Université de Montréal

L'IMPORTANCE DE LA VOIE DE SIGNALISATION AU NIVEAU DE LA BARRIÈRE HÉMATO-ENCÉPHALIQUE

THE IMPORTANCE OF THE HEDGEHOG SIGNALING PATHWAY AT THE LEVEL OF THE BLOOD-BRAIN BARRIER

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Résumé en Français

La barrière hémato-encéphalique (BHE) protège le système nerveux central (SNC) en contrôlant le passage des substances sanguines et des cellules immunitaires. La BHE est formée de cellules endothéliales liées ensemble par des jonctions serrées et ses fonctions sont maintenues par des astrocytes, celles ci sécrétant un nombre de facteurs essentiels. Une analyse protéomique de radeaux lipidiques de cellules endothéliales de la BHE humaine a identifié la présence de la voie de signalisation Hedgehog (Hh), une voie souvent liées à des processus de développement embryologique ainsi qu'au niveau des tissus adultes. Suite à nos expériences, j'ai déterminé que les astrocytes produisent et secrètent le ligand Sonic Hh (Shh) et que les cellules endothéliales humaines en cultures primaires expriment le récepteur Patched (Ptch)-1, le co-récepteur Smoothened (Smo) et le facteur de transcription Gli-1. De plus, l'activation de la voie Hh augmente l'étanchéité des cellules endothéliales de la BHE *in vitro*. Le blocage de l'activation de la voie Hh en utilisant l'antagoniste cyclopamine ainsi qu'en utilisant des souris Shh déficientes (-/-) diminue l'expression des protéines de jonctions serrées, claudin-5, occcludin, et ZO-1. La voie de signalisation s'est aussi montrée comme étant immunomodulatoire, puisque l'activation de la voie dans les cellules endothéliales de la BHE diminue l'expression de surface des molécules d'adhésion ICAM-1 et VCAM-1, ainsi que la sécrétion des chimiokines pro-inflammatoires IL-8/CXCL8 et MCP-1/CCL2, créant une diminution de la migration des lymphocytes $CD4^+$ à travers une monocouche de cellules endothéliales de la BHE. Des traitements avec des cytokines pro-inflammatoires TNF-α and IFN-γ *in vitro*, augmente la production de Shh par les astrocytes ainsi que l'expression de surface de Ptch-1 et de Smo. Dans des lésions actives de la sclérose en plaques (SEP), où la BHE est plus perméable, les astrocytes hypertrophiques augmentent leur expression de Shh. Par contre, les cellules endothéliales de la BHE n'augmentent pas leur expression de Ptch-1 ou Smo, suggérant une dysfonction dans la voie de signalisation Hh. Ces résultats montrent que la voie de signalisation Hh promeut les propriétés de la BHE, et qu'un environnement d'inflammation pourrait potentiellement dérégler la BHE en affectant la voie de signalisation Hh des cellules endothéliales.

Mots-clés : radeaux lipidiques, microdomaine, barrière hémato-encéphalique, jonctions serrées, astrocyte, cellule endothéliale, endothélium, perméabilité, voie de signalisation hedgehog, sonic hedgehog, sclérose en plaques, neuroimmunologie, neuroinflammation

Résumé en Anglais

The blood-brain barrier (BBB), composed of tightly bound endothelial cells (ECs), regulates the entry of blood-borne molecules and immune cells into the CNS. Recent studies indicate that the Hedgehog (Hh) signaling pathway in adult tissues plays an important role in vascular proliferation, differentiation and tissue repair. Using a lipid membrane raft-based proteomic approach, I have identified the Hedgehog (Hh) pathway as a signaling cascade involved in preserving and upkeeping BBB functions. My study shows that human astrocytes express and secrete Sonic Hh (Shh) and conversely, that human BBB-ECs bear the Hh receptor Patched-1 (Ptch-1), the signal transducer Smoothened (Smo) as well as transcription factors of the Gli family. Furthermore, activation of the Hh pathway in BBB-ECs restricts the passage of soluble tracers *in vitro*. By blocking the Hh signaling *in vitro* and by using Shh knock-out (-/-) embryonic mice, I demonstrate a reduced expression of TJ molecules claudin-5, occludin and ZO-1. Hh activation also decreases the surface expression of cell adhesion molecules ICAM-1 and VCAM-1, and decreases BBB-ECs secretion of pro-inflammatory chemokines IL-8/CXCL8 and monocytes chemoattractant protein 1 MCP-1/CCL2, resulting in a reduction of migrating $CD4^+$ lymphocytes across human BBB-EC monolayers. *In vitro* treatment with inflammatory cytokines TNF-α and IFN-γ, upregulates the production of astrocytic Shh and the BBB-EC surface expression of Ptch-1 and Smo. In active Multiple Sclerosis (MS) lesions, in which the BBB is disrupted, Shh expression is drastically upregulated in hypertrophic astrocytes, while Ptch-1 and Smo expression is down-regulated or left unchanged, suggesting that a deregulation in the Hh signaling pathway may prevent the barrier stabilizing properties of Hh. Our data demonstrate an anti-inflammatory and BBB-promoting effect of astrocyte-secreted Hh and suggest that a pro-inflammatory environment disrupt the BBB by impacting, at least in part, on Hh signaling in brain ECs.

