Title:

Antibody approaches to treat brain illnesses

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Keywords:

Blood-brain barrier; central nervous system disorders; brain drug delivery; recombinant antibodies; bispecific antibodies, Molecular Trojan Horses (MTH)

Getting into the brain

Central nervous system (CNS) disorders affect up to 1 billion people worldwide [1], which corresponds to around 13% of the global health burden, surpassing both cardiovascular disease and cancer [2]. Alzheimer and other dementias are estimated to constitute 3 % of total deaths among neurological disorders [3]. It is estimated that 47 million people worldwide live with dementia in 2015 and assuming that the age-specific prevalence rate remains stable, this number is expected to double every 20 years [4]. This has an incredible burden on health care and society as the costs of care for dementia alone in 2015 are estimated at US$818 billion worldwide [5]. The low success rate observed for the CNS therapeutics are mainly related to the incomplete understanding of the brain and its many functions, the organ's susceptibility for off target side effects and a shortage of validated biomarkers for assessing therapeutic efficacy. Moreover, the key drawback in many cases is associated with a low efficiency of drug delivery into the brain [6, 7]. The ability to achieve consistent targeted delivery to the CNS remains a major, largely unmet challenge in the application of numerous small molecule and biopharmaceutical drugs. Among the largest obstacles to effective CNS delivery is the blood-brain barrier (BBB) (Figure 1), formed by tight junctions between brain endothelial and epithelial cells that limit the transfer of therapeutic molecules between the blood and the interstitial fluid of the CNS (Box 1) [8-11].

The delivery of pharmacologically active molecules and especially macromolecules to the brain is challenged by the barrier properties. The BBB has been known for more than a century and is now recognized as a dynamic interface that, by regulating the exchange of substances between

blood and brain, maintains optimal conditions for neuronal and glial function. Cerebral capillaries comprise approximately 95 % of the total area of the barriers between the blood and brain and, therefore, are the main entry route for molecules into the CNS. Apart from limiting the entry of therapeutics the BBB is also responsible for limiting the entry of immune cells and immune mediators into the CNS and is therefore considered an immune privileged site. Therefore the immune responses occurring in the brain are different from those in the peripheral immune system [12]. The BBB limits not only the penetration of antibodies, immune mediators and immune cells from the systemic circulation; but also lacks the lymphatic vessels in the parenchyma to drain antigens and immune cells from the CNS to peripheral lymph nodes. In addition, the features of this immune privilege site include inability of microglial and astroglial cells to maintain immune responses, the scarcity of dendritic cells in the parenchyma and low levels of major histocompatibility complex expression and delayed, reduced or absent responses in the brain [13]. Although being an immune privilege site the CNS is not immune isolated, instead is a collection of CNS-driven mechanisms that actively regulate T-cell responses within the CNS [13]. The recognition of CNS as immune privilege and a better understating of the BBB are leading to the development of new immune-based therapies.

Box 1. The Blood brain barrier

The BBB is a unique, selective barrier formed by endothelial cells that line cerebral capillaries, together with perivascular elements such as closely associated astrocytic-end-feet processes, perivascular neurons and pericytes (Figure 1). Their interaction in the normal state and their coordinated response to injury made these cells a functional unit - neurovascular unit [9]. Cerebral endothelial cells, in contrast to the systemic endothelium, are tightly connected to each other by tight junctions (TJ) and adherence junctions, consequently lacking fenestrations. Among others, proteins of the claudin family, i. e., claudin 3, 5 and 12, occluding, and junction-adhesion molecules (JAMs), which are expressed at opposing cell surfaces, form tight junctions by means of hemophilic interactions. In this manner TJ strands “zip” adjacent endothelial cell and close the paracellular space for access of blood-borne molecules [14]. A number of cytoplasmic accessory proteins, including zonula occludens protein 1 (ZO-1), ZO-2 and cingulin, link the transmembrane proteins to the actin cytoskeleton allowing the paracellular transport to be modulated in response to different stimuli [15, 16]. Importantly, the presence of the tight junctions divides the plasma membrane of the vascular endothelial cells into two separate domains that is the apical membrane, which faces the blood, and the basolateral membrane, which faces the brain tissue. Therefore tight junctions are also responsible to assure specific composition of each domain [14].

To form the neurovascular unit, regulation of BBB integrity and dynamics under physiological and inflammatory conditions, is promoted by multiple basal lamina proteins, matrix metalloproteases (MMPs) and their inhibitors [17]. Furthermore, molecular factors released

from glial cells also contribute to the robustness of the integrity of the BBB. Pericytes regulate directly the BBB controlling vascular permeability and restricting transcytosis. Lastly, stability of the BBB is also provided by interactions of astrocytes, which cover more than 99 % of the basal capillary membrane [18].

Transcytosis of drugs and molecules, from the blood to the brain, is tightly regulated comprising specific mechanisms (Figure 1). Receptor-mediated transport processes are limited in brain endothelial cells (BEC) restricted by the low number of vesicles present in the barrier. BEC's have reduced number of vesicles when compared to other endothelial cells [19, 20].

Strategies for crossing the blood-brain barrier

In the past two decades several strategies have been developed to get therapeutic molecules into the brain. Methods such as osmotic and chemical disruption are far from ideal but the use of endogenous BBB transports systems is a safe alternative [21-24]. The ideal method for transporting drugs across the BBB should be controlled and should not damage the integrety of the BBB [22]. The existing endogenous BBB transporters can be classified into three categories depending on the kind of transport they are associated to: carrier-mediated transport (CMT), active efflux transport (AET) and receptor-mediated transport (RMT) (Table 1 and Figure 1). CMT and AET systems are responsible for the transport of small molecules between blood and brain, such as glucose, amino acids and nucleosides [23]. On the other hand, RMT systems are responsible for the transport across the BBB of certain endogenous large molecules. Examples of these molecules are insulin, transferin, low-density lipoprotein, insulin like growth factor

