Plasmalemmal vesicle associated protein-1 is a marker for the inflamed blood-brain barrier in an animal model for multiple sclerosis

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Summary
The blood-brain barrier (BBB) is crucial in the maintenance of central nervous system (CNS) homeostasis and its barrier function is maintained through the continuous interaction with astrocyte endfeet projected onto the brain capillaries. Disruption of the function of the BBB has been described for a variety of neurological disorders, including multiple sclerosis (MS). Characterized by focal demyelinating lesions throughout the CNS, MS pathology includes massive infiltration of leukocytes, axonal damage and neurodegeneration. BBB disruption is often found to precede lesion formation, but the pathological substrate for this damage remains unclear.

The animal model for MS, experimental autoimmune encephalomyelitis (EAE), reflects many of the pathological changes observed in MS, including BBB dysfunction and infiltration of immune cells into the CNS. We assessed the expression of a known brain endothelial damage marker and permeability regulator, plasmalemmal vesicle associated protein (Plvap), in EAE pathology. Secondly, we investigated whether alterations in astrocyte-BBB interaction were associated with Plvap. We show that Plvap expression is absent from the adult mouse CNS, but is induced on the neurovasculature in animals with EAE in areas of immune infiltration. However, enhanced Plvap expression in EAE pathology does not correlate with a decrease in perivascular astrocyte endfoot interaction with the BBB, as detected by potassium inwardly-rectifying channel 4.1 (Kir4.1). In contrast to EAE pathology however, we show that astrocytes in active lesions in post-mortem tissue of MS patients show markedly increased expression of Kir4.1 and the gap junction protein Connexin 43 (Cnx43), two proteins mainly expressed in perivascular astrocyte endfeet in normal appearing white matter (NAWM). Furthermore, the increased gene expression of Kir4.1 and Cnx43 in immune-activated primary human astrocytes in vitro supports the findings from our post-mortem analysis.

Altogether, we show that endothelial expression of Plvap in the CNS of EAE animals reflects BBB disruption and that the markers for astrocyte endfeet are highly expressed by astrocytes in MS lesions.
PLVAP is a marker for the inflamed blood-brain barrier in EAE

Introduction
The blood-brain barrier (BBB) maintains a tight control of central nervous system (CNS) homeostasis, which is crucial to ensure normal neuronal function. The BBB is formed by highly specialized endothelial cells that are interconnected through tight junction (TJ) and adherens junction (AJ) complexes, thereby effectively creating a structural barrier that restricts the entry of cells and large molecules into the CNS. The BBB endothelium is further characterized by low pinocytic activity and minimal transendothelial transport. Furthermore, the CNS endothelium expresses a wide range of uptake and efflux transporters, dedicated to the uptake of nutrients, the release of metabolic waste, and the efflux of harmful lipophilic compounds. The CNS endothelium relies on surrounding abluminal cell types to induce and maintain these specific barrier functions. Both pericytes and astrocytes have a close interaction with the CNS endothelium and are crucial in BBB regulation during development and in the adult CNS. An important aspect of astrocytic support of the BBB is the regulation of ion and water homeostasis at the abluminal side of the CNS endothelium. Astrocyte endfeet that terminate in the glial basement membrane on the endothelial basement membrane of the CNS endothelium form clusters of ion and water channels in the endfoot membrane called orthogonal arrays of particles (OAPs). The formation of these OAPs is regulated by the extracellular matrix molecule agrin and they are characterized by the incorporation of potassium inwardly-rectifying channel 4.1 (Kir4.1) and the waterchannel aquaporin 4 (AQP4). Astrocytes are furthermore in constant communication with each other via gap junctions (GJ) formed by connexin 43 (Cx43) transmembrane channels, mainly expressed in perivascular astrocyte endfeet.

