Central Nervous System Delivery of Antibodies

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Antibodies, otherwise known as immunoglobulins (Igs), are glycoprotein molecules produced by plasma cells and are mostly found in blood and lymphoid tissues. The primary function of antibodies in vivo is to recognize and neutralize infectious agents, such as pathogenic bacteria and viruses. Antibodies are directed against various antigens and play a pivotal role in the defense mechanism of higher vertebrates and are also involved in autoimmune diseases and allergies.

intranasal delivery scaffolds VHH VNAR Fc receptor mucosal transport

1. General Overview on Immunoglobulin Structures

Antibodies, otherwise known as immunoglobulins (Igs), are glycoprotein molecules produced by plasma cells and are mostly found in blood and lymphoid tissues. The primary function of antibodies in vivo is to recognize and neutralize infectious agents, such as pathogenic bacteria and viruses. Antibodies are directed against various antigens and play a pivotal role in the defense mechanism of higher vertebrates and are also involved in autoimmune diseases and allergies. They are well-characterized molecules because of their considerable use in research, diagnostics and therapy [1]. Antibody-based therapy, with currently more than 100 approved monoclonal-derived products, has emerged as a class of novel therapeutics for various diseases [2]. This type of therapy has grown to become the dominant product class within the biopharmaceutical market and their market is still rapidly growing. Thanks to antibody engineering techniques, it is now possible to generate a specific antibody against almost any protein or peptide antigen [3][4]. Hence, antibodies can be used as therapy for many human disorders, including central nervous system (CNS) diseases, such as Parkinson's disease [5], multiple sclerosis [6], amyotrophic lateral sclerosis [7] and Alzheimer's dementia [8][9].

Igs are Y-shaped molecules with a distinct proteolytic fragmenting pattern: after an IgG antibody is digested with the papain protease, for instance, it is cleaved into two distinct fragments: (i) a fragment antigen binding (Fab) with around 45 kDa and (ii) the crystallizable fragment (Fc) with approximately 50 kDa [10]. IgG antibodies consist of two identical light chains (LC) and two identical heavy chains (HC). Each HC and LC consists of two regions (**Figure 1**a) [11][12]: the variable (V) region and the constant (C) region which are located at the N-terminus and the C-terminus of the antibody molecule, respectively [13]. In humans, the HC consists of a variable (VH) domain and up to four constant domains (CH). The HCs are stabilized by varying numbers of disulfide bonds at the so-called hinge region. The hinge region is located between the first and second constant domain of heavy chains (CH) and is responsible for the flexibility of the two fragment-antigen-binding arms of antibodies.

