Current Topics

Recent Advances in Research on Vascular Permeability to Establish Novel Therapeutic and Drug Delivery Strategies for Intractable Diseases

Review

Claudin-5: A Pharmacological Target to Modify the Permeability of the Blood–Brain Barrier

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Claudin-5 is the dominant tight junction protein in brain endothelial cells and exclusively limits the paracellular permeability of molecules larger than 400 Da across the blood–brain barrier (BBB). Its pathological impairment or sustained down-regulation has been shown to lead to the progression of psychiatric and neurological disorders, whereas its expression under physiological conditions prevents the passage of drugs across the BBB. While claudin-5 enhancers could potentially act as vascular stabilizers to treat neurological diseases, claudin-5 inhibitors could function as delivery systems to enhance the brain uptake of hydrophilic small-molecular-weight drugs. Therefore, the effects of claudin-5 manipulation on modulating the BBB in different neurological diseases requires further examination. To manipulate claudin-5 expression levels and function, several claudin-5 modulating molecules have been developed. In this review, we first describe the molecular, cellular and pathological aspects of claudin-5 to highlight the mechanisms of claudin-5 enhancers/inhibitors. We then discuss recently developed claudin-5 enhancers/inhibitors and new methods to discover these molecules.

Key words tight junction; blood–brain barrier; claudin-5; drug delivery system; vascular stabilization

1. INTRODUCTION

The neural and glial microenvironment of the brain is regulated and maintained by the blood–brain barrier (BBB), which is one of the strongest cellular barriers in humans. The BBB is formed by microvascular endothelial cells (ECs) lining cerebral capillaries that are surrounded by pericytes and astrocytic endfeet. Unlike peripheral microvascular ECs, which have open paracellular spaces for passive diffusion of molecules between the blood and the organ interstitium, cerebral microvascular ECs have well-developed tight junctions (TJs) to seal the paracellular space and undergo minimal pinocytosis activity to prevent uncontrolled entry of blood-borne molecules, including drugs, into the brain. Approximately 98% of all developed small-molecular-weight drugs are not able to cross the BBB.[1)] Overcoming the restriction of drug permeation into the brain by the BBB is a difficult challenge for chemical therapies designed to treat central nervous system (CNS) diseases.

TJs consist of mesh-like strands of multiple intramembrane particles that are mainly composed of claudin (CLDN) family members. CLDN has 27 family members in mouse and human, and CLDN oligomerizes in a heterophilic and homophilic manner in a *cis*- (within the plasma membrane of one cell) and *trans*- (across the plasma membranes of adjacent cells) interaction manner. The strength and complexity of TJ strands are determined by the combination of CLDN family members, which are expressed in a tissue-specific manner. Among CLDN family members, CLDN-5 is predominantly expressed in ECs, especially brain ECs,[2)] and therefore it is thought that the paracellular permeability of the BBB is largely determined by the expression levels of CLDN-5. An initial study using CLDN-5 knockout (KO) mice clearly showed that expression of CLDN-5 in the BBB is essential for preventing the entrance of molecules with molecular weights between 400 and 800 Da in the brain.[3)] The size-selective modulation of BBB permeability has an advantage in comparison to clinically performed methods for increasing BBB permeability, namely intra-carotid hyperosmolar mannitol administration. Mannitol administration completely disrupts the BBB by withdrawing water from ECs and enabling the entrance of blood-borne proteins into the brain.[4,5)] Due to the side-effects associated with mannitol-induced BBB disruption, hyperosmolar mannitol administration is only generally used for severe clinical situations, such as the delivery of anti-cancer drugs to patients with brain tumors, and the drawing of free water from brain parenchyma in patients with brain injury-induced cerebral edema.[6)] Thus, it is thought that size-selective modulation of BBB permeability with pharmaceuticals targeting CLDN-5 could represent a novel therapeutic system to treat brain tumors and other CNS diseases.

Increased BBB permeability and downregulation of CLDN-5 can be observed in advanced, incurable stages of many CNS diseases. The pathogenic alteration of BBB permeability can be mediated by changing the brain microenvironment, such as in the presence of excess amyloid-β deposition,[7)] neurotransmitter release[8)] and/or the entry of blood-borne factors such as immune cells.[9)] Blood-borne factors themselves or their receptors in ECs represent potential therapeutic targets.

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Of note, several studies suggest that increased paracellular permeability of the BBB mediated by changing the neural and glial microenvironment is an early pathogenic event in some primary psychiatric disorders and dementias.[10–13] A molecule capable of normalizing BBB permeability can be expected to become a novel therapeutic that prevents the progression of some CNS diseases.

Some previous studies proposed that inhibiting CLDN-5 expression/function would be a novel technique to deliver small-molecular-weight drugs into the brain. Conversely, molecules that can selectively enhance CLDN-5 expression could be used as a TJ stabilizer to treat psychiatric diseases and dementia. Currently, CLDN-5 is considered a promising drug target for the treatment of numerous CNS diseases. This review focuses on CLDN-5 protein and its role in the BBB under physiological and pathological conditions. Furthermore, this review summarizes newly developed CLDN-5 enhancers/inhibitors and recent techniques for the development of CLDN-5 modulating molecules.