(IGF) and leptin (Table 1). In RMT, molecules in circulation may bind to specific receptors on the luminal surface of brain. Upon binding, the receptor-ligand complex is internalized into the endothelial cell by receptor-mediated endocytosis. The ligand molecule may then be transported across the abluminal membrane of the endothelial cell into the brain [25]. Taking in consideration these properties and advantages, several academic groups and pharmaceutical companies have been exploring RMT to develop molecules that can efficiently cross the BBB and deliver a therapeutic CNS drug into the brain. These molecules, known as Molecular Trojan Horses (MTH), can be either peptides or proteins ligands that target RMT systems (e.g. receptor-binding sequences of insulin) or monoclonal antibodies (mAbs) that specifically target RMT receptors [24, 26-28]. mAbs have long been an integral tool in basic research due to their high specificity and affinity for target antigens (Figure 2a). Moreover, for the past two decades mAbs have had substantial effects on medical care for a wide range of diseases, as shown by the growing list of therapeutic mAbs on the market, as well as in clinical trials, and the possibility to engineer new recombinant antibody constructs as therapeutics (Figure 2b) [29]. For these reasons, recombinant antibodies against RMT receptors have a promising potential to be used as “carriers” for therapeutic delivery into the brain. This manuscript will review the advances in the design and engineering of BBB-crossing antibodies using RMT receptors and their high potential for treatment of CNS disorders.

Benefiting from brain natural portals

The transferrin receptor (TfR) is one of the most intensively studied receptors among those known to undergo RMT and explored to develop MTH´s [25]. The TfR is highly expressed on brain endothelial cells and is responsible to maintain the brain iron homeostasis through the entry of iron-bound transferrin (Tf) by endocytosis [30, 31]. The murine antibody OX26, developed against the rat TfR, was the molecule originating the "Molecular Trojan horse" hypothesis that was formulated at William Pardridge group at University of California, Los Angeles (UCLA) [32]. This antibody binds to an extracellular epitope of the TfR that is distinct from the transferrin binding site therefore not limiting the effects on normal Tf transport, and preventing competition for binding sites between the drug targeting and the natural ligand [32]. The BBB-targeting and drug delivery properties of OX26 has been validated *in vivo* quite extensively by fusing several therapeutic protein molecules to OX26, such as the brain-derived neurotrophic factor (BDNF), the nerve growth-factor (NGF) and the epidermal growth factor (EGF) [33, 34]. For example, fusion of OX26 with the BDNF neuroprotective agent was tested for improvement of stroke symptoms. The data obtained showed that rats given the conjugated OX26-BDNF had a 243% increase in motor performance relative to BDNF alone, showing high potential for treatment of stroke symptoms [35]. In another study, OX26 was conjugated to NGF in a rat model of Huntington disease and it was demonstrated that the fusion molecule prevented the degeneration of cholinergic striatal acetyltransferase-immunoreactive neurons [36]. Since OX26 does not cross-react with the mouse TfR, other mAb, 8D3, has been developed and tested as a BBB-targeting vector in mouse models. In this case, the high brain uptake of 8D3 was demonstrated when fused with the glial-derived neurotrophic factor (GDNF). In fact,

149 the uptake by the mouse brain was 3.1 ± 0.2 % injected dose (ID)/g brain, at 60 min after
150 intravenous injection of 1mg/kg dose [37].

151 Over the past years, others mAbs against the TfR have been developed and their BBB-targeting
152 properties improved by different antibody engineering approaches. For instance, a very elegant
153 study was performed to correlate the binding affinity between anti-TfR antibodies and TfR with
154 RMT efficiency [38]. For that, alanine mutations were introduced into the complementarity-
155 determining regions of a high-affinity mouse anti-TfR^{A} antibody (*K*d = 1.7 nM) to generate
156 variants with a range of lower binding affinities for TfR (Kd= 6.9 nM, 65 nM and 111 nM
157 respectively for variants anti-TfR^{B}, anti-TfR^{C} and anti-TfR^{D}). *In vivo* biodistribution studies
158 performed in mice demonstrated that there is a correlation between antibody affinity and brain
159 uptake for trace doses of anti-TfR. However, when therapeutic doses were administered an
160 inverse effect was observed and low-affinity antibodies presented an increase in brain antibody
161 concentration when compared with the high affinity antibody. Importantly, it was observed
162 that the high-affinity antibody (anti-TfR^{A}) could not detach from the TfR and remained largely
163 associated with the vasculature. On the other hand, lower-affinity antibodies could detach from
164 the TfR and were able to cross the BBB and be found in neuronal cell bodies [38]. Indeed, the
165 lowest-affinity variant, anti-TfR^{D}, exhibited a roughly 3-fold increase in brain uptake compared
166 with parental anti-TfR^{A} antibody when administered in mice at high doses of 20–50 mg/kg and
167 measured 24 h postinjection. Therefore, modest changes in affinity can affect brain uptake,
168 being low-affinity antibodies more prone to cross the BBB when administrated at therapeutic
169 doses.

170 The insulin receptor (IR) is responsible for the import of blood borne insulin into the brain and
171 is the second most studied receptor to develop MTH´s. Like TfR, IR is highly expressed on brain
172 endothelial cells and it can undergo RMT across the BBB endothelium [39]. Extensive research
173 using the 83-14 mouse mAb against the human IR as a RMT delivery vector has been
174 performed. Importantly, the 83-14 mAb is effective in Old World primates, so it can be
175 evaluated in non-human primate studies. Studies in Rhesus monkeys have shown that the total
176 uptake of 83-14 mAb was ~4%, which corresponds to 0.04% ID/g brain tissue 3 hours after
177 intravenous administration [39]. Moreover, both the chimeric antibody and the fully humanized
178 form of the 83-14 mAb have been created and shown to be able to transport a large number
179 therapeutic drugs across the BBB, such as Glial-derived neurotrophic factor (GDNF) to treat
180 Parkinson's, and stroke; tumor necrosis factor inhibitor (TNFR) for stroke; arylsulfatase A (ASA)
181 for metachromatic leukodystrophy; and N-sulfoglucosamine sulfohydrolase (SGSH) for
182 Sanfilippo type A syndrome [40, 41]. Some of these mAbs are already proceeding to clinical
183 trials by Armagen, a spin-off Company of Pardridge's laboratory.
184 The TfR and the IR are two well validated receptors that are in vogue but a drawback related to
185 these RMT targets is that they are also highly and broadly expressed in other tissues and are
186 implicated in metabolically critical cellular functions, creating safety risks, as recently reported
187 [42, 43]. Thus, over the past years researchers have been focusing substantial effort on
188 identifying new BBB RMT targets that may have better BBB specificity. One example is the FC5
189 single-domain antibody (VHH) that was selected by a functional panning of a llama VHH phage-
190 display library for its ability to internalize human brain endothelial cells (BEC) and to
191 transmigrate an *in vitro* BBB model [44]. Moreover, *in vivo* biodistribution data demonstrated