BBB dysfunction has been associated with the pathophysiology of various CNS disorders, including multiple sclerosis (MS). MS is a chronic demyelinating disease of the CNS, characterized by the appearance of focal demyelinating lesions. BBB damage precedes the destructive neuroinflammatory changes in MS lesions, like activated leukocyte infiltration and axonal damage. The early loss of BBB function during neuroinflammation in MS patients can be detected by the increased permeability to small contrast enhancing molecules (Gadolinium) and advanced MRI techniques. In the animal model for MS, experimental autoimmune encephalomyelitis (EAE), BBB alterations are comparable to those found in MS pathology. These alterations include the loss of efflux transporter expression, TJ-complex degradation, and the facilitation of immune cell passage through chemokine and adhesion molecule expression. These changes reflect an overall loss of BBB integrity, which may further exacerbate the inflammatory events leading to MS lesion formation.

One of the characteristics of the CNS endothelium is the absence of plasmalemmal vesicle associated protein (Plvap). Plvap is a transmembrane protein associated with transendothelial transport and the caveolae of fenestrated endothelial cells. In the CNS, endothelial expression of Plvap in the angiogenic brain vasculature is silenced during murine BBB differentiation. Interestingly, endothelial Plvap re-expression has been described in CNS pathologies that involve the loss of BBB integrity, like acute ischemia and brain tumors. In mice, endothelial Plvap expression was shown after spinal cord injury (SCI) and in a transgenic model for Alzheimer’s disease (AD). Furthermore, Plvap has been shown to enhance leukocyte migration across immune-activated endothelial cells. Considering the involvement of Plvap in leukocyte transmigration and as a marker for BBB damage, we investigated the expression of endothelial Plvap in EAE animals. As Plvap expression was
reported to coincide with the loss of astrocyte-markers in SCI\textsuperscript{20}, we furthermore analyzed the distribution of astrocyte endfeet markers in EAE brain tissue. We show here that endothelial Plvap expression is a marker for endothelial cell activation after immune cell infiltration in EAE, but does not correlate with decreased astrocyte endfeet coverage of the endothelium. We furthermore show that the expression of astrocyte endfeet-specific proteins Cx43 and Kir 4.1 is highly increased in active MS lesions, which does not correlate with the expression pattern of Cx43 and Kir4.1 during neuroinflammation in EAE. The use of Plvap as a marker of BBB damage in MS warrants further investigation.

**Materials and methods.**

*Experimental autoimmune encephalomyelitis*

Induction of chronic EAE in FVB mice was described previously\textsuperscript{23}. In short, EAE was induced by subcutaneous immunization with 200 µg recombinant myelin oligodendrocyte glycoprotein emulsion mixed 1:1 with Complete Freund’s Adjuvant (CFA; Difco Laboratories) containing 500 µg of heat-killed *Mycobacterium tuberculosis* H37Ra (MBT; Difco). Control (CFA) animals were injected with saline mixed with CFA containing 500 µg of heat-killed MBT. All animals were additionally intraperitoneally injected with 200 ng pertussis toxin derived from *Bordetella pertussis* (Sigma, Zwijndrecht, The Netherlands) in 200 µL saline at the time of, and after 24 hr following immunization. Mice were killed at day 29 using O\textsubscript{2}/CO\textsubscript{2}, after which brains were dissected, snap-frozen in liquid nitrogen and stored at -80°C before cryosectioning embryonic brains used for the assessment of developmental Plvap expression were dissected from embryos derived from pregnant C57B/6 mice that were killed after 13.5 or 18.5 days of gestation. The maternal brain was used as the adult time point. All experimental procedures were reviewed and approved by the Ethical Committee for Animal Experiments of the VU University Medical Center (Amsterdam, The Netherlands).

*Post-mortem MS brain tissue*

Brain tissue from 4 patients with clinically diagnosed and neuropathologically confirmed MS was obtained at rapid autopsy and immediately frozen in liquid nitrogen (in collaboration with The Netherlands Brain Bank, coordinator Dr. Huitinga). The Netherlands Brain Bank received permission to perform autopsies, for the use of tissue and for access to medical records for research purposes from the Ethical Committee of the VU University medical center, Amsterdam, The Netherlands. White matter samples were selected on the basis of post-mortem magnetic resonance imaging. All patients and controls, or their next of kin, had given informed consent for autopsy and use of their brain tissue for research purposes.