Key words: lipid membrane raft, DRM, microdomain, blood-brain barrier, tight junction, astrocyte, endothelial cell, endothelium, permeability, hedgehog pathway, sonic hedgehog, Multiple Sclerosis neuroimmunology, neuroinflammation

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LIST OF ABBREVIATIONS

Ab:	Antibody
ACM:	Astrocyte conditioned media
AJ:	Adherens junction
AKAP12:	A-kinase anchoring protein 12
ALCAM:	Activated leukocyte cell adhesion molecule
Ang:	Angiopoitein
AngII:	Angiotensin II
APC:	Allophycocyanin (fluorochrome)
BBB:	Blood-brain barrier
Boc:	Brother of Cdon or Biregional cell adhesion molecule-related/downregulated by oncongenes binding proteins
BDNF:	Brain-derived neurotrophic factor
BMP:	Bone morphogenic protein
BSA:	Bovine serum albumin
C-terminal:	Carboxyl-terminal
CAM:	Cell adhesion molecule
CCL:	CC chemokine ligands
CD:	Cluster of differentiation
Cdon:	Cell adhesion molecule-related/down-regulated by oncogenes
CNS:	Central nervous system
CSF:	Cerebrospinal fluid
CXCL:	CXC chemokine ligand
Cy3:	Cyanine (fluorochrome)
DAB:	3,3-diamino-benzidine
Dhh:	Desert Hedgehog
DMEM:	Dulbecco's Modified Eagle's Medium
DRM:	Detergent resistant membrane
EAE:	Experimental autoimmune (allergic) encephalomyelitis
EC:	Endothelial Cell
ECM:	Extracellular matrix
EGF:	Epidermal growth factor
ELISA:	Enzyme linked immunosorbent assay
EPC:	endothelial precursor cell
Eph:	Ephrin receptor
FBS:	Fetal bovine serum
FGF:	Fibroblast growth factor
FITC:	Fluorescein (fluorochrome)
GDNF:	Glial-derived neurotrophic factor
GFAP:	Glial fibrillary acidic protein
Gli-1:	Glioblastoma-associated oncogene homolog 1
Glut-1:	Glucose transporter-1
HDL:	High density lipoprotein
HEK:	Human epithelial kidney

Hh:	Hedgehog
Hip:	Hedgehog interacting protein
HS:	Human serum
HUVEC:	Human umbilical vein endothelial cells
HRP:	horseradish peroxidase
hrShh:	human recombinant sonic hedgehog
ICAM:	Intercellular Adhesion Molecule
ICS:	Intracellular stain
IFN-β:	Interferon beta
IFN-γ:	Interferon gamma
IgG:	Immunoglobulin G
Ihh:	Indian Hedgehog
IL:	Interleukin
IP-10:	Interferon protein-10
JAM :	Junctional adhesion molecule
-/-:	Knock-out
LC-MSpect:	Liquid chromatography-mass spectrometry
LDL:	low density lipoprotein
MAGI:	Membrane-associated guanylate kinase inverted
MAGUK:	Membrane-associated guanylate kinase
MBP:	myelin basic protein
MCAM:	melanoma cell adhesion molecule
MCP-1:	Monocyte chemoattractant protein-1
MHC I:	Major histocompatibility complex, class I
MIP-1α:	Macrophage inflammatory protein-1alpha
MOG:	Myelin oligodendrocyte glycoprotein
MS:	Multiple Sclerosis
mRNA:	Messenger ribonucleic acid
MRP-1:	Multiple drug resistance protein-1
MMLV:	Murine moloney leukemia virus
N-cadherin:	Neural cadherin
N-terminal:	Amino-terminal
NAWM:	Normal appearing white matter
NSC:	Neural stem cell
OPC:	Oligodendrocyte precursor cell
qPCR:	Quantitative real-time polymerase chain reaction
PBS:	Phosphate buffered saline
P-glycoprotein	Permeability glycoprotein
PDGF:	Platelet-derived growth factor
PDZ:	PSD-95/discs-large/ZO-1 domain
PFA:	Paraformaldehyde
PECAM-1:	Platelet/endothelial cell adhesion molecule-1
PLP:	Proteolipid protein
PNS:	Peripheral nervous system
Ptch-1:	Patched-1
PCR:	Polymerase chain reaction