192 that the brain uptake of phage-displayed FC5 was very promising showing an accumulation of
193 2.9 % ID/gram of tissue [44]. FC5 was subsequently shown to recognize a heavily glycosylated
194 protein (Cdc50A) expressed on the luminal surface of brain endothelial cells and underwent
195 actin- and PI3 kinase-dependent transcytosis via clathrin-coated vesicles[45, 46].
196 A TfR and IR-independentBBB-targeting antibody was discovered through a nonimmune yeast
197 surface display of a human single-chain fragment variable (scFv) antibody library library. From
198 this library a panel of scFv antibody fragments were identified and found to be internalized by
199 BECs via an endocytosis system. The analysis of cognate receptor indicated that the selected
200 scFv´s recognize a receptor different from TfR and IR [47]. Identifying new RMT systems specific
201 to brain can be very valuable targets to design and engineer improved BBB vectors that could
202 provide a more selective delivery into the brain.
203
204 **Engineering improved BBB-crossing and therapeutic antibodies for brain therapy**
205 Over the past years, progress in antibody engineering has led to the generation of different
206 types of molecules of biotechnological and clinical interest that differ in size and shape,
207 including the attractive bispecific antibody (bsAb) format. bsAbs are essentially artificially
208 designed antibodies that combine two antigen-recognizing elements into a single construct
209 (Figure 2c). A bsAb molecule can address simultaneously different targets involved in different
210 pathophysiological processes and thereby increase therapeutic efficacy. This dual specificity
211 property of bsAbs has been successfully explored to develop therapeutic antibodies mainly for
212 cancer therapy and inflammatory diseases [48]. Recently, bsAbs have emerged as promising
213 scaffolds to deliver therapeutic antibodies into the brain [49, 50]. In these cases, the antibody is

engineered to incorporate one arm with specificity against a BBB RMT receptor, which drives their transmigration across the BBB, and the other arm against a CNS therapeutic target to produce the pharmacological effect (Figure 2c and Figure 3a). Essentially, bsAbs can be generated by fusion of antibody fragments such as Fabs, scFv or single domain antibodies (sdAb) into the N- or C-terminal of a convention IgG molecule or by heterodimerization strategies such as the “knobs-into-holes” technology developed by Genentech. The first BBB-bsAbs consisted in the generation of bsAbs molecules for therapy of Alzheimer's disease [34, 51, 52]. For that, an anti-beta amyloid (Aβ) scFv was fused to the C-terminal of each heavy chain of TfR or IR mAbs (Figure 3b) [34, 51, 52]. The TfR-scFv bsAb was shown to be the most promising approach in Alzheimer transgenic mice models. With these studies it was demonstrated for the first time that active tetravalent bsAbs could be successfully generated where: i) the head of the bsAb molecule binds to the receptor and enables influx from blood to brain; ii) the tail of the fusion antibody binds the β amyloid plaque and induces plaque disaggregation; and (iii) the midsection of the fusion antibody (CH2-CH3 constant region) binds to the neonatal Fc-receptor (FcRn) that is well expressed on the BBB and enables a reverse transport of the fusion antibody from brain back to blood. With these studies, William Pardridge and his team opened the “road” to develop improved therapeutic antibodies for brain disorders. Currently, there are others BBB-bsAbs being developed and evaluated. One of such examples is a humanized bsAb that was developed by Genentech (Figure 3c) using the knobs-into-holes heterodimerization method which consists in a bsAb that targets the TfR and BACE1, an enzyme that cleaves the pathogenic form of amyloid β (Aβ) in Alzheimer disease. Using a human TfR knock-in mouse, they demonstrated that the anti-TfR/BACE1 bsAb could

236 cross the BBB and reduce brain Aβ in a TfR affinity–dependent fashion. Importantly,
237 intravenous dosing of monkeys with this bsAb also reduced Aβ both in cerebral spinal fluid and
238 in brain tissue, and the degree of reduction correlated with the brain concentration of the bsAb
239 molecule [53]. These results demonstrate that bsAbs that use the TfR RMT pathway can
240 robustly and safely deliver therapeutic antibody across the BBB in the primate brain [53]. An
241 alternative design of TfR-targeting bsAb was also recently developed by Roche, where anti-TfR
242 Fab fragments were fused to the C-terminal of an Aβ- binding IgG molecule to obtain
243 monovalent or bivalent modes of binding activities (Figure 3d). With this approach it was
244 demonstrated with *in vitro* and *in vivo* studies that the monovalent binding mode facilitates
245 transcellular transport, whereas the bivalent binding mode leads to lysosome sorting. Indeed,
246 they demonstrated that the monovalent Fab-bsAb variant is able to increases Aβ target
247 engagement in a mouse model of Alzheimer disease by 55-fold compared to the parent
248 antibody [54]. Another, promising and different platform for engineering BBB-crossing bsAbs
249 was recently developed using the FC5 VHH antibody that was previously isolated against an
250 alternative receptor [44, 55]. In this approach, the FC5 antibody was engineered as a mono-
251 and bivalent fusion with the human Fc domain (Figure 3d). Systemic pharmacological potency
252 was evaluated in the Hargreaves model of inflammatory pain using the BBB impermeable
253 neuropeptides dalargin and neuropeptide chemically conjugated with both FC5-Fc fusion
254 proteins. In contrast to the TfR bsAb developed by Roche, both mono and bivalent FC5-Fc
255 variants showed a similar rate of transcytosis and therapeutic efficacy, despite their differences
256 in binding affinities and valency [55]. This underscores the importance of alternative RMT

carriers and antibody scaffolds that can be engineered and developed as a platform to enhance CNS delivery.