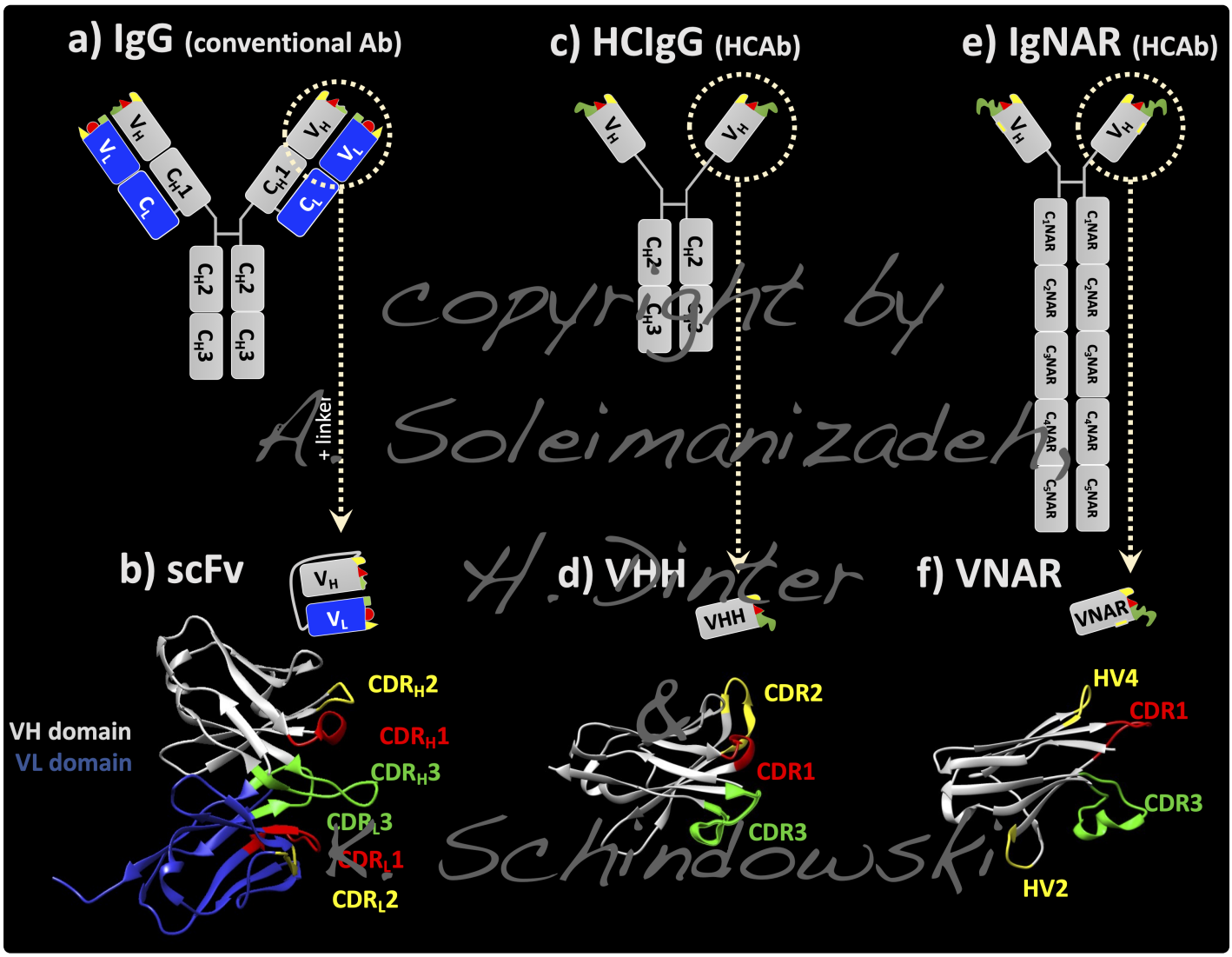

Figure 1. Schematic representation of scaffolds derived from human IgG or heavy-chain-only antibodies (HCAbs). The variable fragment (Fv) of a conventional IgG (**a**) can be fused with a polypeptide linker to form an scFv (**b**). Isolated VH or VL domains can also be expressed as human-derived sdAbs. The isolated VH of a (**c**) camelid heavy chain IgG (HCIgG) is termed VHH (**d**) and has a more compact structure compared to a VH derived from conventional IgG. Although the shark immunoglobulin new antigen receptor (IgNAR) is the largest antibody (**e**), its isolated VH domain, VNAR (**f**), is the smallest sdAb and provides the most compact structure. Crystal structures of a representative (**b**) scFv (6S2I; [14]), (**d**) lama VHH domain (1I3V; [15]) and (**f**) VNAR (1SQ2; [16]) were retrieved from PBD. CDR regions were indicated with Kabat numbering using AbRSA tools [17]; indices in the scFv refer to HC and LC, respectively. The structures were visualized using UCSF Chimera software.

The variable domains of antibodies have an immunoglobulin fold, designating a protein domain structure, first discovered in immunoglobulin constant and variable domains, which consists of two β-sheets packed against each other [18]. The binding of these two domain sheets is facilitated via several disulfide bonds and hydrophobic interactions [19]. This constitution is essential for the function of antibodies through the formation of potential binding sites at the loops at the end of the structure. These hypervariable loops are called complementary determining regions (CDRs), which consist of hypervariable regions and appear in antibodies and T cell receptors.

The amino acid sequence in these loops is highly variable, which leads to the production of several antibodies that have the ability to bind to a variety of antigens [19]. Each variable region in the HC and LC domains has three CDRs between four framework (FR) sequences. The FR region with a highly conserved sequence plays an essential role in the conservation and stability of the structure of the domain antibody [20][21].

Most paratopic regions of an antibody molecule are located in the CDRs of VH and VL domains. Paratopic regions, or antigen-binding sites, refer to the parts of an antibody that recognize an antigen and bind to it. Each antigen binds to the antibody noncovalently through highly specific interactions. The affinity of the antibody refers to the strength of the reaction of a single epitope to a paratope. There are two identical antigen-binding sites on each full-length antibody molecule; thus, they can interact with two equivalent antigens at the same time. This increases the binding strength of the antigen–antibody complex. The resulting accumulated strength of multiple affinities is called avidity [22]. CDR3 is the most extended loop out of the other CDR loops and plays a paramount role in antigen binding [21]. The length of CDR3 exhibits vast diversity in different species [23][24].

