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1 Targeting tight junctions to fight against viral neuroinvasion

2
3 Raphael Gaudin^{1,2,*}, Diana Brychka^{1,2}, Gregorius J. Sips³, Vanesa Ayala-Nunez^{4,*}

4
5 ¹ Institut de Recherche en Infectiologie de Montpellier (IRIM), CNRS, Montpellier, France

6 ² University of Montpellier, Montpellier, France

7 ³ Public Health Service Rotterdam-Rijnmond, Rotterdam, The Netherlands

8 ⁴ Swiss Federal Laboratories for Materials Science and Technology (Empa), St. Gallen,
9 Switzerland

10
11 *Correspondence: vanesa.ayala@empa.ch (V. Ayala-Nunez) and raphael.gaudin@irim.cnrs.fr
12 (R. Gaudin)

13 14 **Keywords**

15 Blood-brain barrier, central nervous system, neuroinvasion, neurotropic virus, tight junctions

16 17 **Abstract**

18 The clinical impact of viral neuroinvasion on the central nervous system (CNS) ranges from
19 barely detectable to deadly, including acute and chronic outcomes. Developing innovative
20 therapeutic strategies is important to mitigate virus-induced neurological and psychiatric
21 disorders. A key gatekeeper to the CNS is the neurovascular unit (NVU), a major obstacle to
22 viral neuroinvasion and antiviral therapies. The NVU isolate the brain from the blood through
23 firm sealing operated by the tight junctions (TJs) of endothelial cells. Here, we make the
24 thought-provoking assumption that TJs can be targets to prevent or treat viral neuroinvasion
25 and resulting disorders. This review aims at defining the conceptual diverse mode of actions
26 of such approaches, evaluate their feasibility and discuss future challenges in the field.

28 **Tight Junctions of the NVU: a ticket to the CNS**

29

30 Infectious diseases that affect the **central nervous system (CNS)** (see Glossary) are commonly
31 observed in clinical practice [1]. Neurotropic viral infection can cause acute or chronic
32 syndromes, including encephalitis, meningitis, meningoencephalomyelitis, Guillain-Barré-like-
33 syndromes, or stroke. In the case of viro-induced encephalitis, the incidence ranges from 1.5
34 to 7 cases/100,000 inhabitants/year, excluding epidemics [2]. This condition is severe, with
35 high mortality rates, as exemplified by a Danish study, estimating at 18.6% the cumulative
36 mortality rate of patients with herpes simplex encephalitis [3]. In the US for instance,
37 encephalitis hospitalizations result in 5.6% mortality, among which infectious agents account
38 for about 40% of death [4]. These studies highlight the importance of finding potent cures to
39 fight neurotropic viral infections.

40

41 Neurotropic viruses have to cross different cellular barriers to reach the CNS, a system well
42 protected against microbial invasion. Some viruses invade the CNS through direct viral
43 crossing of the **neurovascular unit (NVU)**, whilst some others might gain access via the choroid
44 plexus epithelia (the blood– cerebrospinal fluid (CSF) barrier), or non-hematogenous routes
45 of entry via transport within/along nerves.

46

47 The NVU encompasses a specialized group of blood capillaries and microcapillaries that
48 vascularize the CNS. The NVU is composed of brain endothelial cells forming these capillary
49 tubes, surrounded by pericytes, astrocytes, microglia, and neurons. This term is used to
50 address the fact that this group of cells does not form just a physical barrier that functions as
51 a passive filter, but that are an actively and dynamically regulated barrier that results from the
52 interaction of all its elements. The NVU is responsible for cerebral homeostasis through the
53 maintenance of a highly selective **blood-brain barrier (BBB)**.

54

55 The main strategies for viruses to directly cross the NVU are: 1) passive diffusion, where
56 viruses freely diffuse in between endothelial cells (e.g. dengue virus [5]), 2) infection of BMECs
57 and release of virus on the basal side (e.g. West Nile virus [6]), 3) transcytosis across non-
58 infected BMECs (e.g. Venezuelan- and Western equine encephalitis viruses [7]), and 4) blood-
59 to-tissue transmigration of leukocytes that serve as virus carriers (“Trojan horse” strategy)

60 (e.g. Zika virus [8]–[10]). Viruses may take advantage of one or several of these pathways,
61 simultaneously or sequentially. In most studies however, a single pathway is investigated due
62 to the extremely challenging methodological approaches required to isolate these events.
63 Further *in vitro* research is therefore required to decipher the molecular mechanisms
64 underlying viral crossing of the BBB.

65

66 One of the main biological properties of the NVU is its low permeability, as the **brain**
67 **microvascular endothelial cells (BMECs)** forming the vessels selectively restrict free
68 movement of ions, solutes, and cells between the brain and the blood, contributing to the
69 maintenance of the CNS microenvironment. **Tight junction (TJ) proteins** expressed on BMECs
70 play a major role in controlling vascular impermeability, as they contribute to the stability of
71 the unfenestrated tight structure formed by these cells (Box 1). Hence, virus hijacking of TJs
72 expressed on brain endothelial cells is a relevant strategy to invade the brain. In fact, viruses
73 from at least nine families of DNA and RNA viruses have been reported to use TJ proteins in
74 their benefit [11]. As a consequence, TJs are subcellular structures that we must consider as
75 potential targets to fight against neurotropic viral infections. To this end, a detailed strategy
76 on how to effectively target TJs to prevent or treat viral infection of the CNS remains to be
77 established (see Box 2. Clinician's Corner).

