PERSPECTIVE

# Are claudin-5 tight-junction proteins in the blood-brain barrier porous?

The capillaries of the brain are particularly special, as they are not simply conduits for blood, but are primarily responsible to ensure that the neurons function in a strictly regulated homeostatic interstitium. Brain endothelial cells (BECs) express a plethora of ion channels on its luminal and abluminal surfaces, namely: potassium ($K^+$) channels (i.e., Kir2 and Kv1), chloride ($Cl^-$)/bicarbonate ($HCO3^-$) channels, as well as a number of ion-solute exchangers (Redzic et al., 2011). These channels essentially prioritize vectorial transendothelial transport, especially for the regulation of $K^+$ flux across the blood-brain barrier (BBB) (Redzic et al., 2011). The differences between the $K^+$ concentration of the brain interstitium and plasma is only 2 mM to 4 mM, but the maintenance of this ionic concentration difference provides a constancy for the neuronal resting membrane potential, their associated firing thresholds and the preservation of a constant level of neuronal excitability. The stability of the interstitial environment surrounding the brain's neurons is the foundational essence of our persona, it is the basis for the continuity of our making of intellectual decisions and the stability of our psychological essence. Furthermore, pathologies emanating from paracellular (PC) tight junction (TJ) permeability have been implicated in psychiatric disorders, epilepsy, multiple sclerosis, neuroinflammation, stroke and traumatic brain injury (Greene et al., 2019). Thus, the regulation of permeability across the BBB is of interest from a physiological, psychological and clinical perspective.

**The brain capillary:** The cerebromicrovasculature is comprised of specialized endothelial cells, responsible for modulating the constituents of the brain's interstitial fluid (ISF), mitigating against chemical instability in the neuronal milieu. These cells are contiguous, interconnected by TJ protein complexes at their apicolateral membranes which in essence provides the occlusion of the PC spaces, restricting the flux of solutes and ions to transendothelial transport at the apical and basolateral membrane domains (Gunzel and Yu, 2013). In fact, the TJs are mandatory in conferring the characteristic establishment of one of the most impermeable epithelia (> 1000–3000 $\Omega \cdot cm^2$) (Rajagopal et al., 2019), which, in turn, reflects on the "tightness" of the PC space. Any relaxation or compromise of the PC impermeability would counter the efficiency of the transcellular regulation of the brain interstitium. The main TJ proteins of the BBB are occludin and claudins 1/3, 5 and 12 (Gunzel and Yu, 2013). In BECs, claudin-5 concentrations of mRNA are almost 600-fold greater than claudin-3 (Gunzel and Yu, 2013). So dominant and integral are the claudin-5 proteins to the brain function that claudin-5-knockout mice never survive for more than 10 hours after birth (Rajagopal et al., 2019).

**Are BEC claudin-5 porous to ions?** Recently, there have been a number of reports indicating the theoretical possibility that the TJ protein of the brain may indeed be porous in nature (Gunzel and Yu, 2013; Rajagopal et al., 2019). The family of claudins have been reported to possess both barrier-forming and pore-forming properties (Yu et al., 2009; Gunzel and Yu, 2013; Haseloff et al., 2015; Hladky and Barrand, 2016; Rajagopal et al., 2019). Krause et al. (2008) categorized claudins into those which bestow greater impermeability to the PC space (claudins-4, -5, -8, -11, -14, and -19) and those which bestow greater permeability to PC pathways (claudins-2, -15 and -16).

The recent theoretical findings are largely based on the work of Yu et al. (2009), who conducted molecular fluorescence and electrophysiological experimentation on TJ pore-formation. They investigated the porosity of claudin-2 inserted into Madin-Darby Canine Kidney cells by demonstrating increased conductance and permeability and also used "conductance scanning" to deduce pore-formation in claudin-2 TJ proteins. Others have used various experimental models of knockout-mice to deduce the importance of TJs to the homeostatic regulation of the brain. Claudin-5 is a tetraspanning protein, with two extracellular loops (ECLs), ECL 1 and 2, localized within the PC spaces of the BECs which largely influences BBB resistance/impermeability. However, the ECL1 in claudin-5 possess two cysteines which function in PC tightening and its ECL2 does not have a pore-forming function, but rather a narrowing and holding function (Haseloff et al., 2015). Given the historical and robust impermeability of the BBB, the prevailing view is that claudins-5 bestows upon the BEC's PC spaces a high level of ionic and solute impermeability (**Figure 1A**).