*Immunohistochemistry*

For immunohistochemical stainings of human brain sections, 5 µm cryosections were air-dried and fixed in acetone for 10’. Sections were incubated in PBS with 0.1% (wt/vol) bovine serum albumin (BSA) overnight at 4°C with primary antibodies. For the detection of proteolipid protein (PLP), major histocompatibility complex class II (MHCII), Connexin 43, and Kir4.1, slides were incubated with EnVision Kit rabbit/mouse-labeled horseradish peroxidase (DAKO, Glostrup, Denmark) for 30’ at room temperature. Peroxidase activity was demonstrated with 0.5 mg/ml 3,3’-diaminobenzidine tetrachloride (Sigma, St Louis, MO, USA) in PBS containing 0.02% hydrogen peroxide. Between incubation steps, sections were
thoroughly washed with PBS. After a short rinse in tap water sections were incubated with hematoxylin for 1’ and extensively washed with tap water for 10’. Finally, sections were dehydrated with ethanol followed by xylol and mounted with Entellan (Merck, Darmstadt, Germany).

For immunohistochemistry of mouse brain sections, cryosections were fixed as above and then incubated in 5% normal donkey serum (NDS) in PBS for 15’, followed by overnight incubation with antibodies against Kir4.1 or CD45 in 1% NDS in PBS. Detection of the primary antibody was performed by a 30’ incubation with donkey anti-rabbit Alexa488 (Invitrogen Carlsbad, CA, USA) in 1% NDS in PBS. Sections were then blocked with 1% block buffer component of Tyramide Signal Amplification (TSA)-kit (Invitrogen Carlsbad, CA, USA) with 5% normal rabbit serum (NRRS) in PBS for 15’, followed by an overnight incubation with anti-Pvlp in 1% NRRS in PBS. Anti-Pvlp was detected by a biotin labeled rabbit anti-rat antibody (Invitrogen Carlsbad, CA, USA) in 1%NRRS for 30’, followed by Avidin-Horseradish peroxidise and subsequent TSA according to manufacturers protocol (TSA kit). Sections were then blocked with 5% normal rat serum (NRS), incubated with anti-PECAM1Alexa647 in 1%NRS in PBS. Slides were thoroughly washed with PBS between all incubation steps and embedded with DAPI (Invitrogen Carlsbad, CA, USA)-containing vinsol mounting medium. For the detection of Pvlp expression in embryonic and adult mouse brains, all incubation steps before blocking with TSA block buffer were omitted and anti-PECAM1Alexa488 was used to detect PECAM1. Fluorescence analysis was performed with a Leica DM6000 microscope (Leica Microsystems, Heidelberg, Germany). All primary antibodies are listed in table 1.

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<th>Antibody</th>
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<tr>
<td>PECAM1</td>
<td>Rat</td>
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**Primary astrocyte cell culture and qPCR**

Primary astrocytes from control human brain tissue were isolated and cultured as described previously29. Astrocytes were grown to confluency in 24-well plates and incubated with the Toll-like receptor 3 ligand polyinosinic-cytidylic acid (poly I:C; 50 μg/ml, Amersham Pharmacia Biotech, Piscataway, NJ) for 24 hours. RNA isolation was performed using Trizol (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s protocol. CDNA was synthesized with the Reverse Transcription System kit (Promega, Madison, WI, USA) following manufacturer’s guidelines and RT-qPCR was performed as described previously29. Primer sequences used are as follows, describing the forward and reverse sequence respectively: KCNJ10 (Kir4.1) CTCCTAATCTCTGCCGG – GGTCTGACTGTAATACCTTG G JGAI (Cx43) GAGTCTAATCTCTGCCGT – GCTCCAGTACCACCATGT GAPDH CCAAGTTGTCATGGGATG – GGTGCTAAGCAGTTGGGTGGT. Data analysis was performed using Graphpad Prism software.
(v5.01 Graphpad Software, La Jolla, CA, USA). Results are shown as mean with standard error of the mean, and statistical analysis was performed with unpaired, two-tailed Student t-test.