2. MOLECULAR ASPECT: STRUCTURE AND FUNCTION OF CLDN-5

The CLDN family members are tetra-transmembrane spanning proteins. CLDN-5 has a large extracellular loop (ECL) composed of 4 β-strands and an extracellular helix (ECH1) and a small ECL composed of a β-strand and a helix extended from transmembrane domain 3 (ECH2) (Fig. 1). ECH1, ECH2 and β4 are required to form *cis*-interactions, while the two flexible loops located between β1 and β2, and ECH2 and β5 are required to form *trans*-interactions. Newly synthesized CLDNs are initially recruited into non-junctional plasma membrane, then translocate to the junctional area following dimerization/oligomerization by *cis*-interactions, and finally form *trans*-interactions at cellular clefts, resulting in the formation of TJ strands.[14,15] In brain ECs, CLDN-5 is mainly localized at TJs and non-junctional CLDN-5 can also be observed.[16] To date, no disease associated amino acid sequence variations in CLDN-5 resulting from single-nucleotide polymorphisms (SNP) in human have been reported; however, future genome-wide association studies might identify rare SNPs.

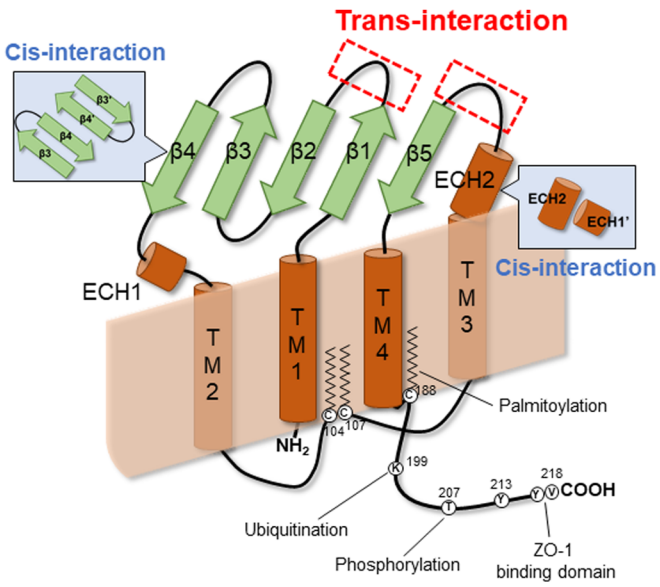

Fig. 1. Schematic Illustration of CLDN-5

The secondary structure of CLDN-5 is shown with highlighting of important amino acids and domains. The α-helices are represented by cylinders and β-sheets are represented by arrows. The domains associated with *trans*-interaction are highlighted by regions boxed with red dashed lines and the domains associated with *cis*-interaction are highlighted by text boxes. The amino acids associated with post-translational modifications are shown with their positions. ECH, extracellular helix; TM, transmembrane domain. (Color figure can be accessed in the online version.)

The intracellular N-terminus of CLDN-5 is very short (<5 amino acids). However, an N-terminally extended form of CLDN-5 (isoform 1, total length 303 amino acids) is registered in the National Center for Biotechnology Information (NCBI) database. An upstream AUG codon has been predicted to be functional using a prediction tool[17]; however, the major transcription initiation site of *CLDN5* is located between AUG codon of the long and short isoforms (see database https://dbtss.hgc.jp/). Therefore, the predominant mRNA variant (variant 4) contains only an open reading frame for the shorter isoform (isoform 2, total length 218 amino acids) consisting of a single exon. Notably, only the shorter CLDN-5 isoform has been detected by Western blotting. Forced expression of the longer CLDN-5 isoform showed that the longer isoform was not translocated to plasma membrane efficiently.[18] Some pathological conditions might enhance the transcription of the longer isoform, but no examples have been reported to date.

The C-terminal intracellular domain of the CLDN family controls their junctional localization and cellular half-life.[15] CLDN-5 can be palmitoylated at C188,[19,20] which can promote efficient localization into TJs.[20] Almost all CLDN family members (including CLDN-5) have a YV-motif at their C-terminus. The YV-motif binds to the N-terminal PDZ domain of zonula occludens proteins (PDZ1) of the ZO family of TJ scaffolding proteins (Fig. 2). ZO family members connect junctional membrane proteins with the actin cytoskeleton. The phosphorylation of T207, and possibly Y213, disturbs CLDN-5-ZO-1 interactions and causes internalization of CLDN-5.[21–24] Rho-associated kinases (ROCKs) can phosphorylate T207 of CLDN-5. Moreover, the ubiquitination status of K199 determines the destiny of internalized CLDN-5, whether it is recycled to the plasma membrane or transported to proteasomes.[25] Furthermore, CLDN-5 can be degraded by other degradation pathways, such as the ubiquitin-independent autophagosomal and lysosomal pathways.[25,26] The estimated half-life of endogenous CLDN-5 in ECs is very short (<90 min), but may be greatly changed by certain experimental conditions.[25,27]

3. CELLULAR ASPECT: EVIDENCE SUPPORTING CLDN-5 EXPRESSION AND LOCALIZATION IN BRAIN ECS

3.1. Other Junctional Proteins Required for Correct Junctional Localization of CLDN-5 Among the CLDN family members, CLDN-5 is predominantly expressed in brain ECs, with CLDN-12 and -25 are also expressed at much lower levels.[2,28] Neither CLDN-12 nor -25 contain a ZO-1 binding domain, indicating that they do not compete with CLDN-5 for interactions with ZO-1.[29] The presence of other CLDN family members in brain ECs have also been reported; however, the experimental evidence for their presence remains controversial.[30–33] These CLDNs can form *cis*-interaction with