The four examples presented above show the significant progress that has been made in the development of BBB-crossing bispecific antibodies and their potential for brain targeting and drug delivery. In addition, the data obtained refute the role of the neonatal Fc-receptor (FcRn) in the antibodies efflux from brain to the blood. This mediated reverse transcytosis across the BBB to pump out antibodies that contain the Fc domain may be an advantage for some disease targets- such as the disaggregation and clearance of beta amyloid in the brain of Alzheimer patients. Nevertheless, in other diseases this fast elimination of antibodies might limit their therapeutic potency and therefore this efflux route and its mechanism should be better evaluated in future studies. While other challenges remain, BBB-crossing bispecific antibodies are considered to be one of the most promising strategies for engineering therapeutic antibodies for brain therapy.

Antibodies towards the medicine of tomorrow

First generation antibodies took a long time to reach commercial potential, but are nowadays an important component of the therapeutic options. Motivated by the clinical and commercial successes of therapeutic antibodies for oncology and inflammatory diseases there is an increasing interest in these molecules to treat brain illnesses. For instance, immunotherapy for Alzheimer's disease has greatly incentivated the development of therapeutic antibodies against Aβ and tau protein (Table 1). These antibodies generated against different epitopes of Aβ proceeded to human trials based on the assumption that elimination of brain amyloid deposits

would promote elimination of circulating amyloid. The mechanism of reduction of CNS Aβ levels is still not clear. One hypothesis, the "peripheral sink hypothesis", considers that systemic antibodies bind Aβ in the blood, drawing monomeric Aβ from the brain to the blood, thus reducing the accumulation in the brain [56]. The alternative hypothesis considers the direct action of antibodies in the CNS. Antibodies binding directly to Aβ might target the protein for phagocytosis through the Fc receptor [57]. Alternatively, antibody binding to Aβ could alter its conformation so that less fibrillar aggregates are formed [58]. The hypothesis that Aβ oligomers are interesting as therapeutic targets further contributes to the optimism of having immunotherapies defeating Alzheimer's in the future [59].

Several anti-Aβ antibodies have been tested in clinical trials: Bapineuzumab, Solenezumab, Gantenerumab, Crenezumab, Ponezumab and Aducanumab (Table 1) among others, are in early clinical trials. Some of these immunotherapeutic approaches have failed to show significant clinical benefits in mild to moderate AD patients being treated, for reasons that are not exactly known. Two hypotheses for the lack of success are: (1) poor uptake of the antibody to the CNS across the BBB; (2) administration of antibody too late in the progression of disease [57]. For example, the first clinical trials for Solenezumb showed no difference between patients in the treated group vs placebo. However, when the data was reanalyzed a slight improvement was found in patient's whose symptoms were mild when the trial initiated [60]. In this sense, the lack of biomarkers to identify early stages of disease is one of the biggest problems to be overcome in modern medicine. Nonetheless, the vital point in the development of antibodies for brain diseases is to understand if antibodies administered peripherally are delivered across the BBB in sufficient amounts and have the capacity to produce a central

301 therapeutic effect. However, some of the failures of these mAbs described were also due to
302 safety issues – both cerebral microhemorrahages as well as meningoencephalitis. This may be
303 due to mAbs accumulation in the brain due to low reverse transport from brain back to blood.
304 Therefore, the main challenge of pharmaceutical companies and academic groups have to
305 tackle in the near future is the understanding of antibodies influx and efflux from the blood to
306 the brain and back to be able to develop safer and effective therapeutic antibodies for CNS
307 diseases.

308

309 **Concluding remarks and future perspectives**

310 In summary, there is still a long away in the development of antibodies for brain illnesses.
311 Several approaches have been explored, such as conventional antibodies, but there are still
312 new avenues to come. Results to date with conventional antibodies (Table 2) have been mixed
313 with no conclusive data on the exact mechanism they exert their function. In addition, some
314 antibodies that were discontinued in Phase 3 due to lack of clinical benefit were found to have
315 some therapeutic effect in early cases of disease, e.g. Solanezumab and Gantenerumab. In fact,
316 these antibodies are being investigated in a Phase 2/3 trial aiming to prevent dementia in
317 people who inherited autosomal-dominant mutation in APP, presenilin-1, or presenilin-2, but
318 have no clinical symptoms. Nevertheless, the challenges of getting these monoclonal antibodies
319 into the brain at therapeutic doses and have a safe clearance from the brain remains.

Optimism towards therapeutic antibodies to the CNS has been kept alive by recent evidence of highly improved efficacy achieved by linking a therapeutic antibody with an antibody that undergoes transport across the BBB. Clearly the techniques mentioned above show that there are novel methods of re-engineering with “old’ and emerging “new” MTH for the BBB (Table 2 and Figure 2 and 3). These methods will improve brain penetration and present huge benefits, by increasing the delivery of the injected dose and reducing the amount of antibody needed for injection thus reducing drug exposure and improving safety. These bispecific antibodies have been validated in pre-clinical models, with some on their path to clinical trials.

Acknowledgements:

The authors thank Portuguese Funding scheme (Fundação para a Ciência e Tecnologia - FCT) for financial support (grants SFRH/BPD/94466/2013; IF/01010/2013); and Marie Skłodowska-Curie Research and Innovation Staff Exchange (MSCA-RISE), call H2020-MSCA-RISE-2014, Grant agreement no: 644167 - INPACT: Innovative peptides against cancer and pathogenic bacteria, with advances in science, biopharmaceutical drug development, product market targeting, training, and communication. 2015-2019.