Results
Microvascular Plvap expression is downregulated during CNS development, and absent in the adult CNS.
We first assessed the neurovascular Plvap expression (previously known as mouse endothelial cell antigen (MECA)-32, now known to be Plvap or PV-1) in the brains of control

![Image of immunohistochemical analysis](image)

**Figure 1.** Plvap expression in CNS endothelium is silenced during development.
Immunohistochemical analysis of PECAM1 (green) and Plvap (red) in two time points of embryonic CNS development and adult brain showing that at E13.5, all PECAM1-positive neurovasculature is positive for Plvap. At E18.5 a minor portion of all PECAM1-positive vasculature is positive for Plvap. In the adult CNS, no immunoreactivity for Plvap is observed in PECAM1-positive vasculature. Scalebar indicates 50μm.
animals at different days of gestation. Immunohistochemical analysis for both Plvap and the pan-endothelial marker platelet/endothelial cell adhesion molecule (PECAM)1 during two different stages of CNS development (E13.5 and E18.5) as well as in the adult CNS are shown in figure 1. At E13.5, all PECAM1-positive vascular structures in the developing CNS are positive for Plvap, indicating an undifferentiated BBB. During the course of CNS development, BBB-differentiation takes place resulting in the loss of Plvap-immunoreactivity on the PECAM1-positive microvasculature. In the adult CNS, Plvap-immunoreactivity is absent from the microvasculature. These data show that the vascular expression of Plvap is downregulated during CNS development, indicative of Plvap expression as a marker of an undifferentiated BBB.

**Neurovascular Plvap expression reflects a state of BBB-disruption in the CNS of adult mice with experimental autoimmune encephalomyelitis (EAE).**

To investigate neurovascular Plvap expression during EAE we performed immunohistochemical analysis of Plvap, leukocyte marker CD45, and PECAM1 in brains of EAE mice compared to control mice that received complete Freund’s adjuvant (CFA), shown in figure 2. Plvap expression, co-stained with PECAM1, could only be detected in the vasculature in the brains of EAE mice, not in control animals. Interestingly, Plvap was exclusively expressed

![Image of immunohistochemical analysis](image)

**Figure 2.** Plvap is expressed in the CNS microvasculature of EAE animals and is associated with immune cell infiltrates.

Immunohistochemical analysis of Plvap expression (red) and CD45 (green) in EAE versus CFA treated animals show that Plvap is only detected in PECAM1 (blue)-positive microvasculature in the CNS of EAE animals and correlates with the detection of CD45-positive extravasated immune cells. CD45 and Plvap were not detected in the CNS of CFA treated animals. Scalebar indicates 25 μm.
in brain endothelium surrounded by CD45-positive immune cells. These data suggest that re-expression of Plvap is induced in the adult brain vasculature by the presence of infiltrated immune cells, which is indicative of BBB-disruption.

**Astrocyte coverage of P lvap-positive microvasculature in the CNS of EAE mice is comparable to that of control mice.**

Astrocyte endfeet coverage of the CNS microvasculature is an important regulator of BBB-maintenance in the adult CNS. We therefore investigated the possibility of decreased astrocyte endfeet coverage of the CNS microvasculature as the underlying cause for the re-expression of P lvap. Astrocyte endfeet where visualized by immunohistochemical detection of Kir 4.1, shown in figure 3. In the CNS of control mice, Kir4.1 expression is mainly localized in astrocyte endfeet surrounding the PECAM1-positive microvasculature. In the CNS of EAE mice, PECAM1-positive microvascular structures that show expression of P lvap do not show a marked alteration in astrocytic Kir4.1 immunoreactivity compared to control animals. These data demonstrate that in the MS animal model EAE, vascular expression of P lvap is not related to a decreased presence of perivascular astrocyte endfeet.
The expression of astrocyte endfeet markers Kir4.1 and Cx43 is highly increased in active MS lesions, and can be mimicked in vitro. In order to investigate the perivascular presence of astrocyte endfeet in MS, we assessed the expression pattern of both Cx43 and Kir4.1 in well-characterized active lesions in post-mortem human brain tissue sections. The upper two panels of figure 4A show the classification of the lesion. The demyelinated area is shown by the absence of myelin-component proteolipid protein (PLP), the detection of MHC class II molecules (LN3) throughout the lesion reflects activated macrophages and microglia. The expression of Kir4.1 and Cx43 within the active lesion (boxed area) is highly increased compared to the surrounding peri-lesional white matter as well as compared to normal appearing white matter (NAWM) further away from the lesion. In NAWM, both Kir4.1 and Cx43 immunoreactivity are predominantly localized to astrocyte endfeet and some occasional astrocyte cell bodies. Surprisingly, in an active lesion, Kir4.1 immunoreactivity is markedly increased in hypertrophic astrocytes, whereas Cx43 immunoreactivity shows a more punctuate, parenchymal staining pattern. This may reflect enhanced interaction between processes of neighbouring astrocytes. These findings were further corroborated in our in vitro model for astrogliosis previously described to closely resemble reactive astrocytes in vivo. Using primary human astrocytes isolated from white matter of non-neurological controls, we investigated the mRNA expression levels of Cx43 and Kir4.1. The mRNA expression level for both genes was increased upon activation of human cultured astrocytes with the Toll-like receptor 3 ligand, polyI:C, compared to untreated astrocytes. Combined, these data show that human astrocytes respond to inflammation by increasing the expression of Kir4.1 and Cx43. However, the mainly perivascular expression pattern observed in NAWM, is no longer apparent in active MS lesions. It is therefore conceivable that normal function of these proteins at the BBB is altered due to the abnormal and mainly parenchymal (Cx43) and somatic (Kir4.1) localization in reactive astrocytes.