2. CNS Drug Delivery with Full-Length IgG, scFv and sdAb

The delivery of drugs which can reach the CNS in sufficient concentrations is still a significant challenge in the treatment of CNS diseases [25]. One of the most selective barriers surrounding the CNS is the BBB formed by endothelial cells with tight junctions (TJ) and adherence junctions (AJ). The BBB does not only exclude large molecules; the FcRn expressed in the BBB and the blood–cerebrospinal fluid (CSF) barrier also contributes to the fast elimination of full-length IgGs from the brain [26]. Excluding immunological proteins, such as antibodies, from the brain is an important function of the BBB, preventing proinflammatory signs, e.g., swelling. Since the brain is surrounded by bony structures, swelling can lead to severe tissue damage. Therefore, the CNS is also referred to as an immune-privileged zone. Hence, smaller proteins, such as nanobodies and antibody derivatives without an Fc domain, should have a better potential for neurological and psychiatric indications [27].

Several drug delivery strategies have been introduced to cross the BBB: a paracellular route or passive diffusion by disruption of the BBB, a transcellular route by enhancing transcytosis using molecular BBB shuttles or direct delivery bypassing the BBB with intrathecal, intraventricular or intranasal delivery [28][29][30]. While nanobodies have been often discussed in relation to crossing the BBB, the number of experimental validations is rather low. However, the results of one study indicated that, following intravenous injection, two VHHs targeting extracellular amyloid deposits and intracellular tau neurofibrils bypassed the BBB in transgenic mouse models [27]. This is a promising achievement which could make the early-stage diagnosis of brain-related diseases, such as Alzheimer's disease, possible. Furthermore, a VHH fusion protein with green fluorescent protein was designed to target glial fibrillary acidic protein, a protein expressed on astrocytes. This VHH was able to cross the BBB in vivo and reached the astrocytes after intracarotid and intravenous injections [31]. The authors discussed the possibility that the basic isoelectric point of a VHH could play a role. More recently, in a study conducted by Miyashita et al., a VHH was delivered into cultured neurons to target and neutralize botulinum neurotoxins. Interestingly, when this nanobody was applied in mouse models with lethal doses of neurotoxins, the duration of muscle paralysis decreased and the mice were rescued within hours from systemic toxicity by restoring the function of the muscles [32]. Nevertheless,

the transport mechanisms across the BBB or into the neurons have not been elucidated in all of the above-mentioned studies.

The active transport of molecules, such as proteins and peptides, through the BBB is mediated by three mechanisms: adsorptive-mediated transcytosis (AMT), transporter-mediated transcytosis, and receptor-mediated endocytosis (RMT) [33]. The human insulin receptor is known to shuttle insulin over the BBB by transcytosis. In addition, IgG antibodies binding this receptor have been shown to be shuttled simultaneously to the CNS [34]. Several studies have shown so far that mAbs targeting insulin receptors are effective in animals to transport a model drug across the BBB [35][36][37]. Engineering of receptor-binding molecules and reduction of size and complexity is believed to improve transcytosis and thereby brain delivery [36][38][39]. Additionally, several sdAbs were shown to be transported into the CNS via this BBB shuttle; for instance, a patented camelid IGF1R (insulin-like growth factor 1 receptor)-specific VHH was able to transmigrate across the BBB by RMT in in vivo and in vitro models [40]. In a study conducted by Muruganandam and his group, a naïve llama library was used to pan against human cerebromicrovascular endothelial cells (HCEC) [41]. Two selected VHHs, FC5 and FC44, were shown to selectively bind HCEC and transmigrate across them via the RMT mechanism and were able to reach the brain in mouse models after IV injection. The transport of FC5 across human brain endothelial cells was polarized, charge independent and temperature dependent. FC5 was taken up by HCEC via clathrin but not caveolin-1 mediated pathways [42]. Finally, Lundbeck in collaboration with Ossianix established the VNAR shuttle TXP1 against the transferrin receptor (TfR1), which can be delivered to the brain by bypassing the BBB via RTM [43].