78

79 Here we review the available evidence supporting the feasibility of targeting TJs as host-
80 targeting agents (HTA) and discuss future challenges in the field. We focus on prophylactic
81 strategies that can be used to prevent viral neuroinvasion, but we also propose possible TJ-
82 related therapeutics to treat an already established CNS infection. A summary of the discussed
83 drugs is presented in Table 1.

84

85 **Strategy 1: Keeping the gate closed by preventing TJs alterations**

86

87 Endothelial impermeability disruption plays an important role in viral neuroinvasion by
88 opening the door for viruses to cross the endothelial barrier (Box 3). Hence, strategies that
89 modulate the expression of TJs to maintain NVU integrity can be an alternative to prevent
90 virus-induced alterations. The timing of permeability modulation is obviously critical, as, for

91 different viruses, CNS invasion takes place during different phases of an infection and differs
92 in duration.

93

94 NVU damage can be a direct cause of infection of the different cells of the NVU or by their
95 exposure to viral proteins, but it can also be indirectly caused by the host inflammatory
96 responses. **Matrix metalloproteinases (MMPs)** are a common mediator of this damage, and
97 are considered as part of the inflammatory response to CNS infections. These enzymes can
98 degrade the TJs between BMECs and also proteins from the extracellular matrix, modulating
99 disruption of the endothelial barrier and leukocyte transmigration [12]. Thus, inhibition or
100 inactivation of MMPs could be an indirect way of preventing vascular disruption by avoiding
101 the degradation of TJ proteins [12].

102

103 Antioxidants have been proposed as a therapeutic approach considering the role of reactive
104 oxygen species (ROS) in viral infection and the fact that they activate MMPs [13]. Antioxidant
105 enzymes like Cu/Zn superoxide dismutase or glutathione peroxidase prevented MMP-2 and -
106 9 mediated degradation of the TJ-associated protein claudin-5 triggered by HIV-1 gp120,
107 hence maintaining NVU integrity in a rat model [14]. In the same line, memantine, a NMDA
108 receptor inhibitor currently used as a treatment of Alzheimer's and Parkinson's disease [15],
109 also attenuates HIV-1 gp120-induced oxidative stress and NVU damage. Memantine prevents
110 claudin-5 and occludin MMP-mediated degradation in rats exposed to gp120 [16]. Although
111 the impact of memantine on HIV replication is unknown, this drug was recently shown to
112 inhibit the ion channel activity of the E protein of SARS-CoV-2 [17].

113

114 Peroxisome proliferator-activated receptor-gamma (PPAR γ) agonists, which down-regulate
115 the expression of different MMPs were tested in mice and in human BMECs infected with HIV-
116 1 or treated with Tat [18], [19]. They reduced vascular leakage by preventing a decreased
117 expression of **JAM-A, occludin, and ZO-1** by endothelial cells. PPAR γ agonists, such as the
118 thiazolidinediones, are used as treatment of type 2 diabetes, but they have not been
119 evaluated for their possible neuroprotective effect against viral infections in humans. The
120 antibiotic minocycline was an interesting candidate to treat virus-induced NVU damage via
121 MMP modulation. In mice, it reduces JEV-induced NVU damage by significantly decreasing the
122 activity of MMP-9 [20]. Besides, *in vitro* minocycline inhibits HIV replication in macrophages

123 and in macaques, it suppressed HIV-1 viral load in the brain and reduced the severity of
124 encephalitis [21]. However, when evaluated in a critical trial to assess its efficacy for the
125 treatment of HIV-associated cognitive impairment, no cognitive improvement was observed
126 [22].

127
128 Modulating MMPs, ROS and PPAR γ represent indirect ways to control for TJs integrity of the
129 NVU, but direct mitigation strategies should also be considered. For instance, neurotropic
130 picornaviruses, such as Poliovirus (PV) and the enterovirus A71 (EV-A71), have been shown to
131 alter NVU integrity, which results in enhanced virulence and promotes viral neuroinvasion
132 [23], [24]. The mechanism of action is mediated by virus-receptor interactions and thus,
133 strategies to prevent the interaction between a virus particle and its host cell receptor, such
134 as virus-targeting antibodies or soluble antagonist peptides, are likely to have dual effects on
135 both infection efficiency and neurovirulence.

136
137 In the past three years, several studies revisited the potential of anti-NS1 strategies to
138 attenuate DENV virulence. The viral protein NS1 of DENV induces systemic vascular leakage,
139 including BBB permeability [5]. The mechanism of action has been linked to major protease
140 activations, leading to endothelial glycocalyx and tight junction degradation [25], [26]. The
141 presence of NS1 antibodies correlates with lower disease severity in dengue patients [5], [27],
142 and robust protection against DENV-induced hemorrhage, coagulopathy, viremia, and
143 mortality [27], [28]. However, the development of anti-NS1 therapies to weaken DENV
144 severity has recently been challenged as researchers found that the pathogenic role of NS1 is
145 DENV strain dependent [29], the potency of DENV NS1 to permeate the NVU has been shown
146 to be much less effective than the NS1 protein of another Flaviviridae member west Nile virus
147 (WNV) [30]. WNV being a “true” neurotropic virus, one could envision to use anti WNV NS1
148 antibodies to treat WNV-induced encephalitis.

149
150
151 Regulating the immune response is an important strategy to prevent virus-induced disruption
152 of TJs. By modulating the activity of pro-inflammatory cytokines like TNF- α , IL-1, IL-4, IL-6, IFN-
153 γ , and IL-13, it would be possible to influence the expression level, the structure, and the
154 localization of TJ proteins [31].