PTX:	Pertussis toxin
PVDF:	polyvinylidene fluoride
RANTES:	Regulated upon activation, normal T cell expressed and secreted
Robo:	Roundabout
RT:	Room temperature
SANT-1:	Smoothened Antagonist-1
SEM:	Standard error of mean
SH3:	src homology domain 3
Shh:	Sonic Hedgehog
Smo:	Smoothened
SSeCKS:	src-suppressed C-kinase substrate
SVZ:	Subventricle zone
T cell:	thymus-matured lymphocyte
$CD4^+$ T cell:	T helper cell expressing CD4
TEER:	Transendothelial electric resistance
TfR:	Transferrin receptor
TGF-β:	Transforming growth factor- beta
TJ:	Tight junction
TNF-α:	Tumor necrosis factor-alpha
TSP:	Thrombospondin
UEA-I:	*Ulex europaeus agglutinin* I
UNC:	Uncoordinated genes
VCAM-1:	Vascular Cell Adhesion Molecule-1
VE-cadherin:	Vascular endothelial-cadherin
VEGF:	Vascular endothelial growth factor
vWF:	von Willebrand factor
WB:	Western blot
Wnt:	Combination of Wingless (Drosophila) and int-1 (mouse, now called Wnt)
ZO-1:	Zona occludens-1

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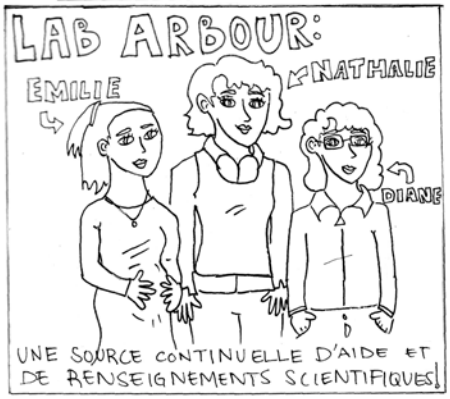
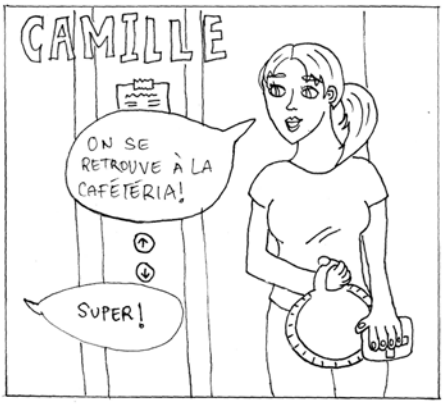
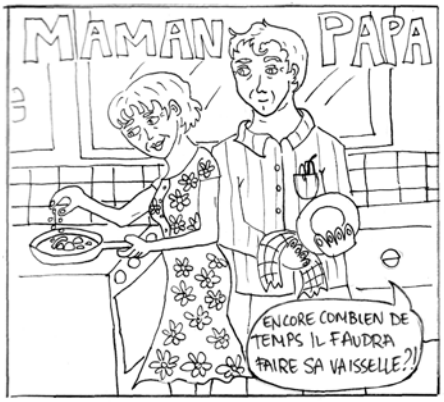
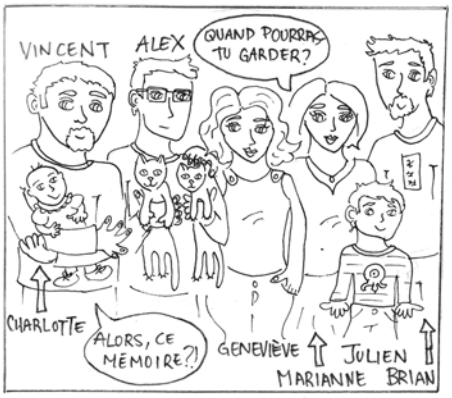
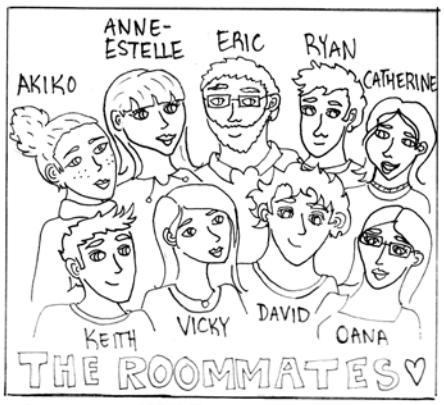
J'aimerais dédier ce mémoire à tous ceux qui m'ont aidé durant ma Maîtrise. I would like to dedicate this thesis to everyone who has helped me during the course of my Masters.











Merci! Thank you!

LITERARY REVIEW

The vascular system is composed of blood vessels that pervade through the entire body, transporting blood and providing nutrients, oxygen, and hormones to the tissues, while removing carbon dioxide and waste metabolites. Vascular development is typically divided into two separate stages. The first stage, vasculogenesis, is defined as the *de novo* formation of endothelial tubes from newly differentiated endothelial precursor cells (EPC), or angioblasts. Aggregations of two or more angioblasts initiate the process of vascular tube formation, also known as tubulogenesis[1]. Lumens are formed by fusion of intracellular vacuoles with cell membranes, enlarging the enclosed space between the cells and generating the appearance of clear slit-like spaces between angioblasts. Establishment of apical-basal cell polarity and interactions between angioblasts and surrounding extracellular matrix (ECM) are also important steps in establishing early vasculature[2-4] The later stages of vasculogenesis include the formation of vascular channels and capillary plexus, which are then remodeled into a circulatory network via angiogenesis[5]. This second separate stage of vascular development is thus the subsequent growth, elaboration and remodeling of existing blood vessels to form a mature vasculature and is also, in general, the process of new blood vessel formation in the adult organism[4,6,7]. Once angiogenesis has ceased, vessel maturation begins.