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Table 1. Classification and list of endogenous blood-brain barrier transporters.

Carrier-mediated transporters (CMT)	Glucose transporter (GLUT1)
	Large neutral amino acid transporter (LAT1)
	Cationic amino acid transporter (CAT1)
	Mono carboxylic acid transporter (MCT1)
	Concentrative nucleoside transporter (CNT2)
	Choline transporter (CHT)
	Nucleobase transporter (NBT)
Active efflux transporters (AET)	Adenosine triphosphate binding cassette (ABC pr P-gp)
	ABC transporter, subfamily C (ABCC)
	ABC transporter, subfamily G (ABCG2)
	Organic anion transporter (OAT or SLC22)
	Organic anion-transporting polypeptide (OATP or SLC21)
	Glutamic acid amino acid transporter (EAAT or SLC1)
	Taurine transporter (TAUT or SLC6)
Receptor-mediated transporters (RMT)	Transferrin receptor (TfR)
	Insulin receptor (IR)
	Insulin-like growth factor receptor (IGF1R)
	Insulin-like growth factor receptor (IGF2R)
	Leptin receptor (LEPR)
	Fc fragment of IgG, receptor, transporter, alpha or FCGT
	Scavenger receptor, class B

354 **Table 2 – List of some therapeutic antibodies for brain illnesses.**

Name	Molecule	Target	RMT delivery	Indication	Outcome	Company	Refs
Conventional antibodies for brain therapy and Clinical outcomes							
Bapineuzumab	Humanized IgG1	N-terminal region of Aβ (Recognizes fibrillar and soluble Aβ)	-	Alzheimer's	Antibody binds and clear excess Aβ and activates microglial phagocytosis and cytokine production. Phase 3 trails reveal no significant between-group differences.	Jansen Pfizer	[61]
Solanezumab	Humanized IgG1	Mid domain of Aβ peptide (Recognizes soluble and monomeric Aβ)	-	Alzheimer's	Sequestration of Aβ, shifting equilibria between different species of Aβ, and removing small soluble species of Aβ that are directly toxic to synaptic function. Phase 3 trails reveal no significant between-group differences.	Eli Lilly	[62]
Gantenerumab	Human IgG1	Both N-terminal and central region of Aβ	-	Alzheimer's	Combination of anti-Aβ with BACE inhibitor resulted in lowering amyloid plaque load and plaque number. In march 2014, Phase 3 trial started with 1000 patient with a clinical diagnosis of mild AD.	Roche	[63, 64]
Crenezumab	Humanized IgG4 With reduced risk of Fc γ receptor-mediated activation of microglia	Aβ aggregated forms, including oligomeric and fibrillar	-	Alzheimer's	Binds with high affinity to multiple forms of Aβ, protected against Aβ1-42 oligomer-induced cytotoxicity, and increase uptake of neurotoxic Aβ oligomers by microglia. Phase II studies demonstrate that crenezumab treatment in people with mild-to-moderate AD demonstrated a trend toward slowing the decline of cognitive abilities	Roche	[65]
Ponezumab	Humanized IgG	Aβ40	-	Alzheimer's	Phase 2 trials with patients with mild to moderate Alzheimer's confirmed adequate safety and showed a plasma Aβ40 increase with treatment, suggesting a peripheral sink effect. The study with paients with mild to moderate AD, however, showed no effect on the primary endpoints of change in brain or CSF Aβ burden.	Pfizer	[66]
Aducanumab	human IgG1	Conformational epitope found on Aβ.	-	Alzheimer's	In early stage trial Aducanumab reduced Aβ in the brain. Drug improved cognition in patients with early signs of the disease 54 weeks after starting treatment. It Has proceeded to	Biogen	[67]

					phase III clinical trials.		
Re-engineered antibodies for brain therapy and pre-clinical results							
HIRMAb-GDNF cTfRMAb-GDNF	Recombinant IgG	Glial-derived neurotrophic factor	HIR and TfR	Parkinson's, stroke, addiction	Demonstration of brain uptake but no information on neuroprotection	ArmaGen	[37, 68]
HIRMAb-TNFR cTfRMAb-TNFR	Recombinant IgG	Tumor necrosis factor inhibitor	HIR and TfR	Stroke	Significant reduction in hemispheric, cortical and subcortical stroke volumes	Armagen	[69-72]
HIRMAb-ScFv cTfRMAb-ScFv	Recombinant IgG	Aβ plaque binder	HIR and TfR	Alzheimer's	57% and 61 % reduction in amyloid peptide in the cortex and hippocampus	Armagen	[51, 52, 73]
HIRMAb-ASA	Recombinant IgG	Arylsulfatase (ASA)	HIR	Metachromatic leukodystrophy	High ASA enzyme activity	Armagen	[74]
HIRMAb-SGSH	Recombinant IgG	Sulfoglucosamine sulfohydrolase (SGSH)	HIR	Sanfilippo type A syndrome	Suggest normalization of brain SGSH enzyme activity	Armagen	[75]
Anti-TfR/BACE1	Bispecific	BACE1 (β-site β-amyloid precursor protein cleaving enzyme 1)	TfR	Alzheimer's	Moderate brain uptake of anti-TfR/BACE1 with modest Aβ reduction in the brain in comparison with CSF.	Genentech	[38, 43, 53, 76]
Anti- Aβ mAB31	IgG-sFab	Aβ plaque binder	TfR	Alzheimer's	Significant reduction of plaque number (small plaques).	Hoffmann-La Roche	[54]
Bi-FC5-hFc-Dal	Bispecific	neuropeptide dalargin	Glycosylated protein (Cdc50A)	Inflammatory pain	Improved serum pharmacokinetics (increased circulation half-life) contributing for improved BBB-delivery. Bi-FC5-hFc-Dal induced dose-dependent inhibition of thermal hyperalgesia in Hargreaves model.	Biogen	[55]