155

156 Since TNF- α also plays a critical role in the control of viral infection, the depletion of this
157 cytokine might be counterproductive for controlling the infection [32]. Still, the administration
158 of antibodies against IP-10 (cytokine that induces the production of TNF- α) to JEV-infected
159 mice minimized the decrease in TJ proteins, restored NVU integrity and prevented CNS
160 damage [33]. Antibodies against IP-10 were tested in a phase 2 clinical trial against ulcerative
161 colitis, where they were proven to be effective and safe [34]. Currently, TNF blockers are
162 widely used to treat chronic inflammatory diseases in patients.

163

164 IL-6 is a common inflammatory mediator induced by different viruses. For example, during JEV
165 infection astrocytes can produce IL-6, VEGF and MMP-2/-9 which are responsible for ZO-1 and
166 claudin-5 degradation respectively [35]. Blocking IL-6 production with TLR3 blockers or STAT1
167 inhibitors has been associated with preventing claudin-5 downregulation and subsequent
168 inhibition of HIV-1-induced NVU dysfunction and increased monocyte transmigration [36],
169 [37]. Anti-IL-6 antibodies can attenuate ischemia-related increases in endothelium
170 permeability by modulating the expression of claudin-1 and occludin [38]. Interestingly,
171 tocilizumab and sarilumab, two types of anti-IL-6 receptor antibodies used to treat
172 rheumatoid arthritis and cytokine release syndrome, have recently been investigated in the
173 context of COVID-19 [39], [40]. However, biologically, IL-6 has both pro-inflammatory and anti-
174 inflammatory effects and is also involved in antiviral responses, and blocking its activity may
175 increase the chances of acquiring a secondary infection [41].

176

177 In the case of IFN- γ , neutralizing monoclonal antibodies blocking this pro-inflammatory
178 cytokine during reovirus or JEV infection attenuated vascular leakage and morphological
179 changes within the vasculature in mice [42], [43]. These data suggest that IFN- γ can be a
180 potential therapeutic target to prevent NVU disruption. Of note, however, because IFN- γ is
181 also an important player of adaptive immunity, further work is required to assess the potential
182 of this antiviral strategy in clinical settings.

183

184 Type I IFNs are a response to the sensing of viral RNA, which boost antiviral immune defenses
185 through the induction of interferon-stimulated genes (ISGs). Among its pleiotropic effects, IFN
186 was shown to promote Rac-1-dependent TJ formation and enhance NVU integrity to prevent

187 viruses from migrating across the endothelium [44]. Accordingly, IFN treatment reduces WNV-
188 induced vascular leakage in a mouse model [45]. Pegylated IFN- λ , a type III IFN that modulates
189 ZO-1 and claudin-5 in a STAT1-independent manner, protected mice against WNV
190 neuroinvasion and lethality [46]. Pegylated IFN- λ is being evaluated in clinical trials for HCV,
191 HDV and COVID-19 treatment. In this context, the effect of IFN on preserving NVU integrity
192 may be secondary, but it could have an additive contribution to its beneficial effects. Besides
193 IFNs, the usage of growth factors such as exogenous TGF- β 1 and human recombinant
194 hepatocyte growth factor diminishes vascular disruption by normalizing the expression level
195 of TJ proteins [47], [48].

196

197 Transcriptional regulation of TJ coding genes could specifically be targeted to increase their
198 expression. Enzastaurin (LY-317615), an inhibitor of protein kinase C β (PKC β), stabilizes the
199 compromised NVU via up-regulation of claudin-5 mRNA, resulting in inhibition of
200 transendothelial T-cell migration and possibly by suppression of cytokine production [49]. This
201 was shown *in vitro*, where treatment of BMECs with this inhibitor induced a fivefold increase
202 in the expression of claudin-5 mRNA [49]. Glucocorticoid receptor ligands and estrogen
203 receptor ligands can also up-regulate the mRNA and protein expression of occludin and
204 claudin-5 in BMECs [50], [51].

205

206 As a remote player of importance, the gastrointestinal microbiome has also been considered
207 as a modulator of NVU integrity. Gut bacteria transform nutrients into several metabolites,
208 like short-chain fatty acids, trimethylamines, amino acid derivatives, and vitamins, that have
209 been reported to modulate NVU integrity and/or brain function [52]. There are several
210 mechanisms by which gastrointestinal microbes and microbe-associated metabolites may
211 influence NVU integrity (for an extensive review, see [52]). For instance, probiotics influence
212 pro-inflammatory cytokine expression and can affect TJ protein expression and composition
213 (ZO-1, occludin, claudin-1, claudin-3, claudin-4, and claudin-5) in a mouse model of colitis [53].
214 This concept was later reinforced in germ-free adult mice (which display increased vascular
215 permeability), for which transplantation of fecal microbiota of pathogen-free donor mice
216 strengthened NVU integrity [54]. Considering the potential role of the gastrointestinal
217 microbiome on NVU function, some therapeutic approaches, such as prebiotics, probiotics, or
218 by fecal microbial transplantation are under consideration [52]. Whether this therapeutic

219 approach could be applied to lower the impact of viral infection onto the brain are lines of
220 research which are worth investigating further.

221

222 **Strategy 2: Blocking viral crossing of an intact non-disrupted NVU**

223

224 Viral neuroinvasion does not necessarily require NVU disruption (Figure 1). Viruses like HIV-1
225 and reoviruses have been proposed to invade the CNS not only by altering the expression of
226 TJs, but by directly using them [55], [56]. In this case, the use of inhibitors that would directly
227 bind and block the activity of these TJs is an alternative.