1.0 THE BLOOD-BRAIN BARRIER (BBB)

1.1 Unique BBB properties

Maturing endothelial cells (ECs) of the central nervous system (CNS) attain unique properties compared with those present in other organs, such as high resistance intercellular complexes, low pinocytosis and specialized transport systems, leading to the formation of the blood-brain barrier (BBB), or barriergenesis [8,9]. During this final developmental step of the brain vascular system, cerebral and spinal cord capillaries form a continuous cellular barrier, restricting the movement of electrolytes, xenobiotics and circulating immune cells between the systemic circulation and the CNS parenchyma in order to maintain an optimal milieu for neuronal functions. Low transcellular passage is a result of the loss of cell fenestration, and tightly adhering

junctions between BBB-ECs permit additional low paracellular diffusion[10]. These optimal BBB properties are achieved through intricate cellular interactions between BBB-ECs and perivascular glial cells, creating a dynamic neurovascular unit.

In order to promote an optimal milieu for neuronal functioning, BBB-ECs also express highly active metabolic enzymes and numerous polarized transporters which regulate oxygen and nutrient transport to the CNS[8,11]. In fact, BBB-ECs contain abundant mitochondria, as compared to non-CNS ECs, reflecting the high energy demand required to achieve barrier functions[8]. These active transport systems include nutrient transporters (specific for glucose, amino acids, nucleoside, fatty acid, minerals and vitamins), peptides and protein transport systems (oligopeptide transporters, absorptive and receptor mediated endocytosis) as well as various ion transporters[8,11,12]. Thus, expression of BBB-specific transporters, such as glucose transporter-1 (Glut-1), sodium/glucose co-transporters and transferrin receptor (TfR), are necessary for proper nutrient transport from the blood to the CNS[11]. Many drug efflux pumps, such as P-glycoprotein, and Multiple drug resistance protein-1 (MRP-1), carry toxic substrates from the interstitial space of the CNS back to the blood and thus by restricting toxin accumulation within the CNS, provide an extremely important mechanism by which the BBB prevents damage to the brain[13].

Other molecular markers for *in vivo* and *in vitro* BBB-ECs exist, but few are specific to the brain vasculature, as they also reside on peripheral ECs, epithelial cells, or other CNS cells. To name a few, von Willebrand factor (vWF), a large multimeric glycoprotein, is selectively produced in ECs and is involved in coagulation[14]. The plant-derived lectins *Ulex europaeus agglutinin* I and *Lycopersicon esculentum* are known to bind ECs and their uninterrupted staining are commonly used as a marker of EC and BBB integrity[15,16]. Other BBB-EC markers include tissue transglutaminase, Neurothelin/HT7/EMMPRIN, and γ-glutamyl transpeptidase (γGT), an enzyme involved in the catalytic transport of amino acids[8,17-20]. Antibodies directed against caveolin-1 also bind to ECs *in vitro* and *in situ* and can be used as a reliable marker of human CNS vessels[21]. Indeed, caveolin-1 is expressed throughout the CNS vasculature, is not vessel size-dependant nor substantially affected by inflammation and is only weakly expressed by perivascular astrocytes[22,23].

1.1 Tight and adherens junctions

Intercellular tight junctions (TJs) and adherens junctions (AJs) between BBB-ECs gradually restrict the passage of blood-borne molecules and cells from the blood to the CNS as the junctions mature[24]. TJ complexes are the main protein structures responsible for the barrier properties and are located at the apical, or luminal, plasma membrane between adjacent BBB-ECs. They are viewed to polarize the cell into its apical and basolateral domains, in addition to providing high transendothelial electrical resistance (TEER) and restrictive permeability[25,26]. TJs consist of at least three known types of integral/transmembrane proteins including occludin, claudins and the junctional adhesion molecules (JAMs)[8,11,25-27]. Occludin does not seem to be functionally required for the structural integrity or formation of the TJs as the occludin knock-out (-/-) animals developed normal TJs and functional BBB. However, other developmental abnormalities during development in the occludin -/- mice, such as aberrations in brain function and inflammation of the gastric epithelium, suggest that occludin may modulate TJs by other means[28]. Claudins, on the other hand, are members of a large family of proteins (over 20 claudins) that share structural homologies with occludin and are reported to be essential TJ components[29]. Claudins create an impermeable seal between the cells through homophilic and heterophilic binding of their extracellular loops between adjacent cells. Claudin-1, -3, -5 and -12 are expressed in BBB-ECs and contribute to cerebral vessel impermeability[30,31]. In particular, claudin-5 is an essential TJ protein as *in vivo* permeability experiments in claudin-5 deficient mice demonstrated selective loss of BBB integrity to small molecules. Although the general morphology of blood vessels was not altered in these claudin-5 -/- animals, these mice died within hours of birth[31]. Claudin-3 has also been observed to be selectively lost from BBB TJs under pathological conditions where BBB integrity is compromised, such as in experimental autoimmune encephalomyelitis (EAE) and in glioblastoma multiforme[30,32]. This suggests that claudin expression and function might be regulated by pro-inflammatory cytokines and in pathological conditions. The third protein reported to be part of TJ complex is JAM-1,

