy, intranasal delivery, and these potential targets reveal substantial knowledge gaps, underscoring the promising prospects of intranasal gene therapy in the context of AD treatment. Currently, two leading hypotheses regarding the origin of AD are the tau protein hypothesis and the Aβ hypothesis. These hypotheses have evolved from the discovery of amyloid-like proteins and tau NFTs in the brains of AD patients. Numerous enzymes and proteins involved in pathways related to the Aβ and tau proteins, such as BACE1, have the potential to become therapeutic targets for AD. Additionally, various genes associated with AD, such as ApoE4[214] and TRIM11,[215] participate in the metabolism of Aβ and tau through diverse mechanisms.

Despite advancements in understanding the interplay among various genes and proteins in AD progression, particularly interactions among Aβ, tau, and these proteins, considerable knowledge gaps persist. The development of brain proteomics in AD patients has unveiled numerous potential therapeutic targets and the potential of individualized gene or protein therapy. However, due to the large molecular weight of biologics, challenges in penetrating biological barriers, and the susceptibility to degradation in the systemic circulation, there are currently very few clinically effective biologics available for AD treatment. Even the limited number of mAbs that have received FDA approval or are undergoing clinical trials exhibit significant side effects and restrictions. Consequently, the development of intranasal biologics for AD treatment is highly needed, albeit challenging. Several challenges must be addressed to establish a more effective intranasal biologic delivery strategy for AD treatment with higher clinical translational potential.

First, further research into potential gene or protein targets related to AD is essential, particularly regarding the complex interplay between these targets and known AD-related signalling or metabolic pathways, such as ApoE4 and TRIM11.

Then, while the advantages of intranasal delivery for biologics are appealing, overcoming its limitations is necessary by the development of suitable and effective drug carriers. Whether it is widely used liposomes or various polymer nanoparticles, exosomes, viral vectors, and other carriers, their roles, functional principles, toxicity, and delivery mechanisms in intranasal delivery require further exploration.

Third, although the physiological structure and pathways to reach the CNS of intranasal delivery have been specified in numerous studies, there is limited research that specifically examined biologics and their carriers. The interactions of biologics and their carriers with different cells during intranasal delivery and potential factors, such as molecular weight, surface charge and hydrophilicity of carriers, which may influence the rate of intranasal distribution after administration, as well as their biodistribution and bioimaging after entering the brain, warrant further exploration to unveil potential biological functions and applications.

Last but not least, in the field of intranasal biologic delivery, insights from fragmented research need to be consolidated and summarized. Although many studies have pointed out several aspects that need attention in intranasal delivery, metabolism of biologic drugs in the brain is not yet a mature field due to the high complexity of brain's physiological environment and CNS disease mechanisms. Some small molecular drugs and endogenous neurochemicals can be metabolized through brain cytochrome P450 enzymes (CYPs), such as antidepressants, antipsychotics, and dopamine, but brain metabolism of biologics remains consideration. Lymphatic pathway is also involved in clearance of drugs in the brain

through the CSF transports harmful metabolites into deep cervical lymph nodes from the brain. Hence, many practical situations require researchers to design and further develop based on the nature of the actual biologics, formulations, physiologic and pathologic factors.

In summary, exploring intranasal gene therapy for AD is a promising avenue, but many questions and challenges remain. A deeper understanding of the genetic and protein targets involved in AD, improved intranasal formulations, investigations into cellular interactions, and consolidations of research findings are essential for advancing this field.

Conflicts of interest

There are no conflicts to declare.

Abbreviations

Aβ	Amyloid-beta
AD	Alzheimer's disease
Ajs	Adherence junctions
ARIA-E	Amyloid-associated imaging abnormality oedema
ARIA-H	Amyloid-associated imaging abnormality haemorrhage
ASOs	Antisense oligonucleotides
BBB	Blood–brain barrier
BTB	Blood-tumour barrier
BNDF	Brain-derived neurotrophic factor
CDP	Cystine-dense peptide
CDX	Candoxin
CNS	Central nervous system
CPP	Cell-penetrating peptides
CSF	Cerebrospinal fluid
CYPs	Cytochrome P450 enzymes
DY647	DyLight 647
EGF	Epidermal growth factor
EPR	Enhanced permeability and retention
HPMC	Hydroxypropyl methylcellulose
ICV	Intracerebroventricular
IDUA	α-L-Iduronidase
JAMs	Junctional adhesion molecules
IgG	Immunoglobulin G
LNPs	Lipid nanoparticles
mAbs	Monoclonal antibodies
MPIOs	Micron-sized paramagnetic iron oxides
MPS I	Mucopolysaccharidosis type I
MRI	Magnetic resonance imaging
NGF	Nerve growth factor
NLC	Nanostructured lipid carriers
ONFs	Olfactory nerve fibres
PCL	Polycaprolactone
PEG	Polyethylene glycol
PET	Positron emission tomography
PNPs	Polymeric nanoparticles
P-gp	P-glycoprotein
rAAVs	Recombinant adeno-associated viruses
RVG	Rabies virus glycoprotein
SLN	Solid lipid nanoparticles
SPECT	Single-photon emission computed tomography
TEER	Transepithelial electrical resistance
Tf/TfR	Transferring/transferring receptor
Tjs	Tight junctions
TNFα	Tumour necrosis factor α
WGA-HRP	Wheat-germ agglutinin horseradish peroxidase

Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

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