228

229 TJs used as viral receptors can be specifically targeted to prevent infection of BMECs, but also
230 to prevent transcytosis through the endothelium, although very few TJs expressed on BMECs
231 have been proposed as viral entry factors (Figure 1). *In vitro*, anti-claudin-1 antibodies and
232 claudin-1 miRNAs prevented HCV infection of human BMECs [57], [58]. However, these data
233 should be considered carefully since BMECs are not a usual target for HCV and their role in
234 HCV pathogenesis remains debated.

235

236 Transient interactions between TJs on the leukocyte and TJs on endothelial surfaces have been
237 proposed as a mechanism of virus-induced transmigration of infected cells [56], [59]. In
238 theory, TJ proteins expressed on the membrane of circulating leukocytes can compete with
239 endothelial TJs through their exposed extracellular loops, inducing the opening of the
240 endothelial TJs without disrupting the cellular barrier [60]. Williams *et al.* showed that primary
241 human monocytes exposed to HIV-1 have an increased expression of JAM-A, and that anti-
242 JAM-A antibodies selectively prevented transmigration of these monocytes through an *in vitro*
243 BBB model [61]. Blocking JAM-A with antagonist peptides has also been shown to inhibit
244 leukocyte-BMECs contacts in mice [62]. Glucocorticoid treatment reduces the levels of TJ
245 proteins in immune cells, thus reducing their infiltrative potential [60]. However,
246 glucocorticoids were reported to enhance replication of respiratory viruses *in vitro* and *in vivo*
247 experiments [63], highlighting the potentially detrimental impact of their use and the
248 importance of evaluating the effect of any of these drugs on virus infection.

249

250 **Strategy 3: A transient increase of brain vascular permeability can be therapeutic**

251

252 When the virus has already bypassed the NVU and established an infection in the CNS, can TJs
253 still be considered as targets to treat the infection? Here we evaluate whether temporarily
254 increasing vascular permeability could be useful to allow antiviral drugs to cross it.

255

256 The high impermeability of the NVU conferred by tightly sealed TJs of the brain capillary
257 endothelial cells is the main obstacle for drug delivery to the CNS. Thus, a therapeutic strategy
258 would be to transiently and selectively open the TJs of the endothelial cells for the passage of
259 macromolecules to the brain. This effect should be fast and reversible to minimize side effects
260 or toxicity resulting from a leaky barrier [64], [65]. This strategy could be particularly useful
261 in scenarios in which an acute infection is under control and the NVU is not already severely
262 compromised, e.g. in case of subacute or chronic infections. Of note, this option is relatively
263 daring and it is critical that deeper investigations evaluate the safety of such an approach
264 before proceeding to further developments.

265

266 HIV-associated neurological disorders (HAND) illustrate the potential usefulness of this
267 strategy. Evidence shows that HIV remains active within the CNS, even after efficient
268 pharmacological control of viral replication in the rest of the body. This is attributed to the
269 inability of several antiretroviral therapy to bypass the NVU and reach therapeutic
270 concentrations in the CNS [66].

271

272 An alternative to transiently decreasing endothelial paracellular impermeability is to target
273 specific components of the TJs with siRNAs or with peptidomimetic drugs [64]. A single
274 injection of claudin-5 siRNAs in mice caused no apparent harm and was well tolerated, while
275 it significantly increased endothelial permeability [67]. The peptidomimetic trictide, a
276 synthetic peptide derived from tricellulin ECL2, opens cellular barriers by interfering with the
277 TJ protein interactions [68], but its activity is not specific for brain endothelium and may be
278 problematic for therapeutic use. In contrast, peptidomimetic drugs based on the first
279 extracellular loop of claudin-5 allowed a transient permeabilization of the NVU in mice. After
280 treatment with these peptides, the number of TJ strand structures diminished and claudin-5
281 was downregulated and redistributed from cell-to-cell contacts to the cytosol [69].
282 Remarkably, this treatment has a relatively short temporal activity, ranging between 4 and 12

283 h post-injection [69]. As such, the beneficial/detrimental effect of claudin-5 modulation in the
284 context of neuroinfection deserves to be further investigated.

285

286 Viral and bacterial proteins with binding capacity to TJ proteins may potentially be exploited
287 as vehicles to direct drugs to the CNS or to specifically affect TJs during therapy [65]. Claudin-
288 5-binding mutants of the non-toxic C-terminal domain of the *Clostridium perfringens*
289 enterotoxin (cCPE) reversibly decreased transendothelial electrical resistance (TEER) and
290 permeability of endothelial cells *in vitro*, meaning that such mutants can potentially be used
291 to improve paracellular drug delivery across the BBB [70]. The NS1 protein of flaviviruses could
292 also be considered as a candidate to transiently increase brain endothelial permeability.
293 Although it has not been reported to directly bind to TJs, NS1 disruption of endothelial
294 glycocalyx components results in endothelial hyperpermeability [5]. Interestingly, the
295 different NS1 proteins of different flaviviruses cause tissue-specific vascular leakage in mice.
296 As a consequence, NS1 could provide the means to specifically target the endothelium of a
297 given tissue and decrease secondary effects derived from changing the permeability of the
298 whole vascular system. Protein injection has a relatively limited half-life in the blood. One may
299 expect a transient effect, although the therapeutic window requires to be carefully evaluated
300 in future investigations.

301

302 Lastly, one could imagine to enhance penetration of small molecules, antibodies, plasmids,
303 and viral vectors to the CNS through the use of Focused Ultrasound (FUS), a non-invasive
304 approach for site-specific opening of the brain endothelium [64]. The principle of the method
305 lies in the injection of microbubbles intravenously and apply burst-type ultrasound to a
306 specific region of the brain, which results in a transient disruption of the NVU [71]. FUS-
307 induced NVU disruption is associated with ultrastructural alterations of claudin-5, occludin,
308 and ZO-1 [72]. However, FUS was also shown to induce a sterile inflammatory response
309 accompanied by apoptotic neurons, activated astrocytes and microglia when applied on the
310 NVU [73]. Clinical trials utilizing this technique to improve CNS drug delivery are currently
311 underway [64].