also known as JAM-A. JAM-A localizes within TJs and is involved in the recruitment and organization of the TJs, as its expression enhances the accumulation of zona occludens (ZO)-1 and occludin by linking to the actin cytoskeleton[33]. Recent reports described reduced JAM-A expression during the early phase of BBB breakdown, suggesting that JAM-A contributes to BBB integrity[34]. JAM-2 and -3 are also present in BBB-ECs[11,35,36], but their contribution to TJ complexes and BBB integrity remains unclear.

Occludin, claudins and JAMs interact with a variety of intracellular proteins and may therefore regulate a wide array of signaling pathways essential for TJ assembly, maintenance and regulation. In fact, the cytoplasmic region of transmembrane TJ complexes interacts with a vast number of adaptor proteins that allow binding of actin microfilaments to the TJ complexes while also acting as signaling molecules. ZO -1, -2 and -3 are members of the membrane-associated guanylate kinase (MAGUK) protein family of adaptor proteins that contain many protein-protein interaction domains (such as SH3 and PDZ domains), which can interact with the cytoplasmic tail of TJ proteins and bind to other adaptor proteins, leading to the formation of intricate signaling scaffolding platforms[27]. ZO-1 is important for TJ platform stability as disruption of ZO-1 correlates with loss of BBB properties[15]. In fact, the suppression of ZO-1 and ZO-2 in cultured epithelial cells lead to the cytoplasmic accumulation of claudins and absence of TJ formation[37]. Additional MAGUK proteins expressed by BBB-ECs include MAGUK inverted proteins (MAGI-1 and -3) and multi-PDZ protein (MUPP1)[27]. Well characterized signaling molecules, such as the small GTPases RhoA, Rac and Cdc42 control actin cytoskeleton organization and are also involved in the maintenance of TJs[38-40]. Overall, the expression levels of TJ proteins have been found to decrease in many neurological disorders that are characterized by the loss of BBB permeability integrity, such as in brain tumors, ischemia, and multiple sclerosis (MS)[15,41-46]. Additional putative TJ and intracellular adaptor proteins are still being identified and found to be associated with the junctional complexes, such as EMP-1 (epithelial membrane protein)[47], LYRIC (LYsine-RIch CEACAM1)[48], coxsackie adenovirus receptor (CAR)[49], AF-6[50], cingulin[27,51], CASK[52] and 7H6 antigen[53]. The exact contributions of these molecules are yet to be established.

Another type of junction that contributes to BBB permeability and intermingles with the TJs in ECs is the adherens junction (AJ). AJs of BBB-ECs are primarily composed of platelet-endothelial cell adhesion molecule (PECAM)-1 and vascular endothelial cadherin (VE-cadherin) which interact with intracellular proteins, including the different catenins (isoform α, β, γ), ZO-1, p120 and desmoplankin, therefore creating an association with the actin cytoskeleton[54-63]. In addition, catenins are also crucial signaling molecules which can enter the nucleus and induce gene transcription upon AJ disruption[64]. AJ expression temporally precede the expression of TJs at intercellular contacts, and may be necessary for proper TJ formation as recent reports demonstrated that VE-cadherin expression and clustering modulates the activity of various transcription factors leading to an upregulation of claudin-5 gene transcription in ECs[65]. To summarize, the various proteins involved in the junctional complexes are illustrated in Figure i.