Receptor-mediated uptake and transcytosis can be combined with carrier-mediated targeted drug delivery. Highly specific scFv or sdAb against receptors at the BBB, such as the insulin or low-density lipoprotein (LDL) receptors, are loaded into liposomes or other nano/microparticles to target and guide an encapsulated drug cargo into the specific tissue. Therefore, sdAbs binding to receptors at the BBB and inducing transcytosis are an interesting option for CNS delivery. VCAMelid against mouse vascular cell adhesion molecule 1 (VCAM-1) was used as a carrier to deliver therapeutic protein superoxide dismutase (SOD-1) and a nanocarrier (liposome) into the brain of mouse models in the format of both monovalent and bivalent nanobodies. This delivery was higher when a VHH was used as a targeting receptor [44].

The Rotman group used modified liposomes to deliver VHHs to the brain. In their study, two different formulations of glutathione targeting PEGylated liposomes were used to deliver an anti-amyloid VHH as cargo. The encapsulated VHHs were intravenously injected into a murine Alzheimer's disease model and the presence of VHHs quantified in the CNS. While from 0.001% of the injected dose was found for an unformulated VHH without carrier, the encapsulation in liposome achieved significantly higher levels, from 0.015% up to 0.094% [45]. These results indicate that liposomes with specific formulations could be useful carriers/shuttles for administrating large therapeutic drugs over the BBB into the brain.

2.1. Intranasal Nose to Brain Delivery

CNS drug delivery via the olfactory, trigeminal or optic nerves bypasses the BBB and could thereby dramatically advance the CNS drug delivery. In particular, intranasal nose to brain (N2B) delivery has emerged as an exciting and attractive minimally invasive delivery option (**Figure 2**a). As we have recently shown, after region-specific deposition at the olfactory region, full-length IgGs can use either intracellular or paracellular pathways to cross the epithelial layer (**Figure 2**b). Intracellular pathways across the olfactory epithelium include the endocytosis pathway into epithelial cells and/or olfactory neurons and transcytosis to the lamina propria [46][47]. In the extracellular pathway, molecules use paracellular diffusion through either the olfactory or respiratory tight epithelial junctions to reach the basal lamina [48]. From here, the IgGs are observed along neuronal bundles that project to the olfactory bulb.