312

313 **Concluding Remarks**

314

315 Therapies available for treatment of viral infections of the CNS are very limited, due in part to
316 the low penetration rate of many drugs and potential neurologic toxicity. Here, we are
317 proposing to evaluate alternatives to the classical antiviral strategies used to treat CNS
318 infection (summary in Figure 2). Specifically, TJ-associated proteins expressed on BMECs of
319 the human brain endothelial cells represent attractive therapeutic targets since their role in
320 hematogenous viral neuroinvasion via the NVU is pivotal.

321

322 Toxicity is a relevant issue when targeting a host factor. TJs serve an essential function in
323 different tissues, and ideally, TJ modulation should be specific for the brain endothelium and very
324 transient to avoid causing side effects or toxicity [64]. Hence, it becomes imperative to
325 improve the experimental models to assess toxicity and efficacy. When working *in vitro*, most
326 groups used BMECs without other cells from the NVU compartment. The use of human-
327 induced pluripotent stem cells (hiPSCs), that can be differentiated into all members of the
328 NVU, and versatile animal models, such as zebrafish, are excellent alternatives to study viral
329 brain endothelial crossing [74], [75]. These experimental models could provide physiological
330 insights to study viral neuroinvasion although they remain complex to develop in virology
331 laboratories (for review, see [76]).

332

333 As above-mentioned, using an antiviral treatment targeting brain endothelial TJs requires a
334 precise timing (see Outstanding Questions). Strategies 1 and 2 will only be effective if used in
335 a preventive manner. After the infection in the CNS is established and cell death or tissue
336 damage is apparent, these strategies would have little usefulness, although slowing the
337 continuous infiltration of leukocytes and immune modulators (chronic neuroinflammation)
338 could change the course of the disease. Some viruses, such as HIV, will infect significantly the
339 brain only days to weeks after infection, and in this case, strategy 2 may be very beneficial.
340 Conversely, Strategy 3 should not be considered immediately post-exposure to the virus as it
341 would rather potentiate neuroinvasion.

342

343 Stabilizing TJs to avoid NVU disruption (Strategy 1) might not be the best alternative to treat
344 all viral CNS infections. In the case of rabies virus, brain capillary leakage allows recruitment
345 of immune cells that will attempt to clear the virus or infected cells from the CNS [77]. In this
346 case, tightening the barrier would be counter effective for clearing the infection. This example

347 illustrates the need of identifying the etiological agent in the clinical setting. However, for CNS
348 infections, establishing a cause can be challenging [78], [79].

349

350 A transient NVU disruption via TJ modulation (Strategy 3) would also be an interesting option
351 in combination with antiviral compounds. HIV-infected patients, for example, may benefit
352 from tailored strategies preventing viral neuroinvasion whilst facilitating therapeutic entry
353 since CNS penetration of infected immune cells may cause severe neurological disorders.

354

355 In conclusion, targeting TJs of the brain endothelial cells may sound like a daring approach at
356 first, but this is mostly due to the lack of comprehensive studies on the subject. Going beyond
357 our current knowledge and lifting current conceptual barriers through exploratory research
358 projects shall provide novel therapeutic strategies to be considered with care.

359

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362 (ANRS).

363

364 **Conflict of interest**

365 The authors have no conflict of interest to declare.

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Box 1. Tight junctions of the NVU

The BMECs that are part of the NVU are characterized by their low endocytosis rate, extensive transport machinery, and elevated production of tight junction (TJ) proteins. TJs are located close to the apical membrane of polarized endothelia where they form strands of extensive, overlapping occlusions between BMECs. TJ complexes consist of three groups of transmembrane proteins (Figure 1): claudins, TJ-associated MARVEL proteins (TAMPS, which include occludin, tricellulin and MarvelD3), and the immunoglobulin superfamily (IgSF) membrane proteins (such as JAM-A/-B/-C, coxsackie adenovirus receptor (CAR), and endothelial cell-selective adhesion molecule (ESAM)) [80]. Cytoplasmic scaffolding proteins connecting the cytoskeleton to the TJ transmembrane proteins are part of the TJs. One of the main TJ-associated cytosolic proteins is the zonula occludens family (ZO-1, -2, -3), which plays a major role in maintenance of TJ-barrier functioning by anchoring most of these transmembrane proteins to the actin cytoskeleton and being a scaffold for Rho family GTPases and transcriptional factors [81]. Cingulin is another important cytoplasmic component of TJs that is involved in the formation and regulation of TJ permeability.

TJ proteins are expressed by endothelial cells from all tissues. What makes the endothelial cells of the NVU different from the rest of the vasculature is that 1) they express high levels of TJ proteins and 2) their combination of expressed TJ-associated proteins is unique (Figure 1). The highly expressed TJ proteins form a physical barrier that gives the endothelia of the NVU its impermeability. This tight construction is observed *in vivo* as an electrical resistance of ~ 1500–2000 $\Omega\cdot\text{cm}^2$ in brain capillaries, which is considerably higher than peripheral vessels that have a resistance of 3–30 $\Omega\cdot\text{cm}^2$ [64].