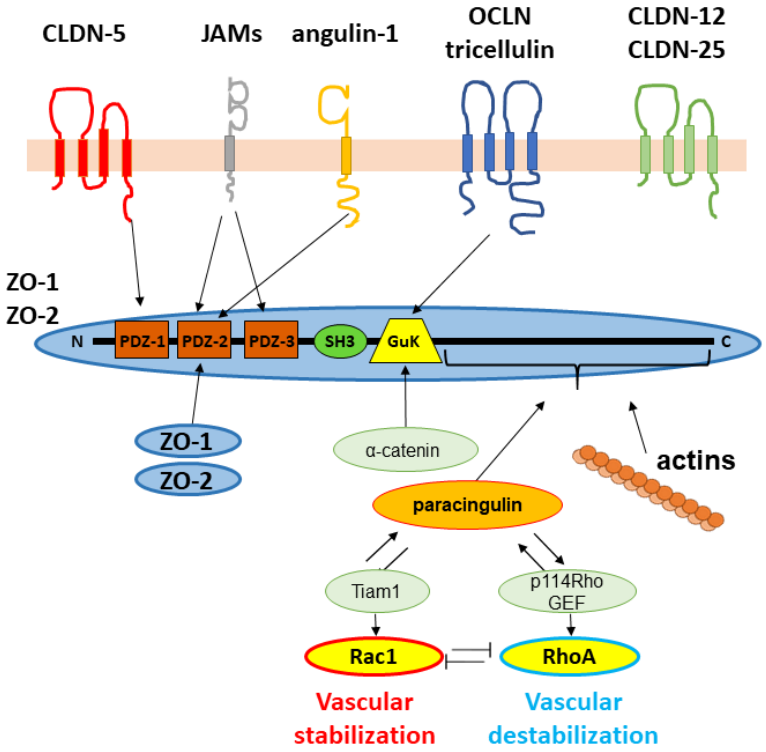

Fig. 2. TJ-Associated Proteins in Brain Endothelial Cells

The TJ proteins present in brain ECs are shown with their associated proteins. Major TJ membrane proteins interact with one of following domains of ZO-1 and -2: postsynaptic density-95/discs-large/ZO-1 (PDZ) domains 1–3, Src Homology-3 (SH3) and guanylate kinase (GuK). CLDN-12 and -25 cannot directly interact with ZOs. ZO family members can dimerize through their PDZ-2 domains, and α-catenin (an accessory protein of VE-cadherin) interacts with GuK. The C-terminal half of ZOs interact with actin and paracingulin, which recruit p114RhoGEF or Tiam1 to induce junctional RhoA or Rac1 activation, respectively. RhoA and Rac1 exert opposing effects on the maintenance of TJs. (Color figure can be accessed in the online version.)

Table 1. BBB Permeability in TJ Protein Deficient Mice

	The paracellular permeability of the BBB	Other key phenotypes	Ref.
CLDN-5	Increased toward molecules <800 Da	Dead within a day of birth	3)
CLDN-3	No change	Increased fluid uptake in ECs	30,33)
CLDN-12	No change	Complicated neurological defects (not mediated by lack of endothelial CLDN-12)	28)
CLDN-25	Increased or no change (predicted by *in vitro* studies)	—	32,106)
OCLN	Increased (at least permeability against Ca^{2+} ion)	Complicated complex phenotypes	42)
Tricellulin	No change	Progressive hearing loss (not mediated by lack of endothelial tricellulin)	47)
LSR	Increased toward molecules <800 Da	Dead before embryonic day 15.5	44)
JAM-A	Increased toward molecules <950 Da	CLDN-5 expression is reduced by 70% in the brain	37,50)
JAM-B	No change	Mice develop less severe EAE	107)
JAM-C	Decreased (predicted by *in vitro* study)	Severe hydrocephalus (not mediated by lack of endothelial JAM-C)	65,108)
ESAM	No change (against albumin levels)	Paracellular permeability of albumin in lung vascular ECs is enhanced	50)

EAE, experimental autoimmune encephalomyelitis.

CLDN-5; however, based on studies using KO mice (Table 1), their contribution to the formation of strong TJs in the BBB is much less than that of other TJ proteins, such as occludin (OCLN) or junctional adhesion molecule-A (JAM-A).[2,28] Importantly, these TJ proteins interact with different binding domains of ZOs (Fig. 2). Of note, the junctional localization of CLDN-5, OCLN, JAM-A and ZO-1 are controlled by vascular endothelial (VE)-cadherin, which is the main adherens junction (AJ) protein in brain ECs, but not *vice versa*, suggesting that VE-cadherin is an upstream regulator of these TJ proteins.[34–37]