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Figure legends:

Figure 1. The brain capillary and the blood-brain barrier (BBB). The blood-brain barrier is formed by endothelial cells that interact with perivascular elements such as basal lamina, astrocytes, neurons and pericytes to form a functional unit. Brain endothelial cells form complex tight junctions (TJ) that seal the paracellular route, only available to small water-soluble agents. Small lipid molecules (MW < 400 Da) are able to cross BBB using transcellular lipophilic pathway. Essential molecules such as glucose, amino acids and nucleosides are transported via carrier-mediated transport (CMT), through transport proteins. Efflux pumps move molecules out from the brain into the blood. Large molecules such as antibodies, lipoproteins, proteins and peptides only get across the BBB by receptor-mediated transport (RMT). In this process a ligand interacts with a specific receptor at the apical plasma membrane (blood) of the endothelial cell. Once bound to ligand, the process of endocytosis is initiated. The receptors for iron transferrin (TfR), insulin, low-density lipoprotein (LDL) and leptin are involved in transcytosis. Finally, Transcytosis might be enhanced through adsorptive mediated endocytosis (AMT) induced by chemically transformed cationic proteins or peptides, relying on nonspecific charge-based interactions.

Figure 2- Schematic representation of the structure of a conventional IgG antibody and antibody constructs of biotechnological and clinical interest. (a) IgG antibodies comprise a pair of identical heavy and light chains linked by disulphide bonds. Light chains contain one constant domain (CL) and one variable domain (VL), while heavy chains contain three constant domains

(CH1, CH2 and CH3) and one variable domain (VH). The variable domains of both the heavy and light chains are responsible for the antigen-binding site of the molecule. The Fc constant region recruits effector functions of the immune system. Constant light (CL) and heavy (CH) chain domains are represented in gray. Variable light (VL) and heavy (VH) chain domains are represented in green. (b) The engineering of antibody fragments that can be generated from an intact conventional IgG: antigen-binding fragment (Fab), single-chain Fv fragment (scFv) and heavy and light chains only (V domains). (c) Example of a therapeutic bispecific blood brain barrier crossing IgG antibody that combine two antigen-recognizing elements into a single construct. The bispecific antibody is engineered to incorporated one arm with specificity against a BBB RMT receptor (BBB arm) and the other arm (therapeutic arm) against a CNS therapeutic target.

Figure 3. Mechanism and examples of therapeutic bispecific blood-brain barrier crossing antibodies scaffolds. a) Schematic representation of the mechanism of a therapeutic bispecific blood-brain barrier crossing antibody. The left side of Figure A shows a conventional CNS therapeutic mAb that cannot bind to the RMT receptor and is unable to cross the BBB. The right side of Figure A shows a bsAb that was engineered to contain one arm with specificity against a BBB RMT receptor, which drives their transmigration across the BBB, and the other arm with a therapeutic function that produce the pharmacological effect when the bsAb encounter the target. **Examples of four platforms of bsAb**. b) bsAb generated by fusing an anti-beta amyloid scFv to the C-terminal of an TfR or IR mAb. c) bsAb generated using the knobs-into-holes heterodimerization method of TfR and BACE1 variable domains. d) bsAb generated by fusing

581 anti-TfR Fab fragments (monovalent or divalent) to the C-terminus of an Aβ- binding IgG
582 molecule. e) bsAb generated by an VHH (FC5) with the human Fc domain.