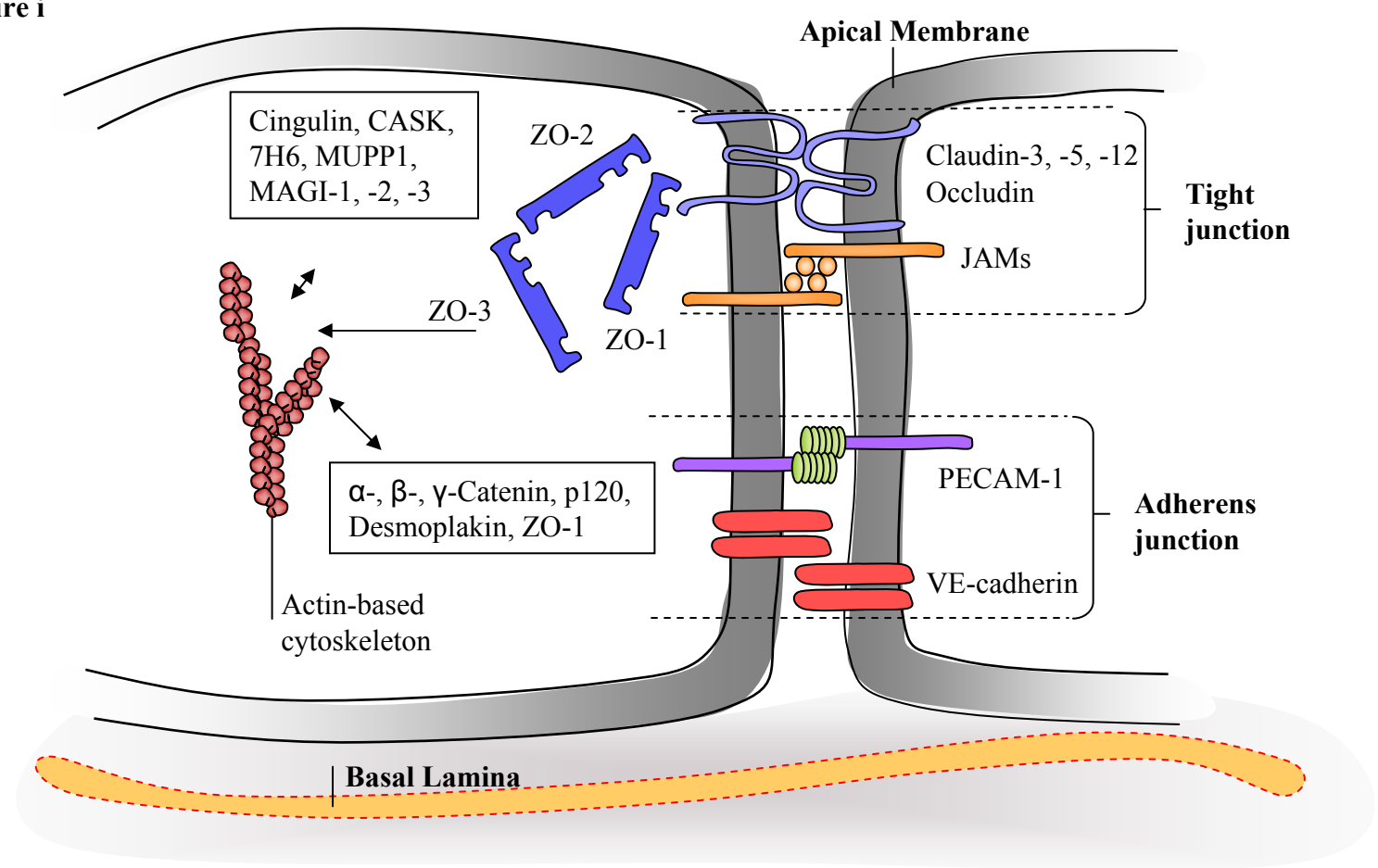


Figure i: *Schematic illustration of the Tight and Adherens Junction of BBB-ECs.* TJs are compromised of the transmembrane proteins occlduin and the claudins, which are similar in homolgy and JAMs. BBB-EC AJ consists mainly of PECAM-1 and VE-cadherin. The cytoplasmic region contains adaptor proetins which link TJ and AJ proteins to the actin cytoskeleton. ZO-1, through its protein-protein domain, can bind the cytosolic tails of transmembrane proteins. With the help of other cytosolic proteins, such as Cingulin, CASK, MAGIs, TJs can be associated to the cytoskeleton. Catenins are the main adaptor protein for AJs, interacting with additional proteins such as desmoplankin, p120 and ZO-1, to stabilize the junctions.

2.0 CUES IN THE VASCULATURE

The cues and receptors most known to be involved in vasculogenesis and angiogenesis are largely specific to the cardiovascular system, such as vascular endothelial growth factor (VEGF) and components of the transforming growth factor (TGF)-β signaling pathway, angiopoietins (Ang)-1 and -2[66]. VEGF and Ang-1 are potent mitogens and survival factors for the vasculature as they stimulate the initially scattered EPCs to proliferate, fuse together and form a primitive plexus of vessels that consequently enlarges and remodels, in the end, to develop into highly conserved organ-specific vascular patterns[7,66]. For precise and functional vascular networks to be reproduced, additional signals assist in the temporal and spatial guidance and proliferation of cells within the developing vasculature, permitting pervasive navigation of blood vessels to their appropriate target sites and to acquire a mature vasculature that is functionally and elaborately adjusted to its organ-specific environment, such as the CNS.