OCLN, which is a member of the TJ-associated MARVEL protein (TAMP) family, is also a tetra-transmembrane protein with 2 extracellular loop domains, but with no sequence similarity to CLDNs. VE-cadherin-mediated signaling promotes junctional localization of OCLN through altering the phosphorylation status of the intracellular domains of OCLN.[35] OCLN can form homophilic *trans*-interactions similar to those observed with CLDN family proteins,[38] but cannot build TJs on its own. OCLN can enhance barrier integrity by increasing the complexity of TJ strands *via cis*-interactions with CLDNs.[38,39] Knockdown of OCLN clearly attenuates TJ barrier function in brain ECs.[40] OCLN KO mice and individuals with an OCLN deletion mutation develop calcium deposition

around the BBB.[41,42]

Tricellulin is another TAMP family member that is also expressed in brain ECs and can increase the complexity of TJ strands *via cis*-interactions with CLDNs.[38] Unlike OCLN, tricellulin can form TJ strands at tricellular contacts in the presence of angulin family proteins, which act to recruit tricellulin to tricellular contacts.[43–45] Tricellular TJs can prevent the passage of relatively large molecules (1–10 kDa) across tricellular contacts of epithelial cells.[46] In brain ECs, while both angulin-1 and -3 are expressed, angulin-3 exhibits much lower expression and poor ability to act as a tricellulin recruiter,[2,45] indicating that CLDN-5, tricellulin and angulin-1 are the main components of tricellular TJs in brain ECs. However, the contribution of tricellular TJs to the maintenance of BBB permeability remains unclear because angulin-1 KO mice show a BBB-related phenotype, while tricellulin KO mice do not.[44,47] One possible explanation for this discrepancy could be that angulin-1 seals tricellular contacts in the absence of tricellulin in brain ECs.[48]

JAMs are type I membrane proteins with two immunoglobulin-like extracellular domains, and are important factors for the regulation and signaling of TJs and AJs. Among JAM family members, JAM-A, -B, -C and endothelial cell-selective adhesion molecule (ESAM) are expressed in brain ECs.[2] Well-developed JAM-A interactions form a close membrane apposition that can act as a weak barrier against molecules larger than 4 kDa even in the absence of TJ strands.[49] Similarly, ESAM functions as an adhesion protein to form stable AJs in ECs in mice.[50]

ZO-1 and ZO-2 are expressed in ECs, and cells lacking both cannot form TJs.[49,51] Both ZO-1 and ZO-2 are necessary for maintaining TJs in brain ECs, but functional differences between them remain unclear. VE-cadherin-mediated signaling is required for junctional localization of ZO-1.[34,35] ZO-1 interacts with cingulin and paracingulin in junctional areas, resulting in the junctional association of the inactivated form of guanine nucleotide exchange factors (GEFs): p114RhoGEF[34] and Tiam1.[52] In brain ECs, the expression of paracingulin is much higher than that of cingulin.[2] p114RhoGEF-mediated junctional RhoA activation loosens TJs by CLDN-5 phosphorylation *via* ROCKs,[22,53] while Tiam1-meidated junctional Rac1 activation inhibits RhoA and stabilizes TJs.[35]

3.2. Junctional Protein-Mediated Signaling to Regulate *CLDN5* Transcription VE-cadherin also regulates mRNA expression levels of CLDN-5. The extracellular domain of VE-cadherin engages in *trans*-, homophilic interactions in a Ca^{2+} dependent manner. Its intracellular domain associates with p120-catenin and *β*-catenin (Fig. 3a). VE-cadherin also binds to ZO-1 *via α*-catenin, which binds to *β*-catenin. Trans-interactions of VE-cadherin induces the accumulation of *β*-catenin at junctional areas, resulting in the prevention of nuclear localization of free *β*-catenin and unphosphorylated FoxO1 *via* Akt-mediated phosphorylation. The transcriptional repressor complex of *β*-catenin and FoxO1 suppresses CLDN-5 transcription by promoter methylation.[54] VE-cadherin interactions also inhibit RhoA activation through the junctional recruitment of the Rac1 activator Tiam1. The transcriptional regulations mediated by Wnt/*β*-catenin signaling *via* an increase in intracellular *β*-catenin in the absence of VE-cadherin destabilization improves TJ strength in ECs.[55,56] *β*-Catenin may interact with different transcription factors