Figure 1

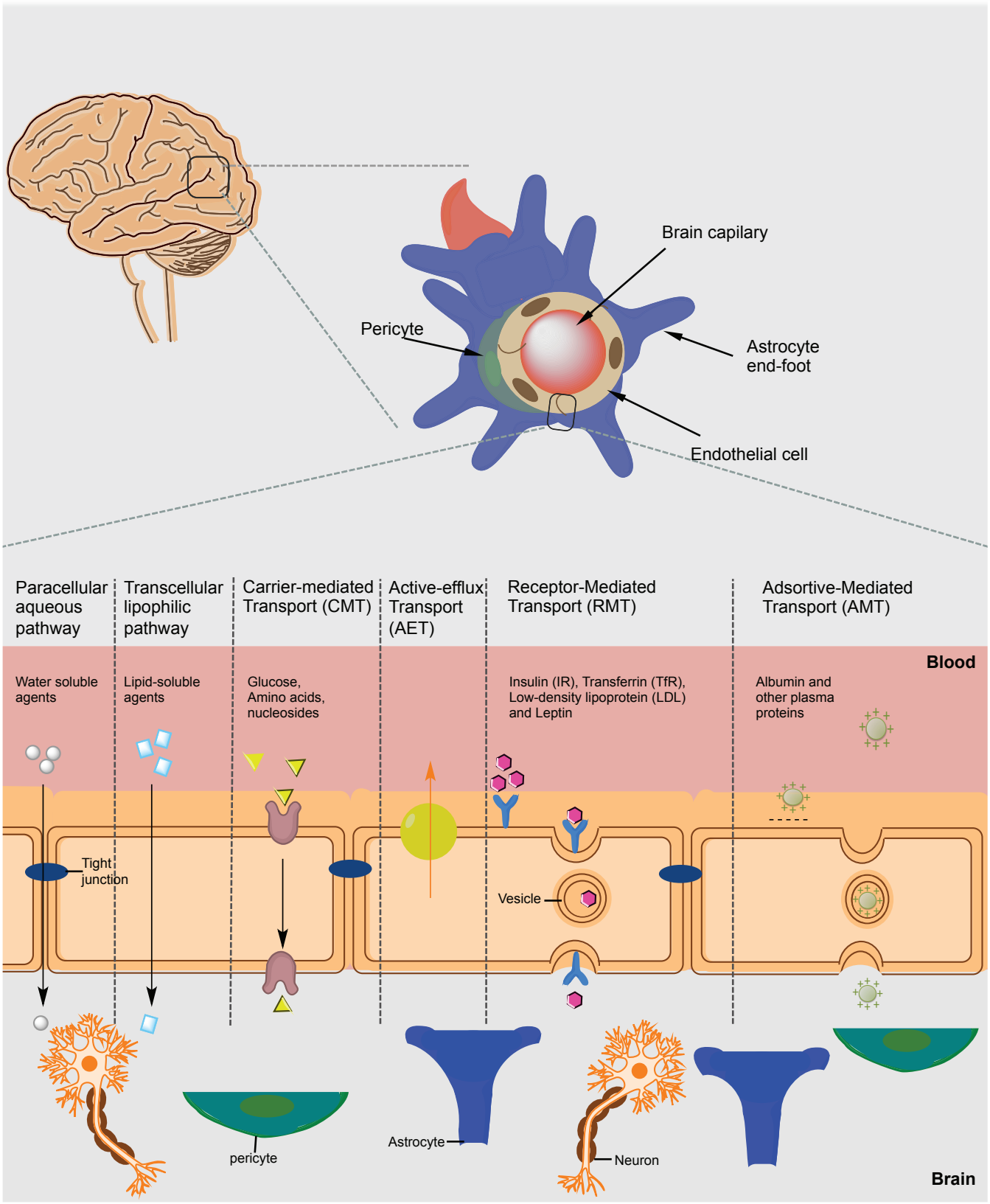

Brain capilary
Pericyte
Astrocyte end-foot
Endothelial cell
Paracellular aqueous pathway
Transcellular lipophilic pathway
Carrier-mediated Transport (CMT)
Active-efflux Transport (AET)
Receptor-Mediated Transport (RMT)
Adsortive-Mediated Transport (AMT)
Blood
Water soluble agents
Lipid-soluble agents
Glucose, Amino acids, nucleosides
Insulin (IR), Transferrin (TfR), Low-density lipoprotein (LDL) and Leptin
Albumin and other plasma proteins
Tight junction
Vesicle
pericyte
Astrocyte
Neuron
Brain