2.1 Glial cues control of the vasculature and blood-brain barrier function

Barriergenesis and the unique EC properties in the CNS are not predetermined by brain-specific EPCs but are induced by the local neural environment during the development of the vascular system. Persistence of a functional BBB throughout adulthood is thus similarly maintained and regulated by numerous brain-derived factors[11,67]. The complex communication and diverse signaling events between BBB-ECs and surrounding astrocytes, pericytes and microglia, allow for regulated BBB functions and promote TJ maintenance, metabolic and specialized transporter expression as well as immune quiescence of the brain ECs[68]. These proximate signals also promote rapid regulation and remodeling of the BBB, a phenomena crucial for maintaining CNS homeostasis in response to physiological and pathological stimuli[8,11,15,21,24,45,69-72]. One relatively important cellular component of the neurovascular unit comprises of pericytes which are believed to play a role in the maturation and maintenance of the BBB by

secreting growth factors and producing ECM constituents[73]. Pericytes contact ECs via N-cadherin-dependent binding[74], and are reported to secrete TGF-β[75], Ang-1 and -2[76,77], and platelet-derived growth factor (PDGF)[78], which promote vascular maturation, integrity and BBB impermeability[76]. Although pericytic abnormalities and dysfunctions have been associated with CNS pathologies such as hypertension, Alzheimer's disease and stroke, their exact nature and function in regards to the BBB remain under investigation[73,76,77].

Other cellular constituents of the neurovascular unit which are crucial inducers of BBB properties are the astrocytes. Astrocyte-BBB-EC interactions are known to regulate vascular proliferation, angiogenesis, transporter protein expression, TJ protein expression and morphology and finally, inflammatory responses in the brain[8,11,32,43,68,71,79-83]. Astrocytic endfeet, separated only by a thin, but compact basal lamina, contact and ensheathe the entire surface of brain and spinal cord blood vessels[84,85]. The importance of astrocytes on BBB properties becomes clear when BBB-ECs are isolated and cultured *in vitro* in the absence of astrocytes. BBB-derived ECs rapidly lose some of their barrier characteristics, such as P-glycoprotein and TfR expression[86,87]. BBB function can be reinstated by co-culturing BBB-derived ECs in the presence of astrocytes or supplementation of astrocyte-conditioned media (ACM)[43,88,89]. Conversely, culturing non-neural ECs in the presence of astrocytes, or astrocyte-secreted factors, induces BBB-specific properties, such as P-glycoprotein and TJ expression[11,68,81,90]. These observations underscore the importance of signals provided by astrocytes and provide evidence that factors needed for reliable BBB function are at least partly soluble and secreted[91]. A number of studies have identified a variety of soluble and contact-dependent factors provided by astrocytes. Taken together, the microenvironment of the healthy brain and cellular interactions within the neurovascular unit provide inductive and maintenance signals for the BBB.

Astrocytes can be induced to secrete classic angiogenic factors that are important in vascular growth. VEGF, primarily acting through Flk1 receptor, is a potent mitogen for endothelial cells[92] and is required for the formation, remodeling and survival of embryonic blood vessels as VEGF deficient embryos develop few or no angioblasts and die prematurely[93]. Later in development and adulthood, astrocyte-derived VEGF can be induced under hypoxic conditions[94] to stimulate angiogenesis and, in consequence,

provokes BBB disruption[95-97]. On the other hand, Ang-1 and -2 bind to the receptor tyrosine kinase Tie-2 on ECs. Whilst Ang-2 activates angiogenesis and is expressed in early phases of BBB breakdown[98], Ang-1 is involved in vascular maturation and quiescence, events that are characteristic of BBB differentiation[99,100]. In fact, Ang-1 causes a time- and dose-dependent decrease in endothelial permeability by upregulating TJ expression[100]. Astrocyte-secreted thrombospondin (TSP)-1 and -2 also have anti-angiogenic properties, inhibiting EC proliferation and has been suggested to promote vascular maturation[101]. Thus, a balance between angiogenic and BBB promoting agents occurs at the level of brain ECs.

Additional potential cues that promote BBB maintenance include members of the fibroblast growth factor (FGF) family which have the ability to decrease EC permeability[102,103]. In fact, FGF-2 and -5 -/- mice have decreased occludin and ZO-1 expression and show defects in barrier functions[103]. Astrocytic secretion of glia-derived neurotropic factor (GDNF) has also been seen to increase BBB integrity via its ligation to EC-expressed GDNF receptor α1[104]. Src suppressed C kinase substrate, also known as SSeCKS, produced by astrocytes, regulates angiogenesis, by decreasing the expression of VEGF and stimulates expression of Ang-1, augments ZO-1 and claudin-1 protein expression and decreases cerebral EC monolayers permeability [81]. Additional novel astrocytic factors, recently identified by Dr. Kim and colleagues, include meteorin and A-kinase anchor protein-12 (AKAP12), both which are capable of regulating barrier development and formation via induction of TSP-1 and-2 expression and reduction of angiogenic factor secretion[101,105]. TGF-β, a well known lymphocyte-produced cytokine with multiple and complex physiological functions, is also known to be secreted by astrocytes and ECs and to induce a downregulation of the level of leukocyte migration across ECs *in vitro* and *in vivo* in a dose-dependent manner[106-108]. Finally, work by Wosik and colleagues have shown that astrocytes secrete angiotensinogen and angiotensin II (AngII) which decreases BBB permeability. Angiotensinogen -/- mice have a diminished expression of occludin and disorganized TJ strands in brain capillaries, supporting the notion that AngII is involved in the formation of BBB-EC TJs[43]. Therefore, the current view is that astrocytes are key regulators of BBB development, maintenance and regulation and that understanding the complex astrocyte-BBB-EC interactions under

physiological and pathological conditions may lead to the development of novel therapeutic strategies.