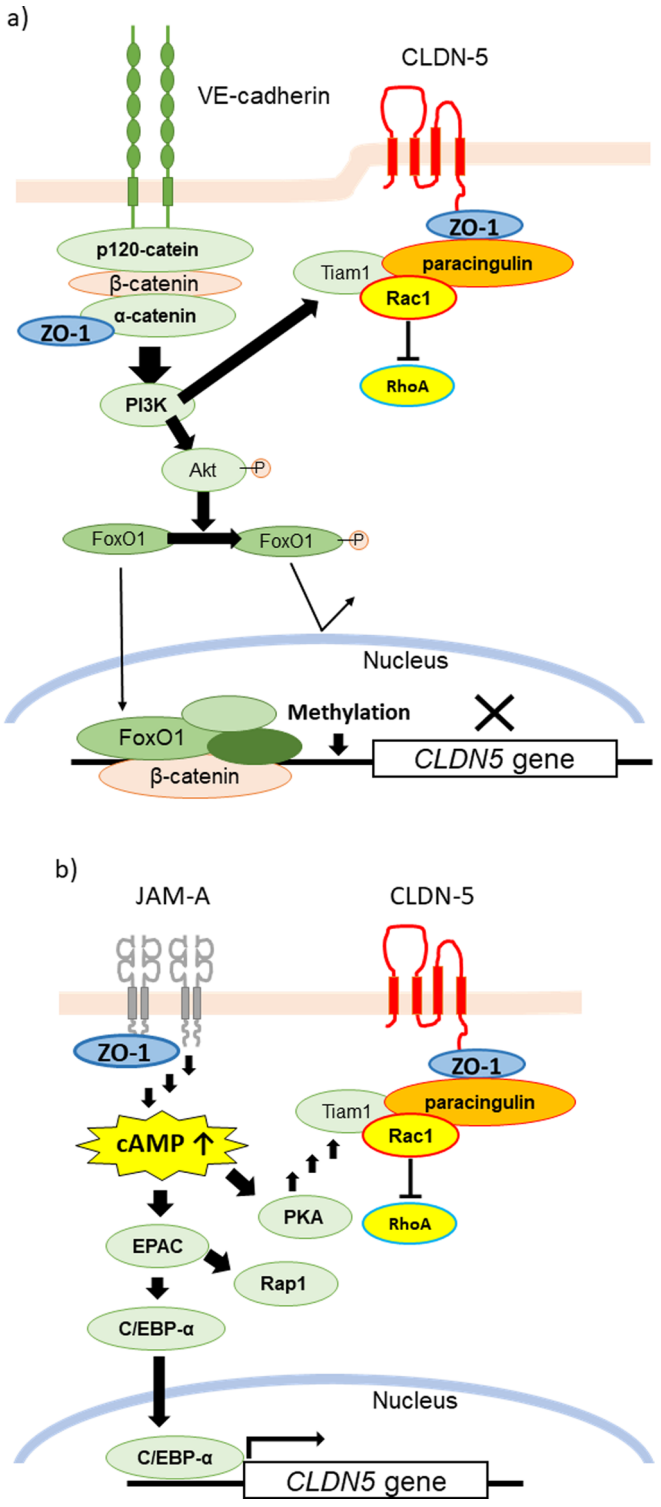

Fig. 3. The Junctional Proteins-Mediating CLDN-5 Regulation

a) The *trans*-interaction of VE-cadherin at junctional areas can induce PI3K activation. PI3K signals maintain CLDN-5 expression/localization in two ways: preventing transcriptional inhibition by phosphorylation of FoxO1 *via* Akt, and preventing post-translational modification by activation of Rac1 *via* Tiam1. b) The *trans*-interaction of JAM-A at junctional areas can induce an increase in intracellular cAMP levels. cAMP can maintain CLDN-5 expression/localization in a protein kinase A (PKA)-dependent and -independent manner. In the PKA-dependent manner, Rac1 activation is induced *via* Tiam1. In the PKA-independent manner, exchange protein activated by cAMP (EPAC) induces Rap1 activation to stabilize the *trans*-interaction of VE-cadherin and enhance the CLDN5 promoter activity by CCAAT/enhancer-binding protein-*α* (C/EBP-*α*). (Color figure can be accessed in the online version.)

depending on the functional condition of the cells. It has been reported that Wnt/β-catenin signaling during angiogenesis suppresses signaling from sphingosine-1-phosphate receptor 1 (S1PR1), which stabilizes junctional localization of CLDN-5 *via* Rac1 and Akt activation; whereas signaling during barrier formation processes do not suppress S1PR1 signaling.[57–60]

Some JAM family members involve *CLDN5* transcriptional regulation. JAM-A-mediated signals can induce an increase in intracellular cAMP levels, which activate CCAAT/enhancer-binding protein-α (C/EBP-α) *via* exchange protein directly activated by cAMP (EPAC) (Fig. 3b). C/EBP-α can enhance *CLDN5* promoter activity.[37] Increased cAMP levels also activate protein kinase A (PKA), which in turn activates Rac1.[61] EPAC-1 is a GEF for Rap1, which stabilizes the *trans*-interaction of VE-cadherin until AJ maturation is complete.[62] The expression level of CLDN-5 in the brain of *JAM-A*-null mice was reported to be almost 30% of that of wild-type mice, despite no observed VE-cadherin abnormalities.[37] ESAM is able to recruit MAGI-1 at junctional areas,[63] which can activate Rap1.[64] JAM-C, which is mainly localized in the intracellular compartment under physiological conditions, inhibits the junctional localization of JAM-A once it is translocated to the cell surface.[65]

4. PATHOLOGICAL ASPECT: CLDN-5-BASED BARRIER TO MAINTAIN BRAIN HOMEOSTASIS

4.1. The Tightness of CLDN-5-Based Barriers under Physiological Conditions Due to the CLDN-5 based barrier in ECs, only hydrophobic small-molecular-weight drugs (<400 Da) with less than 8 to 10 hydrogen bonds can pass through the BBB.[1] The BBB in *CLDN5*-null mice or mice injected with small interfering RNA (siRNA) against CLDN-5 is permeable to molecules less than 800 Da.[3,66] The observed increase in BBB permeability induced by siRNAs lasted at least 3 d, but not beyond 6 d, and did not show obvious side-effects following repeated knockdown.[40,66] However, when the effect of sustained *CLDN5* knockdown was studied using short hairpin RNA (shRNA)-inducible mice, the mice died 2 weeks after initiation of sustained knockdown.[67] Mice with sustained *CLDN5* knockdown showed immunoglobulin deposition in brain and developed seizures and schizophrenia-like behavior before dying. Taken together, although transient increases in BBB permeability toward molecules less than 800 Da does not appear to overtly affect the brain microenvironment, sustained *CLDN* knockdown may affect the brain microenvironment and induce neuroinflammation.