The main TJ protein subset expressed by BMECs are occludin, claudin-3, -5 and -12, JAM-A, ZO-1 and -2, and cingulin (Figure 1) [82]. In particular, claudin-5 is the most enriched protein [83]. This TJ-protein is critical for brain impermeability, which has been observed in claudin-5-deficient mice that die shortly after birth due to brain edema [84].

Box 2. Clinician's Corner

* Viral infections of the CNS are commonly observed in clinical practice with typical clinical presentations of meningitis, encephalitis, myelitis and more rarely meningoencephalitis.

Some infected patients present neurological sequelae such as long-term cognitive impairment, short-term memory loss, psychiatric morbidity and chronic fatigue syndrome [85].

* The most common viruses causing CNS infections in immunocompetent adults are herpes simplex virus (HSV), varicella zoster virus (VZV), and enterovirus. Other important neurotropic pathogens are arboviruses, like Japanese encephalitis virus (JEV), West Nile virus (WNV), tick-borne encephalitis virus (TBEV), eastern equine encephalitis (EEE) and Zika

407 virus (ZIKV), whose emergence or re-emergence have been in a steady increase in recent
408 years [86], [87]. However, there are just seven approved drugs to treat viral CNS infections
409 in humans and they are mainly used against herpes viruses, acyclovir being the most
410 common [88]. Therefore, it is important to develop new alternatives to cope with this
411 situation.

412 * Several neurotropic viruses are able to invade the CNS by modifying the tight junctions (TJs)
413 expressed on the endothelial cells from the BBB. Hence, these TJs can serve as relevant
414 clinical targets to prevent viral neuroinvasion, as well as to allow influx of antiviral drugs.

415 * Targeting of TJs expressed between endothelial cells would result in changes in the BBB
416 permeability. A decrease in permeability can be induced in an acute infection to prevent
417 neuroinvasion. On the other hand, an increase in BBB permeability can be beneficial in
418 chronic infections since it will allow entry of antivirals. Drugs that are being used for non-
419 infectious neurological disorders and target TJs could be considered to be used against virus
420 neuroinvasion.

421 * Patients with a CNS infection may benefit from tailored strategies that prevent viral entry
422 and facilitate penetration of therapeutics, since the CNS can be a difficult-to-treat site of
423 latency [89].

424

425 **Box 3. How do viruses use TJs to cross the NVU?**

426 *1. Digging holes: changes in TJ protein expression and vascular disruption*

427 Clinical observations and autopsy studies of patients with a neurological viral infection have
428 shown that disruption of the NVU can be part of the disease neuropathogenesis [12], [74].
429 NVU alterations have been correlated with - and can be explained by - an altered phenotype
430 of TJ proteins on BMECs (a decreased TJ total protein expression, protein relocalization,
431 altered function, and/or degradation) [12], [74] (Figure 2), which would weaken the vascular
432 structure, resulting in a (temporary) disruption of its permeability. Whether NVU disruption is
433 the cause or consequence of the neurological infection is not clear. One option is that viruses
434 first cross the NVU without altering its permeability and subsequently infect cells from the
435 CNS, which would trigger a series of events that results in BBB damage [6], [90]–[92]. Another
436 option is that BBB disruption happens first, opening the door for the virus to passively diffuse
437 between the BMECs and gain access to the CNS [5], [93]. Either way, BBB disruption can result
438 in the presence of infectious virus particles, immune cells, and inflammatory mediators in the
439 CNS parenchyma, leading to neuronal dysfunction or tissue destruction [94].

440

441 *2. Crossing without disrupting the NVU (TJs as viral receptors and the Trojan horse strategy)*

442 Viruses can cross the NVU without affecting the expression or function of TJs on the BMECs.
443 TJs could be used as receptors or co-receptors to access the brain parenchyma following
444 infection of BMECs or transcytosis [12], [57] (Figure 2). However, direct evidence of viral
445 particles interacting with TJs of the BBB is scarce. The use of leukocytes as Trojan horses
446 (Figure 2) has been proposed for important human neurotropic pathogens like HIV-1, WNV or
447 ZIKV. It can take place before NVU disruption, as it is a physiological process performed by

448 leukocytes during normal immune surveillance. From a mechanistical point of view, the role
449 of TJs on leukocyte transmigration can be explained in two ways: 1) by homotypic interactions
450 between TJ proteins expressed on the leukocyte and their counterpart on BMECs, which
451 would induce an opening of the TJs in a zipper-like manner,[56] or 2) by extracellular vesicles,
452 which may act as a vehicle for transferring TJ proteins from endothelial to immune cells, which
453 would facilitate leukocyte transmigration into the CNS [59], [83].

454

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456

457 **Figure legends**

458

459 **Figure 1. TJs proteins in the NVU.** (A) Comparison between brain capillaries that build the NVU
460 and capillaries from peripheral organs. (Top panel) In peripheral capillaries, the intercellular
461 junctions between endothelial cells are relatively permeable and the cells are often
462 “fenestrated” (i.e. formation of transcellular holes). Diverse solutes in blood can cross this
463 endothelium in between cells or through the cells. (Bottom panel) In contrast, the endothelial
464 cells of the brain capillaries (BMECs) are sealed by TJs that are highly expressed in this tissue.
465 Besides, the endothelial cells are not fenestrated. The TJs form a physical substrate that
466 restricts diffusion of solutes between the blood and brain. The endothelial cells are in close
467 contact with astrocytes, pericytes, microglia and other cells from the NVU, which is important
468 for induction and maintenance of the barrier properties. (B) The main TJ proteins expressed
469 in endothelial cells of the NVU are occludin, claudin-3, -5 and -12, JAM-A, ZO-1/-2, and
470 cingulin, being claudin-5 the most abundant. Created with BioRender.