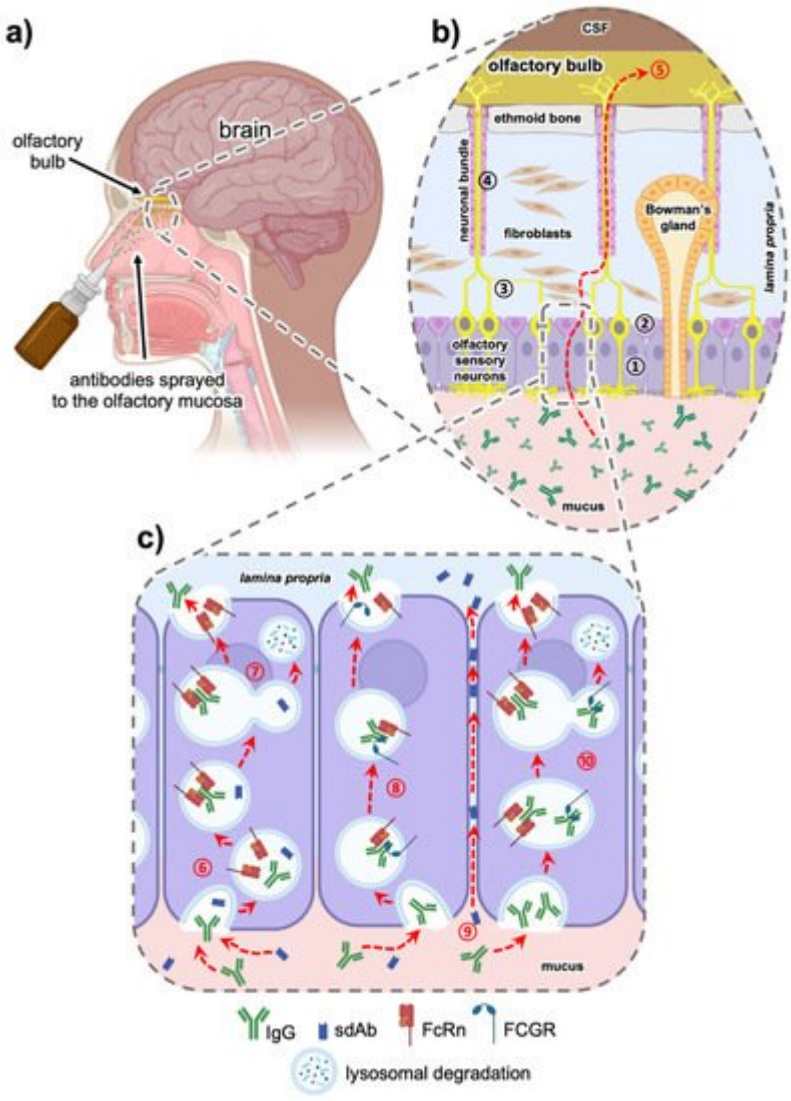

Figure 2. Intranasal delivery via the olfactory region of antibodies and their derivatives. Anatomical overview of the human nasal cavity, with olfactory nerve endings within the olfactory mucosa projecting to the olfactory bulb (**a**). A magnification of the olfactory mucosa is shown in (**b**). The epithelial layer consists of sustentacular ① and basal cells ②. The hallmarks of this epithelium are the olfactory sensory neurons and Bowman's glands. The axons ③ of the olfactory sensory neurons are collected in the neuronal bundles projecting to the olfactory bulb. These bundles are surrounded from olfactory ensheating cells ④ and transverse the ethmoid bone into the cranial cavity. When

drugs such as antibodies are administered to this olfactory region, the proteins can be transported from the nasal mucosal surface to the brain (nose-to-brain) as demonstrated with the red dotted arrow ⑤. The state-of-the-art hypothesis concerning the olfactory transport of full-length IgG antibodies [46][47] or sdAbs (unpublished data) [49] is shown in (**c**): uptake via endocytosis and binding of IgG to neonatal Fc receptors (FcRn) via their Fc domain under acidic conditions ⑥. An SdAb devoid of Fc cannot be bound and is therefore sorted into the lysosomal pathway ⑦. The endosomes containing FcRn-bound IgG transmigrate the polar sustentacular cell where they fuse with the basolateral plasma membrane. At physiological pH, the IgG is released from the FcRn diffuses to the lamina propria. In addition, a transcytosis pathway based on a mixed co-transport of full-length IgG via FcRn and Fc-gamma receptors (FCGR) could be possible ⑧. Due to their smaller size, sdAbs can enter paracellular pathways between the epithelial cells ⑨ to reach the lamina propria. Finally, IgG bound to FCGR only can be sorted to the lysosomal pathway and degraded ⑩. Image created with BioRender.com.

It is well accepted that lipophilic molecules are predominantly transported along transcellular pathways, while more polar molecules appear to have a higher preference for the paracellular pathway [50][51]. However, both pathways are dependent on diffusion, which is the limiting factor when it comes to large molecules, such as antibodies. The impact of molecular size on the probability of crossing an epithelial barrier in the absence of other transport mechanisms is well known [52]. Up to now, intranasal delivery of small proteins, such as insulin [53], scFvs [54] and nanobodies [55], was demonstrated. Benedict et al. showed that administered insulin had a positive effect on mood and also memory in patients with mild cognitive impairment and Alzheimer's disease [56]. Intranasally administered insulin exhibited positive clinical effects on memory [57] and hypothalamic functional connectivity [58]. They found that the intranasal insulin did not enter the blood and did not affect the plasma glucose levels. It can be concluded that intranasal administration decreases the possibility of side effects of systemic application.

Only a few other reports used larger proteins like IgGs and found a rapid distribution to the olfactory bulb and other brain areas, implicating olfactory and trigeminal pathways [59][60][61]. Unfortunately, most intranasal in vivo studies lack pharmacokinetic data, and even if quantitative data are shown, they do not disclose intranasal bioavailability [62]. Therefore, the effects of molecular size and physicochemical properties, e.g., polarity, and active or passive transport mechanisms have hardly been investigated in the context of intranasal delivery. To predict the efficiency of intranasally delivered biopharmaceuticals, it is crucial to estimate the percentage of the dose that is able to cross the nasal mucosa in relation to the bioactivity of the biopharmaceutical.