Discussion
In the present study we describe a new marker for the inflamed BBB during neuroinflammation in EAE. We have confirmed previous reports that early Plvap expression is silenced during BBB development, and absent in the adult CNS microvasculature. Of note, we show that Plvap is re-expressed and exclusively located in the brain vasculature of EAE animals, in particular in areas of inflammation. Brain endothelial Plvap expression was not associated with a decrease in perivascular astrocyte endfeet coverage, as detected by the expression of endfoot-marker Kir4.1. Finally, reactive astrocytes in active MS lesions displayed a markedly increased expression but aberrant localization of Kir4.1 and Cx43 expression. In vitro experiments using primary human astrocytes and a model of reactive gliosis revealed the enhanced expression of Kir4.1 and Cx43 gene transcripts upon astrocyte activation. Our findings are the first to describe a re-expression of Plvap in the CNS vasculature of EAE mice. Plvap expression in the adult CNS microvasculature has been described as a marker for BBB disruption in acute brain ischemia, AD, SCI and malignant brain tumors in both human and animal studies. Since normal Plvap function in endothelial cells involves promoting transendothelial vesicle transport by the formation of endothelial diaphragms, it is surprising that the adult CNS endothelium responds to neuroinflammation by re-expressing this marker for immature brain microvasculature and non-CNS endothelium. The expression of Plvap by BBB endothelium under inflammatory conditions might contribute...
to the extravasation of plasma components into the CNS parenchyma, leading to a toxic environment for surrounding CNS cells. The discovery that Pivap is actively involved in the transmigration of leukocytes and is regulated by the pro-inflammatory cytokine TNFα\textsuperscript{22} could explain the presence of CD45-positive infiltrates surrounding all Pivap-positive vascular structures in our study. To date, there is no validated mechanism described that
PLVAP is a marker for the inflamed blood-brain barrier in EAE

Figure 4. Cx43 and Kir4.1 expression is highly increased in active MS lesions and reactive astrocytes. A. Immunohistochemical analysis of active MS lesion. PLP expression shows the absence of myelin, LN3 reflects MHCII expression on activated microglia and infiltrated macrophages. Kir4.1 and Cx43 immunoreactivity is markedly increased in active MS lesions (boxed area of upper left image) compared to NAWM. Kir4.1 shows parenchymal and intense hypertrophic astrocyte cell body staining, whereas Cx43 shows a parenchymal, punctuate staining pattern. PLP and LN3: scalebar indicates 200 μm; Kir4.1 and Cx43: scalebar depicts 50 μm. B. Primary human astrocytes show significant upregulation of both Kir4.1 and Cx43 expression upon activation by poly I:C, n=4 **p<0.01.