**TJ pore-forming proteins:** TJ pore-formation is largely due to claudin mutations caused by charged residues of its ECLs or the addition of cysteine's covalent modification which are reported to alter PC permeability (Rajagopal et al., 2019).

The nature of the TJ pore is theoretically envisaged as, firstly, being involved in passive processes driven by electrochemical gradients that have been generated through active transcellular transport mechanisms. Secondly, mechanisms to regulate the directional fluxes are absent, viz., it does not rectify or behave as membrane-based channels. Given the variety of claudins (1–30) and the characteristic bouquet of claudins which define the various epithelia utilizing TJs, it is probable that each epithelia may require a non-regulated passage for the movement of ions or water via the PC space. A simple analogy may involve the active transport of $Na^+$ across an epithelium, which creates the electrochemical gradient to drive $Cl^-$ through the PC route which has a set of claudin-based TJs which specifically only allow $Cl^-$ anion permeability (Gunzel and Yu, 2013).

**The case for paracellular ionic impermeability in the brain:** Given the highly restrictive character of the BBB, its strict homeostatic role to especially regulate the ionic milieu ($K^+$, $Na^+$, $Cl^-$, and $Ca^{2+}$), ensuring consistency in neuronal function, it is difficult to reconcile ionic-pore-like features in the claudin-5 based TJ of BECs. In fact, there is little to no experimental data to convincingly support the premise that this is indeed a feature of the current experimental and theoretical understanding of the normative BBB. Instead, a PC pathway in the BBB which is porous to ions would suggest a PC-shunt that would make ionic transcellular regulation quite inefficient. Given the scarcity of supporting experimental evidence, and the lack of theoretical imperative for an ionic claudin-5 pore for the BBB, we caution against the mathematical and computational research which is not directed at resolving a clearly defined physiological position, as it has the potential to steer research down the proverbial *cul-de-sac*. This is the case for Rajagopal et al. (2019) who has postulated numerous mathematical and computational configurations for ionic pores through the claudin-5 TJ.

**A role for aquaporosity in claudin-5?** It is conspicuous that *in vivo* BECs are not known to express aquaporins (AQPs). Endorsing this view, Dolman et al. (2005) have shown that primary rat BEC cultures only express AQP1 after the third passage. Furthermore, in the presence of co-cultured astrocytes, this expression is suppressed. This supports the view that under normal physiological conditions *in vivo* BECs do not express AQP1, or any other AQP (Dolman et al., 2005; Francesca and Rezzani, 2010; Papadopoulos and Verkman, 2013). The main function of AQPs is to facilitate the movement of water in response to osmotic gradients and, therefore, it begs the question: just how does water cross the capillary endothelium of the brain? Although it has been mooted that water crosses the brain endothelium transcellularly, and may also be able to pass through certain ionic channels, the absence of AQPs in the BECs suggests that water is routed across the brain endothelium via the PC pathway. The existence of water permeability across the PC TJs is also consistent with the maintenance of electrical resistivity across this barrier, given that high transendothelial electrical resistivity is related to the impermeability of ionic flux across the PC pathway. Thus, we postulate that water crosses the brain endothelium via the PC route, across the TJ, claudin-5, driven by oncotic and osmotic gradients. This postulate is supported by the elegant theoretically configurations of computer-generated pores within the structure of claudin-5 (Rajagopal et al., 2019). They reported on the *in silico* arrangement of the claudin-5 dimer B pore which allows for the selective translocation of water molecules. We thus support mathematical and computational postulates which allow for the elucidation of an experimental phenomenon.

**Water homeostasis in neural tissue:** Surrounding the endothelial cells of brain capillaries, the perivascular astrocyte end-foot processes abundantly express AQP4. These foot-processes are joined to each other via permeable junctional complexes and essentially envelops the brain capillaries, forming the perivascular space (**Figure 1B**). By extension, the strongly expressed AQP4 on these processes, suggests a role for astrocytes in regulating water homeostasis of the brain's ISF (Francesca