Figure 2

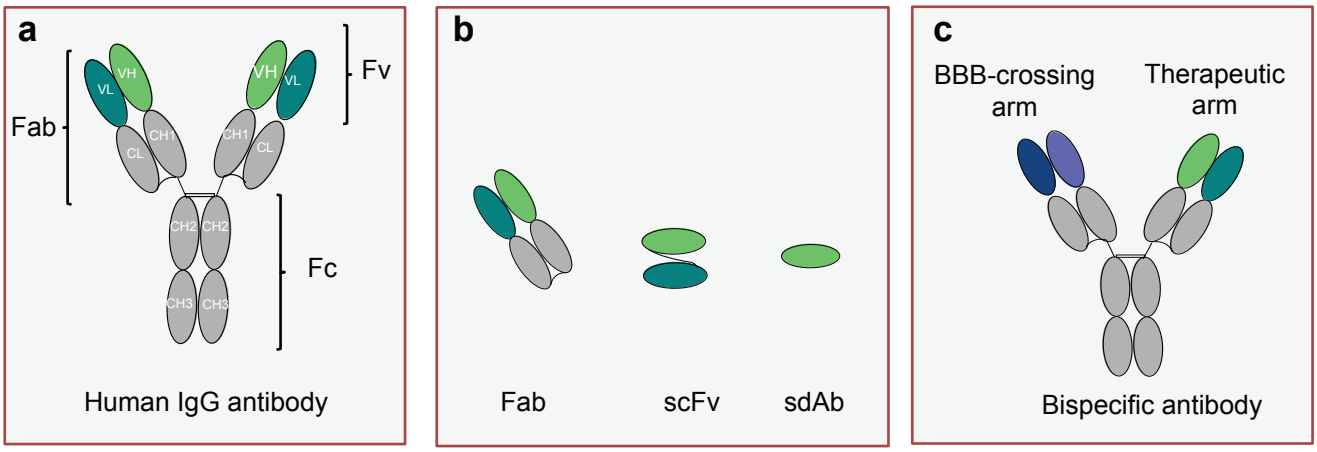
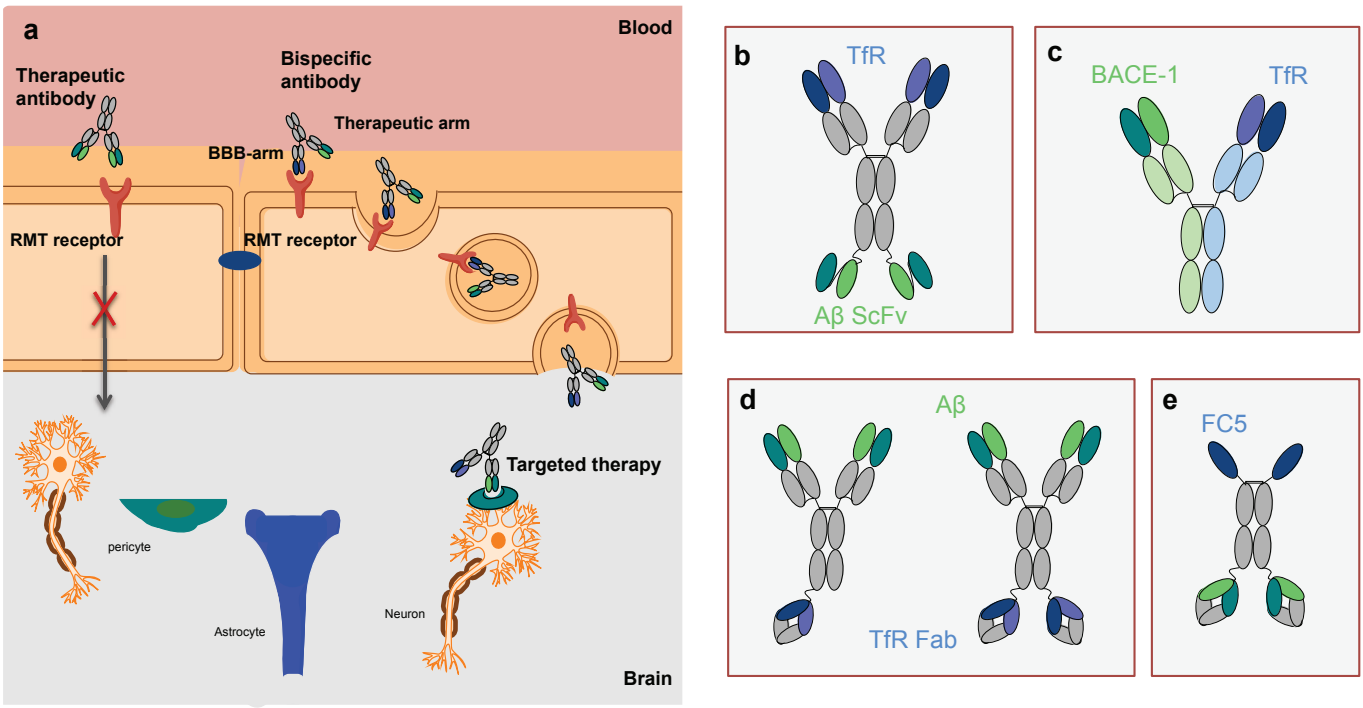
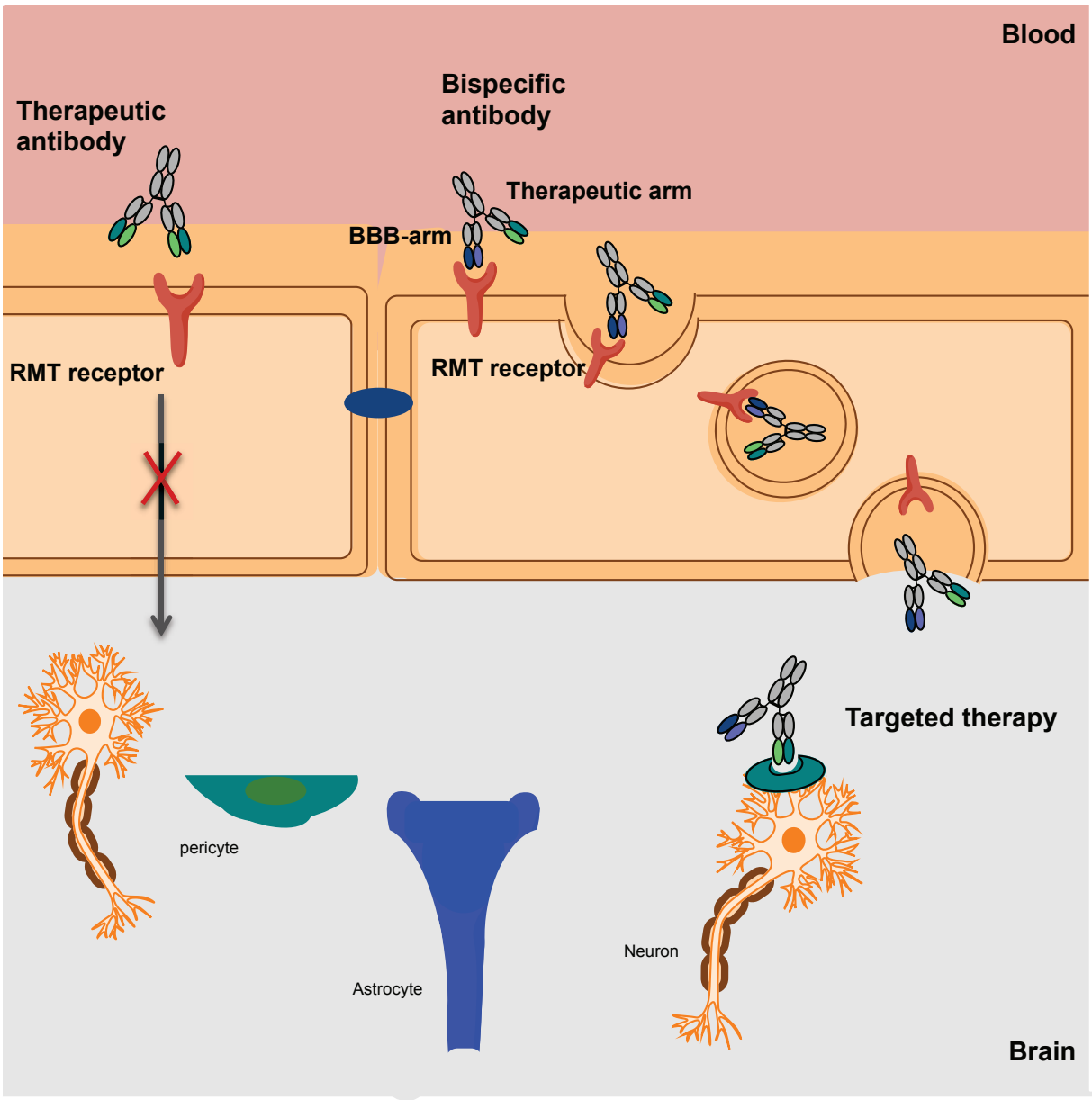

a
VH
VL
VH
VL
Fv
Fab
CH1
CL
CH1
CL
CH2
CH2
Fc
CH3
CH3
Human IgG antibody
b
Fab
scFv
sdAb
c
BBB-crossing arm
Therapeutic arm
Bispecific antibody

Figure 3


a
Blood
Therapeutic antibody
Bispecific antibody
Therapeutic arm
BBB-arm
RMT receptor
RMT receptor
Targeted therapy
pericyte
Astrocyte
Neuron
Brain
b
TfR
Aβ ScFv
c
BACE-1
TfR
d
Aβ
TfR Fab
e
FC5

Key element


Blood
Therapeutic antibody
Bispecific antibody
Therapeutic arm
BBB-arm
RMT receptor
RMT receptor
Targeted therapy
pericyte
Astrocyte
Neuron
Brain