While TJ strands are still observed in the BBB of CLDN-5 KO mice,[3] their composition has not been fully characterized. As implied by the KO and knockdown studies, CLDN-5-based TJs supported by OCLN can form a barrier against molecules less than 4000 Da.[40] JAM-A may function as a paracellular barrier against macromolecules in the BBB even after the complete loss of CLDN-5 and OCLN.[49] It is likely that compensation mechanisms to protect the brain parenchyma occur following the loss of CLDN-5. CLDN-1 expression might be induced by pathological stimuli or CLDN5 suppression in brain ECs.[32,68] Unidentified CLDNs or other TJ molecules may interact with OCLN to form a barrier against molecules less than 800 Da in CLDN-5 KO mice. Compensation also occurs in astrocytes, where endothelial CLDN-5 loss may trigger the induction of CLDN-1 and -4 expression, both of which are not expressed in astrocytes under physiological conditions,[2] to form a TJ-like barrier at astrocytic endfeet (known as the glia limitans).[69,70]

4.2. Loss of the CLDN-5-Based Barrier under Pathological Conditions Increased BBB permeability correlates with the progression/severity of diseases including Alzheimer-related dementia,[10] mood disorders,[12,71] multiple sclerosis[9] and epilepsy.[72] The driver of pathogenesis for these diseases sometimes clearly affects BBB permeability: amyloid-β deposition,[7] inflammatory cytokines and neurotransmitters.[73,74] However, post-mortem brain of patients with cognitive decline in the absence of amyloid-β deposition showed a high methylation status around the *CLDN5* locus.[13] Neuronal activity influences regional BBB permeability in the brain as well as resistance to blood-borne molecule-mediated effects. Chronic peripheral inflammation and extravasated inflammatory cytokines, including tumor necrosis factor α (TNF-α), interleukin-6 (IL-6) and IL-1β, are considered causative factors for major psychiatric disorders induced by social stress; moreover, these cytokines can attenuate CLDN-5 expression *via* multiple pathways, including epigenetic suppression by histone deacetylation 1 (HDAC1).[36] In addition, social stress may negatively regulate the BBB by altering neural cAMP production in the nucleus accumbens and cause brain-site specific CLDN-5 down-regulation.[12,75] Furthermore, brain regions affected by seizures develop microenvironments that accumulate excess glutamate and promote BBB impairment *via* RhoA activation.[76] Although CLDN-5 down-regulation may not always trigger these diseases, it can be considered a risk factor. A CLDN-5 SNP rs10314 located in 3′-UTR, which down-regulates CLDN-5 expression in a microRNA (miRNA)-independent manner, shows weak association with the prevalence of schizophrenia.[67,77,78] This SNP, whose minor allele frequency is 16%, may increase the vulnerability of the BBB toward pathological stimuli. CLDN-5 enhancer-mediated vascular stabilization has the potential to ameliorate these diseases.

5. RECENTLY DEVELOPED CLDN-5 ENHANCERS/INHIBITORS

Over the last 5 years, several molecules have been developed/discovered to be CLDN-5 enhancers or inhibitors. The availability of these CLDN-5 modulators correlates with a deepening understanding of the molecular mechanisms involved in the regulation of TJs in the BBB. In this section, we introduce the relevant findings and discuss their potential and remaining challenges.

5.1. CLDN-5 Enhancers The most intensively studied strategy for stabilizing BBB integrity is to remove the causative factors that induce impairment of the BBB. However, this strategy itself does not always support the restoration of BBB integrity. The molecules introduced in this section can increase BBB integrity even under physiological conditions and have the potential to prevent the progression of some CNS diseases.

Molecules that support VE-cadherin-mediated CLDN-5 localization/expression could be considered CLDN-5 enhancers. Of note, some approved and canonical drugs for mood disorders coincidently exhibit this function (Table 2). GSK-3β is

Table 2. Recently Developed/Studied CLDN-5 Enhancers and Their Mechanisms

Category	Examples	Mechanism	Ref.
cAMP analogues	8-Chlorophenylthio-cAMP	Enhances promoter activity Inhibits ROCK activation	37)
Steroids	Glucocorticoid Dexamethasone	Enhances promoter activity	82)
GSK-3β inhibitors	LiCl Valproic acid SB216763	Extends cellular half-life of TJ proteins	27,109)
HDAC-1 inhibitors	Valproic acid MS-275	Inhibits epigenetic suppression	36,109)
S1PR1 agonists	SAR247799	Inhibits ROCK activation	79)
TGF-β signaling inhibitors	Lavendustin A Sunitinib RepSox	Inhibits TGF-β-mediated tyrosine phosphorylation of VE-cadherin.	24,83)

GSK-3β, glycogen synthase kinase 3β; HDAC-1, histone deacetylase 1; ROCK, Rho-associated protein kinase; S1PR1, sphingosine-1-phosphate receptor 1; TGF-β, transforming growth factor-β.