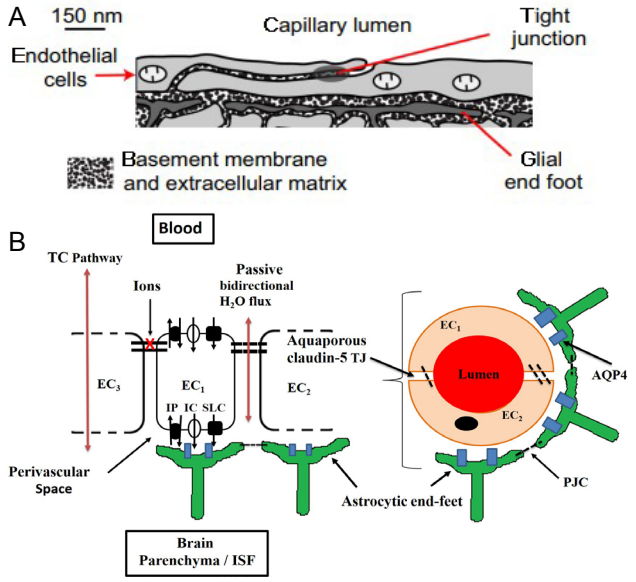


**Figure 1 Anatomical and theoretical postulates for ion and $H_2O$ regulation across brain capillaries.**
(A) A diagram to illustrate the brain capillary endothelium with its TJs occluding the PC space between adjacent overlapping BEC and the close inter-relationship with the perivascular astrocyte end-foot processes (reprinted from Hladky and Barrand, 2016). (B) Ilustration of the relationship between the BEC and the astrocyte-foot processes within the perivascular space. TJs are located apicolaterally, between adjacent EC1 and EC2/EC3. With reference to the degree of impermeability that exists across the BBB. AQP4: Aquaporin-4 on the astrocytic end-feet; BBB: blood-brain barrier; BEC: brain endothelial cells; EC: endothelial cell; IC: ion channels; IP: ion pumps; ISF: interstitial fluid; PJC: permeable junctional complexes localized between adjacent astrocytic foot-processes and TC pathway represent the transcellular regulation of solute and ion transport across the BEC; SLC: solute carriers on the luminal and abluminal membranes of the BEC; TJ: tight junction.

and Rezzani, 2010; Hladky and Barrand, 2016). Further support for this view is based on the evidence that astrocyte foot processes swell during cytotoxic brain edema, and that AQP4 knockout mice have an impaired ability to eliminate water from brain parenchyma (Francesca and Rezzani, 2010). This perivascular space (**Figure 1B**) between the brain capillary and the contiguous foot processes of the astrocytes suggest a homeostatic water/osmotic buffer between the capillary endothelium and the ISF of the brain parenchyma. In the light that we postulated an unregulated aquaporous claudin-5 pore for the BBB endothelium, and given the absence of water regulation via AQPs at the endothelial level, the presence of a homeostatic mechanism for water at the perivascular level of the astrocyte foot processes, makes physiological sense.

**Conclusion:** The BBB achieves homeostasis by establishing highly regulated permeability to ions and blood-borne solutes via the transcellular route. The fundamental absence of an experimental rationale in the literature supporting the idea for the existence of PC barrier-forming TJ (i.e., claudin-5) which is postulated to possess pores responsible for passive ionic flux between the cerebral interstitial and vascular compartments, is difficult to conceive. Given the regulatory ionic channels and protein pumps that exist across the endothelium of the BBB, it is nugatory to suggest the presence of non-regulatory ion pores in the PC spaces of barrier tissue. The passive ionic flux through the postulated non-regulatory claudin-5 pores will negate the well-established transport system established across the TC pathway. Furthermore, the ability of the BEC to preclude the bidirectional flux of select substances across its PC spaces is primarily enabled by the convoluted, non-porous TJ scaffolding, a TC transport system, a lack of fenestra, and the presence of overlapping apical membranes which contribute to mechanically occlude the PC space (**Figure 1A**). To date, the morphological profile of the barrier-forming TJs are not entirely understood. More irrefutable inferences about TJ localization and interaction can only be made upon further nanoscale investigation.

Furthermore, the concept that BEC TJs have pore-forming functions will directly impact the existing theoretical understanding of BBB permeability, and it also becomes an additional factor implicating the etiopathology of neurodegenerative disorders (Greene et al., 2019). We, therefore, caution against postulates that have no grounding in established physiological or experimental supporting evidence. Given this principle, and the absence of AQPs in the *in vivo* brain capillary endothelium, we postulate water may indeed flow through the PC pathways, via the 'theoretical aqua-pores of claudin-5′ (**Figure 1B**). This postulate is further supported by the current evidence that the perivascular astrocytic foot-processes possess high levels of AQP4 with implications for water homeostasis of the brain ISF. Furthermore, the pharmacokinetics involved in the treatment of the central nervous system still requires extensive investigation, due to the restrictive nature of BBB interaction (Upadhyay, 2014). It remains a rate-limiting factor when designing neuropharmaceutics. Thus, the regulation of the permeability across the BBB remains an area requiring intense experimental scrutiny.

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