An scFv against tumor necrosis factor α (TNFα) was previously delivered to the brain via an intranasal route and some pharmacokinetic parameters were determined for different brain regions. TNFα is a well-established and well-studied target with a high clinical impact and application. TNFα is a critical inflammatory cytokine that plays a pathological role in acute and chronic diseases of peripheral organs, such as rheumatoid arthritis and Crohn's disease, as well as diseases of the CNS, such as Parkinson's disease, Alzheimer's disease and multiple sclerosis [63]. The results of these experiments showed that the maximum concentration reaching the brain was 1.1 to 1.2 µg/mg of the total applied scFv (400 µg) [54].

Gomes et al. successfully delivered an anti-transthyretin single-domain antibody intranasally in mice [55]. After delivery of 400 µg of this nanobody in transthyretin knock-out mice, it distributed to all brain areas, but the highest levels were observed in the olfactory bulb and the central parts of the brain. Interestingly, this nanobody was even detected in the spinal cord after intranasal delivery. Moreover, they found that the highest concentration of this antibody reached the brain and cerebrospinal fluid within one to two hours. A comparable pattern of this distribution was shown for transthyretin in wild-type mice.

2.2. The Role of the Fc Domain in Intranasal Transmucosal Delivery—Is it a Friend or a Foe?

Fc-gamma receptors (FCGR) belong to the immunoglobulin superfamily. It is well known that the Fc domain of an IgG molecule binds to FCGR and relevant complement factors, thereby triggering immune effector functions, such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Moreover, Fc-related transport molecules, e.g., FcRn, have been well characterized. Since the nasal mucosa is highly exposed to microbes and viruses, several immune cells armed with different FCGR are resident in this mucosa. Recently, our group has demonstrated that the use of ex vivo mucosa and primary cells arising from this tissue are valuable models for studying the uptake and transport pathway for intranasal delivery [46][64]. The nasal mucosa harbors FcRn and different FCGRs in epithelial, basal and immune cells, but also olfactory ensheathing cells [65][47], facilitating the transport of IgG from the apical to the basolateral side in polarized cells [46][66]. As shown in **Figure 2**c, FcRn-mediated transcytosis in an acidic environment increases the uptake of IgG from the mucosal surface [46][67]. On the other hand, we were recently able to demonstrate that FCGR2 directs IgGs to the lysosomal pathway, such that they are degraded rather than being distributed to the CNS [47].

Currently, it is not clear whether the interaction with Fc receptors is a friend or a foe for intranasal drug delivery, since the interaction of IgG molecules with immune cells is very likely. We have observed that mucosal lymphoid follicles are spared from full-length IgG-immunoreactivity [46]. The suspected underlying mechanism is uptake of the IgGs via FCGR, lysosomal degradation and the presentation of eventually captured antigens via MHC. Though this is a highly important process for mucosal immune surveillance, it is a potential source of adverse effects and immunogenicity for N2B CNS delivery with full-length IgGs. Fc fusion molecules would also become eligible for FcRn recycling [68].

Hence, a potential mitigation strategy could be to use either IgGs with mutations that abolish Fc receptor interaction or to use antibody formats without Fc domains, such as sdAbs. However, it is rather likely that without the transport via the FcRn receptor system the penetration of the mucosa will be rather low, despite the advantage of their smaller size.

3. Conclusions and Outlook

The effect of the size of antibodies and their derivatives on permeability and tissue penetration is unquestionable, and hydrodynamic diameter is the main reason for the absence of transport mechanisms. Smaller antibody formats

have essential characteristics for CNS delivery which can be developed into novel carriers to facilitate drug transport across the BBB. Although sdAbs and other small formats are clearly superior in trans-mucosal delivery under in vitro conditions, pharmacokinetic factors, such as half-life, are important for efficacy in vivo. Based on recent results of in vitro, ex vivo and in vivo studies, the passive diffusion of sdAbs might be countervailed by transport-related processes via the Fc domain involving full-length IgGs in the airway mucosa. The Fc domain interacts with different Fc receptors, such as FcRn or members of the FCGR family. These receptors mediate transcytosis, rescuing full-length IgGs from the endosomal system but also leading to their lysosomal degradation. Nevertheless, sdAbs, being less complex and more stable during aerosolization, have significant potential for inhalative drug delivery, and could be made competitive with reduced manufacturing costs. In conclusion, exploring the potential and limitations of sdAbs for CNS drug delivery is highly relevant in the context of developing treatments for diseases such as brain tumors or neurodegenerative disorders.

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