Table 3. Recently Developed/Studied CLDN-5 Inhibitors and Their Mechanisms

Category	Examples	Mechanism	Ref.
S1PR1 antagonists	NIBR-0213	Activates ROCKs	60)
A2A-AR agonist	Regadenoson (Lexiscan)	Activates ROCKs	88)
LPA receptor ligand	Gintonin	Activates ROCKs	110)
Cadherin peptide	HAV6	Inhibits *trans*-interaction of VE-cadherin	87)
CLDN-5 peptide	C5C2	Inhibits *cis*-interaction of CLDN-5	90)
Anti-CLDN-5 mAb	R9, 2B12	Inhibits junctional localization of CLDN-5	92,93)

A2A-AR, adenosine A_{2A} receptor; LPA, lysophosphatidic acid; mAb, monoclonal antibody; ROCK, Rho-associated protein kinase; S1PR1, sphingosine-1-phosphate receptor 1;

responsible for reducing free β-catenin and its inhibitors could activate Wnt/β-catenin signaling. Increased levels of β-catenin following GSK-3β inhibition stabilized AJs and TJs, and results in increased CLDN-5 half-life.[27] Histone deacetylase inhibitors also counteract β-catenin/FoxO1-mediated *CLDN5* suppression.[36] Recently, an S1PR1 agonist, which enhances Rac1 activity *via* phosphatidylinositol 3-kinase (PI3K) activation and inhibits RhoA-mediated TJ destabilization, is being clinically evaluated for BBB stabilization.[79]

To perform cell-based screening to discover CLDN-5 inhibitors/enhancers, cells should retain most of the *in vivo* functional characteristics of brain ECs. Unfortunately, almost all immortalized human brain EC lines do not have well-developed TJs.[80] Typically, the strength of paracellular permeability, as determined by trans-endothelial electrical resistance (TEER), is lower than $150\,\Omega\cdot cm^2$. This is much lower than the paracellular tightness of the *in vivo* BBB, which is estimated to be $8000\,\Omega\cdot cm^2$ in *in vivo* rat brain ECs.[2,81] The expression levels of junctional proteins are clearly lower in cultured cells even if compounds are used to enhance the promoter activity of the *CLDN5* gene.[37,82] The lack of a suitable *in vitro* model is a clear cause for the delayed development CLDN-5 enhancers/inhibitors. To create improved *in vitro* models of the BBB, human induced pluripotent stem cell (iPS)-derived brain ECs have been developed that are capable of producing suitable TEER values (over $1000\,\Omega\cdot cm^2$). A group conducted chemical screening for CLDN-5 enhancers using human iPS-derived brain ECs that expressed CLDN-5 tagged with a cleavable GFP.[83] Although they failed to discover a novel pathway/target, they showed TGF-β signaling inhibitors could be used as a potential CLDN-5 enhancer.[83] TGF-β signaling inhibits *trans*-interactions of VE-cadherin by phosphorylation of the VE-cadherin intracellular domain, thereby inhibiting the association with scaffolding proteins, p120-catenin and β-catenin.[24] It is still questionable whether these iPS-derived cells are truly brain ECs. A report showed that CLDN-4, which is a non-BBB CLDN, is strongly expressed in human iPS-derived brain ECs and that CLDN-4 clearly contributes to paracellular barrier tightness.[84] Other reports showed that iPS-derived brain ECs produced using a similar induction protocol lacked common endothelial markers and expressed poor Wnt/β-catenin signaling components.[85,86] The authors suggest that these iPS-derived cells may be neuroectodermal epithelial cells having choroid-plexus epithelial cell-like characteristics.[85] Improved human iPS-derived brain ECs will be a promising platform to study CLDN-5 enhancers/inhibitors as well as in furthering basic understanding of TJs in the BBB.

5.2. CLDN-5 Inhibitors Targeting CLDN-5 Regulators In contrast to CLDN-5 enhancers, molecules that disturb VE-cadherin-mediated CLDN-5 localization/expression could act as CLDN-5 inhibitors. For example, an S1PR1 antagonist was able to transiently modulate the BBB to allow the diffusion of molecules less than 1 kDa into mouse brain.[60] Among the CLDN-5 inhibitors summarized in Table 3, ROCKs-mediated CLDN-5 relocalization is the main mechanism used to modulate TJs. Direct inhibition of VE-cadherin *trans*-interaction may induce stronger BBB modulation because it can also induce suppression of CLDN-5 transcription. An E-cadherin derived heptapeptide, which can bind to VE-cadherin, opened the BBB sufficiently to enable albumin permeability.[87] Due to

the low blood half-life of this peptide, the modulation effect lasted only 10 min after administration to mice.

Since the initial enhancer of BBB permeability (the bradykinin analog RMP-7) failed to show a clinical benefit for antitumor drug delivery to the brain in a phase II clinical trial in 2006, only one permeability enhancer (the adenosine A2A receptor agonist regadenoson) has been clinically investigated. Unfortunately, two recent pilot clinical studies of regadenoson also failed to show any benefit to drug uptake in the brain.[88,89] It is possible that increased doses of these enhancers could enhance BBB drug permeability; however, the risk of neurotoxicity associated with blood-borne molecule influx could be also increased. Because these molecules modulate both AJs and TJs, their dose should be minimized in order to avoid breaching the TJs and the consequent passive diffusion of protein-size-molecules. Lastly, no clinical trials have yet been conducted using permeability enhancers targeting CLDN-5 regulators.