471

472 **Figure 2. Strategies to target TJs to prevent or treat neurological viral infections.** (A) Strategy
473 1 is focused on preventing infection of the CNS by avoiding disruption of the NVU induced by
474 virus-related alterations of the TJs. We propose to either inhibit the infection of BMECs,
475 modulate the activity of MMPs, or block the production or inactivate pro-inflammatory
476 cytokines and viral proteins. This will result in preventing changes in the pattern of expression
477 of the TJs connecting the BMECs (reduced expression, degradation, and/or mislocalization) or
478 the inactivation of their function that are triggered by viral infection of the cells, and exposure
479 to viral proteins (e.g. HIV-1 Tat, HIV-1 gp120, DENV NS1) or cytokines (e.g. TNF- α , IL-1, IL-4, IL-
480 6, IFN- γ , and IL-13). An altered TJ phenotype increases vascular permeability, enabling viruses
481 to cross in between endothelial cells via diffusion of single virus particles or through an
482 increased transendothelial migration of leukocytes (Trojan Horse strategy). (B) In Strategy 2,
483 we propose to prevent infection by blocking virus crossing of an undisrupted NVU, where TJ
484 expression on BMECs is not altered and crossing can be mediated by leukocytes as Trojan
485 Horses. It has been proposed that the leukocytes that are infected or carry the virus express
486 TJ proteins on their cell membrane that will interact with the TJs from the BMECs, inducing a
487 temporary opening of the barrier without damaging it. Virus crossing through an undisrupted
488 BBB could also take place via transcytosis, where TJs can function as cell receptors. However,

489 there is no evidence supporting this mechanism. (C) In Strategy 3, we propose to target TJs to
490 temporarily increasing vascular permeability at the NVU to allow antiviral drugs to cross it. Its
491 application is relevant in chronic infections, when the virus has already bypassed the NVU and
492 established an infection in the CNS. Created with BioRender.

493

494 **Table 1.** Compounds, Tight junction-related mode of action, and effect of viral infection.

495

Treatment strategy	Compound	TJ-related mode of action	Effect on viral infection	Experimental model	Applications in patients	Reference
Strategy 1 - Keeping the gate closed by preventing TJs alterations	Cu/Zn superoxide dismutase or glutathione peroxidase	Antioxidant enzymes. Prevents MMP-2 and -9 mediated degradation of laminin and claudin-5.	Protected from HIV-1 gp120-induced BBB damage	Rat model	Treatment of radiation cystitis, osteoarthritis, and Peyronie's disease	[14]
	Memantine	NMDA receptor inhibitor. Prevents claudin-5 and occludin MMP-mediated degradation.	Protected from HIV-1 gp120-induced BBB damage	Rat model	Treatment of spasticity and Parkinson's disease	[16]
	Peroxisome proliferator-activated receptor-gamma (PPAR γ) agonists	Down-regulate MMPs, preventing decreased expression of JAM-A, occludin, and ZO-1.	Reduced HIV-1- or Tat-induced dysfunction of BBB	Mouse model, <i>in vitro</i> BMECs	Treatment of type 2 diabetes	[18], [19]
	Minocycline	Tetracycline antibiotic. Decreases the activity of MMP-9.	Protected from JEV-induced BBB damage, blocks HIV replication in macrophages	Mouse model, macaques	Treatment of different bacterial infections	[20], [21]
	Anti-NS1 antibodies	Monoclonal antibodies against the NS1 protein of WNV	Protected mice against WNV-induced disease	Mouse model	N.A.	[95]
	Anti-IP-10 antibodies	Monoclonal antibodies that reduce the production of TNF- α , preventing reduced expression of occludin, claudin-5 and ZO-1.	Protected from JEV-induced BBB disruption and CNS damage	Mouse model	Clinical trial for ulcerative colitis	[33], [34]
	TLR3 blockers	Inhibitors of dsRNA binding to TLR3, which results in the blockage of expression of IL-6, preventing claudin-5 downregulation, monocytes adhesion and transendothelial migration.	N.A.	<i>in vitro</i> BMECs	N.A.	[36]
	Anti-IL-6 antibodies	Attenuate ischemia-related increases in BBB-permeability and modulate TJs (claudin-1 and occludin) expression.	N.A.	Fetal sheep model	Treatment of rheumatoid arthritis, juvenile idiopathic arthritis and Castleman disease.	[38]
	Anti-IL6R antibodies	Prevent the IL6-caused down-regulation of occludin and claudin-5.	N.A.	Mouse model, <i>in vitro</i> BMECs	Treatment of rheumatoid arthritis, juvenile idiopathic arthritis and Castleman disease. In clinical trials for COVID-19 treatment.	[96]
	Anti IFN-g antibodies	Monoclonal antibodies against IFN- γ . Prevent claudin- and ZO-1-disorganization.	Protected from reovirus-induced BBB-disruption and CNS damage	Mouse model, <i>in vitro</i> BMECs		[43]
	Type I IFNs	Promote BBB-stability by regulating the expression of TJs (ZO-1 and claudin-5).	Reduced WNV-induced BBB-leakage (mouse model)	Mouse model, <i>in vitro</i> BMECs	Treatment of multiple sclerosis	[45]