underlies the silencing of Plrp expression during development. Others have suggested a role for BBB-surrounding pericytes in this process\(^6\). Interestingly, the degradation or regression of pericytes from the BBB has been shown in neurovascular disruption in Alzheimer’s disease\(^28\) and amyotrophic lateral sclerosis\(^39\). There are however no reports that describe the loss of pericyte-endothelial interaction in either EAE or MS pathology. Another possible explanation could be altered astrocyte-endothelial signaling during CNS injury. Earlier studies in SCI\(^35\) reported that endothelial Plrp induction is associated with the loss of perivascular astrocytes. However, our data did not reveal a loss of astrocyte endfeet-specific Kir4.1 immunoreactivity surrounding Plrp-positive microvasculature in EAE. A possible explanation for this discrepancy is the difference in CNS injury between both animal models. Furthermore, the expression pattern of glial fibrillary acidic protein (GFAP) and AQP4, used in the SCI study, and Kir4.1 used in the present study might be subject to differential regulation under neuroinflammatory conditions. The detection of AQP4 and GFAP (astrocytes) and pericytes surrounding the Plrp-positive microvasculature in EAE might therefore be of additional value to find the cause for Plrp expression. Another possible mechanism that requires attention is the reactive astrocyte secretome. Reactive astrocytes have been described to produce a wide range of inflammatory mediators during neuroinflammation, capable of endothelial activation, like interleukin-6 (IL-6)\(^30\), IL-1β\(^31\), and the chemokines C-C motif ligand 2 (CCL2)\(^16\). The downstream effect of these pro-inflammatory mediators on brain endothelial Plrp expression has not been investigated to date.

Astrocytes in neuroinflammatory foci in EAE were previously reported to show loss of connexin 43 (Cx43) expression, which was hypothesized to result in decreased communication between neighbouring astrocytes, and linked to axonal dystrophy\(^29\). The astrocyte endfeet-specific potassium channel Kir4.1, although unaffected in EAE, was shown to be downregulated upon primary human astrocyte activation by IL1β \textit{in vitro}\(^32\). Both Cx43\(^24\) and Kir4.1\(^35\) are described to be essential in the control of BBB homeostasis and integrity. Considering previous reports, it is of interest that we find a marked increase of both Kir4.1 and Cx43 in reactive astrocytes in active MS lesions, as well as increased gene expression in cultured primary human astrocytes upon activation with the Toll-like receptor 3 agonist PolyI:C. The enhanced expression of Cx43 in MS lesions was recently confirmed by others\(^36\), and raises the question whether increased Cx43 expression in reactive astrocytes in MS results in enhanced communication between reactive astrocytes.
Furthermore, a better understanding of Cx43 function is needed to predict if a possible enhanced communication between reactive astrocytes has a beneficial or a detrimental role in the spreading of inflammation in MS pathophysiology. A previous report also showed a different role for increased Cx43 expression in reactive astrocytes, which is the formation of Cx43 hemichannels, resulting in enhanced exchange to the extracellular space\textsuperscript{37}. Increased Cx43 hemichannel formation in this study was associated with promoting neuronal degeneration during NMDA-induced cytotoxicity. The highly increased expression of Kir4.1 in active MS lesions was furthermore not reflected by EAE pathology, and showed intensely stained hypertrophic astrocyte cell bodies, rather than that of perivascular endfeet. This difference in localization might reflect the need for parenchymal potassium buffering in an active lesion, due to the disruption of normal AQP4/Kir4.1 regulated homeostasis at the BBB. The differences in MS and EAE in the astrocytic response to neuroinflammation show that astrocyte behaviour in animal models should be validated in post-mortem studies before pathological changes are attributed to both disease processes.

Finally, our results could imply a general mechanism in which inflammatory conditions reinstate a developmental pathway in the blood-brain barrier leading to the re-expression of markers of the undifferentiated BBB and the consequent loss of its key features. Intriguingly, a recent report shows the involvement of Sonic hedgehog (Shh) expression, a BBB differentiation factor, in MS lesion pathology. Reactive astrocytes show increased expression of Shh, which in turn can act as an immune-dampening molecule and can reinstate barrier functions in the damaged BBB\textsuperscript{18}. The involvement of other BBB-inducing developmental pathways in MS like the Wnt/\beta-catenin pathway\textsuperscript{19} and retinoic acid signaling\textsuperscript{6} and their therapeutic potential warrants further study.

\textbf{Reference List}


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