5.3. CLDN-5 Inhibitors Targeting CLDN-5 CLDN-5 can be considered the most downstream protein for maintaining TJs. Theoretically, molecules that specifically inhibit CLDN-5 function should be capable of modulating BBB permeability without altering AJ function. Therefore, CLDN-5-binding molecules may be the best options to achieve size-selective BBB modulation; however, there are only a few reports describing the development of CLDN-5 inhibitors.

As the extracellular domain of CLDN-5 is necessary for the formation of CLDN dimers/oligomers, peptides derived from the extracellular domain of CLDN can inhibit CLDN–CLDN interactions. Two types of peptide analog have been reported, those that disrupt *cis*-interactions of ECH1–ECH2 and/or β4–β4 (Fig. 1). C5C2 consists of the β4 and ECH1 domains of CLDN-5,[90] and a pentapeptide derived from ECH2 domain of CLDN-5[29] (Fig. 1). Four hours after C5C2 administration to mice, brain uptake of sodium fluorescein (376 Da) was increased by 40%. C5C2-mediated barrier modulation is transient, lasting less than 12 h. The relatively short blood half-life of the peptide is an advantage for transient modulation of BBB permeability, and these peptides will readily distribute to normal peripheral tissues. Of note, these peptides may bind to other CLDN family members and OCLN since CLDNs can form both homophilic and heterophilic *cis*-interactions with their family members.[39,91]

Several monoclonal antibodies (mAbs) against the extracellular domain of CLDN-5 have been developed[92,93] and are now commercially available. The mAbs showed high CLDN-5 specificity. The clone R9, which showed the greatest ability to lower the integrity of the TJs in an *in vitro* model of the BBB, binds the interface of CLDN-5 *trans*-interactions in the second ECL.[94] The mAbs could induce miss-localization of CLDN-5 in brain ECs, probably because incorporation of CLDN-5 into TJs is inhibited by the mAbs.[15,92]

5.4. Advanced Technologies/Tools to Identify CLDN-5 Binders To date, no small-molecular-weight CLDN-5 binders have been developed; however, some small molecule binders against other CLDNs have been developed/screened using chemical/peptide library-based approaches and crystal structure-based *in silico* chemical designs. The crystal structures of several CLDNs (CLDN-3, -4, -9, -15, and -19) have been published since 2012[95–99]; however, the structure of CLDN-5 has not been determined. *In silico* molecular docking-based virtual screening has been conducted to obtain CLDN-4 binding molecules.[100] CLDN-4 binding affinity and CLDN-specificity of the two identified CLDN-4 binders remain to be determined. Future understanding of the crystal structure of other CLDNs will improve screening technologies for CLDN binders. Insights derived from the crystal structure enabled the modification of CLDN-binding specificity of the C-terminal region of the claudin-binding domain of *Clostridium perfringens* enterotoxin (C-CPE), which binds to many CLDN family members.[101] C-CPE mainly binds a flexible loop between the β5 strand and ECH2 of CLDNs and weakly associates with a flexible loop between the β1 and β2 strands of CLDNs.[102] Unfortunately, the original C-CPE cannot bind to CLDN-5, but a mutagenesis strategy based on the crystal structure of CLDNs successfully developed a C-CPE whose binding ability is relatively CLDN-5 specific.[102]

As CLDNs readily aggregate in the absence of a lipid membrane due to their high hydrophobicity, only a few successful methods have been established to solubilize CLDNs with lipid membranes to maintain their intact structure.[19,103,104] Solubilized CLDNs or peptides derived from CLDN ECLs that can partially form correct structures, such as C5C2, can be used as a screening material for phage-display based peptide screening. There are two successful examples: antibody fragments against CLDN-1[104] and a heptapeptide (RTSPSSR) against CLDN-1.[105] The actual screening of CLDN-5 binders using chemical libraries remains challenging, but a method to solubilize CLDN-5 has been reported.[103]

6. CONCLUSION AND FUTURE PERSPECTIVES

CLDN-5 manipulation is expected to be one of the most optimum ways to control the paracellular permeability of the BBB. Advanced understanding of TJ composition in the BBB, post-translational modification of CLDN-5, CLDN-5 regulators, crystal structures of CLDNs and iPS-derived brain ECs support the development of CLDN-5 enhancers/inhibitors. However, proof-of-concept studies for treating CNS diseases using CLDN-5 enhancers/inhibitors remain insufficient. The long-term safety of CLDN-5 enhancers/inhibitors is also unclear. CLDN-5-based drug delivery systems based on transient BBB inhibition may worsen the pathological state of certain psychiatric disorders; however, this remains to be confirmed. Future studies using CLDN-5 enhancers/inhibitors under different pathological conditions will further increase our understanding of the role of CLDN-5 in maintaining the brain microenvironment and may highlight CLDN-5 as a potential therapeutic target for CNS disease.

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Conflict of Interest Trinity College Dublin owns a patent portfolio related to the use of RNA interference (RNAi) to target claudin-5 for BBB modulation. The other authors declare no conflict of interest.

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