	Pegylated IFN- λ	A type III IFN that modulates TJ protein localization (ZO-1 and claudin-5) in a protein-synthesis- and STAT1-independent manner.	Protected against WNV neuroinvasion and lethality	Mouse model, <i>in vitro</i> BMECs	Clinical trials for HCV, HDV and COVID-19 treatment	[46]
	Enzaustarin	Inhibitor of protein kinase C β (PKC β), also known as LY-317615. Stabilizes the BBB by up-regulation of claudin-5 mRNA.	N.A.	Mouse model of experimental autoimmune encephalomyelitis	Clinical trial for brain cancer treatment	[49]
Strategy 2 - Blocking viral crossing of an intact non-disrupted BBB	Anti-claudin-1 antibodies	Monoclonal antibodies	Prevented HCV infection	<i>in vitro</i> BMECs	N.A.	[57]
	Claudin-1 miRNAs	Block claudin-1 protein expression.	Prevented HCV infection	<i>in vitro</i> hepatocytes	N.A.	[58]
	Anti-JAM-A antibodies	Monoclonal antibodies	Selectively prevented transmigration of monocytes exposed to HIV-1.	<i>in vitro</i> BMECs and monocytes	N.A.	[61]
	JAM-A antagonist peptide	Inhibits JAM-A/leukocyte interaction by blocking the interaction of the C2 domain of JAM-A with LFA on neutrophils and monocytes/macrophages.	N.A.	Mouse model	N.A.	[62]
	Glucocorticoids	Decrease claudin-5 protein levels in human T cells, B cells and monocytes.	N.A.	Human blood samples	Treatment of multiple sclerosis, asthma and chronic obstructive pulmonary disease	[60]
Strategy 3 - A transient increase of BBB permeability can be therapeutic	Claudin-5 siRNAs	Transiently decrease claudin-5 expression and increase BBB-permeability in mice (up to 48 h).	N.A.	Mouse model	N.A.	[67]
	Claudin-5 peptidomimetics	Based on the first extracellular loop of claudin-5. Transiently increase BBB-permeability in mice (from 3 to 12 h).	N.A.	Mouse model	N.A.	[69]
	Claudin-5 binding peptides	Claudin-5-binding mutants of the non-toxic C-terminal domain of the <i>Clostridium perfringens</i> endotoxin. Enable a mild and transient opening of brain endothelial TJs.	N.A.	<i>in vitro</i> co-culture of BMECs and astrocytes	N.A.	[70]
	NS1 proteins - flaviviruses	Do not bind to TJs, but disrupt endothelial glycocalyx components, leading to increased BBB-permeability.	N.A.	Mouse model	N.A.	[5]

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499 **Glossary**

- 500 • **BBB (blood-brain barrier):** Group of blood vessels and associated cells (see NVU) that
501 vascularize the central nervous system. It serves as a diffusion barrier that separates
502 the circulation from the brain, and that tightly regulates the crossing of solutes,
503 molecules and immune cells.
- 504 • **BBB disruption:** Reduction in BBB tightness and increase in leakiness derived from the
505 loss of BBB integrity.
- 506 • **BMECs (brain microvascular endothelial cells):** Group of endothelial cells that line the
507 blood vessels forming the BBB. Essential part of the NVU.
- 508 • **Central nervous system (CNS):** Part of the nervous system that consists of the brain
509 and spinal cord.
- 510 • **Claudins:** Main family of tight junction proteins. They have a tetraspan transmembrane
511 structure. More than 20 types have been described, being expressed in a tissue-
512 specific manner.
- 513 • **Claudin-5:** Most enriched TJ protein expressed on endothelial cells from the BBB. It is
514 also expressed on endothelial cells from the lung, liver, kidney and skin.
- 515 • **Junctional adhesion molecule (JAM-A/-B/-C):** Member of the immunoglobulin
516 superfamily of tight junctions. JAMs have a role in cell polarity, endothelium
517 permeability and migration of leukocytes.
- 518 • **Matrix metalloproteinases (MMPs):** Zinc-dependent endopeptidases that can directly
519 degrade the TJs between endothelial cells and proteins from the extracellular matrix,
520 modulating disruption of the BBB and leukocyte transmigration. MMPs are secreted
521 into the extracellular space by BMECs, leukocytes, and CNS resident cells.
- 522 • **Neurovascular unit (NVU):** Structure conformed by BMECs, neurons, microglia,
523 pericytes, astrocytes, and the extracellular matrix. Its elements are in close contact and
524 have both a structural and functional relation.
- 525 • **Occludin:** First tight junction protein to be identified. It has four transmembrane
526 domains. Occludin contributes to TJs stability and barrier function.
- 527 • **Tight junctions (TJs):** Protein structures located close to the apical membrane of
528 polarized endothelia and epithelia where they form strands of extensive, overlapping
529 occlusions between cells. TJs consist of three groups of transmembrane proteins:
530 claudins, TJ-associated MARVEL proteins (TAMPS) (including occludin, tricellulin and

531 MarvelD3), and the immunoglobulin superfamily (IgSF) membrane proteins (such as
532 JAM-A/-B/-C, coxsackie adenovirus receptor (CAR), and endothelial cell-selective
533 adhesion molecule (ESAM)). They contribute to the barrier function of the BBB by
534 tightening up the endothelial layer.

- 535 • **Zona occludens (ZO-1/-2/-3)**: Cytoplasmic accessory proteins of tight junctions that
536 function as protein binding molecules.

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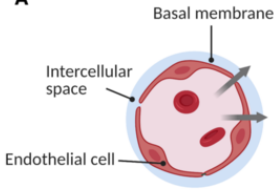
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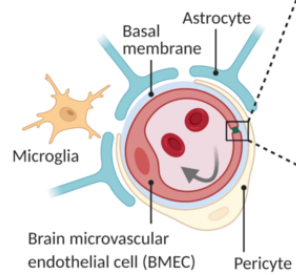
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- 817

A Peripheral capillary



Brain capillary



B

Main TJ proteins in the NVU