2.2 Neural cues control of the vasculature

The search for additional cues that could potentially promote barrier formation can benefit from drawing on similar processes which utilizes signaling molecules. Prior to barriergenesis, vascular development requires directional guidance to establish a precise, conserved branching pattern in the vertebrate body, including those in the brain[9]. Due to the nutrient and oxygen demand of neurons, the development of the vascular system is interconnected to the development of the nervous system. Recent evidence suggests that blood vessels and nerves share common mechanisms to expand, navigate and mature during development, as well as post-natally[109-111]. In fact, some neurons follow the path of blood vessels and inversely, the differentiation and branching of certain blood vessel depends on the emergence of new neural circuits[112,113]. It is now clear, that many guidance cues, first found and characterized for their function in neural development, are recently also recognized to function during vascular development. The vascular network shares much of the extensively studied neuronal navigational methods to guide its blood vessels and many parallels have been drawn between the two systems. In neurons, the growth cone at the tip of the axon is a highly motile structure that explores the microenvironment by extending finger-like filopodia and veil-like lamellipodia. These extensions contain guidance cue receptors that have the potential to activate various signaling cascades affecting the arrangement and composition of the cytoskeleton and therefore impact on the directional movement and elaboration of the growing axon[114]. Recent studies have shown that in nascent capillary sprouts, endothelial tip cells can be seen as the vascular equivalent of the growth cone as they act as sensors, signal transducers and motility devices than can regulate extension of capillary sprouts[109]. Although tip cells proliferate minimally, endothelial stalk cells further down the sprout, divide to generate new vessels along the path chosen by the tip cell[115]. Like the axonal growth cone, the tip cells can extend filopodia and sense guidance cues in ECM or on

neighboring cells that dictate the direction of growth of sprouting capillary. Fusion of tip cells and lumen formation allows the generation of new vessels. Interestingly, both vessels and axons respond not only express but to common cues[109-112].

The long search for morphogens and guidance cues culminated in the identification of several highly conserved, distinct but multifunctional protein families. Members of the Wnt, Hedgehog (Hh), FGF and bone morphogenic protein (BMP) families were shown to act as classical morphogens in a number of different contexts. On the other hand, proteins of the Netrin, Slit, Ephrin and Semaphorin families were found to act as guidance cues for migrating cells and axons. Morphogens are expressed early in development in spatially very distinct patterns. Through the formation of gradients (for example along a developing body- or organ-axis), morphogens can directly induce different cell fates in a concentration-dependent manner. Thus, morphogens govern the pattern of tissue development and the position of various specialized cell types within a tissue[116,117]. Diffusible or cell surface-bound guidance cues can attract, repel or induce turning of growing axons and thus aid in the pathfinding process of neurons[118-120]. With recent studies, axon guidance functions have now been documented for members of each of the three major families of classical morphogens: Hhs, BMPs and Wnts, clearly demonstrating the complexity of these developmental cues[121-123]. In addition to these groups of major signaling molecules being active in early developmental processes, they are utilised repeatedly in a variety of different organs and systems.

Most of the morphogenic/guidance pathways have been studied in other angiogenic processes[109,124-129]. EC differentiation and vascular morphogenesis are dependent on morphogenic factors such as Hh, Wnt and BMP[127,130,131]. Furthermore, Hh has been associated with several stages of vascular development, which will be reviewed later on (section 3.0). In analogy to the growth cone, the extending filopodia of the endothelial tip cell express Netrin-receptor UNC5, Slit-receptor Robo4, and Ephrin-receptors EphA and B, interpreting signals as attractive or repulsive when blood vessels navigate in through environment[129,132,133]. For example, loss of UNC5H2/B or Netrin-1 in mice and zebrafish lead to abnormal vessel guidance and excessive vessel branching, suggesting they provide critical repulsive guidance for navigating blood vessels[124,134]. Netrin-4, however, seemed to be involved in angiogenesis after ischemic injury as it

upregulates in astrocytic endfeet and blood vessels [135]. Similarly, Slit-2 inhibits the migration of Robo4-expressing ECs, while attracting ECs expressing the receptor Robo1[125,133]. EphrinB2 seems to provide a repulsive cue for intersomitic vessels[136]. In addition, Sema4D was seen to actually induce blood vessel formation, tubologenesis on top of EC migration. In fact, Semaphorin receptor Neuropilin-1 has now been recognized as a co-receptor for VEGF isoforms, suggesting complex interactions between vascular and axonal networking[137,138]. In fact, capillary sprouts radially invade the developing neuroectoderm along concentration gradients of VEGF[113]. While angiogenic factors are seen to act as chemoattractant cues, guidance and morphogenic cues can, on the other hand, act as angiogenic factors. Netrin-4 can increase blood-vessel density by enhancing blood vessel proliferation[135] and BMP2 can both play a chemotactic role on angioblasts and microvascular ECs as well as stimulating their proliferation and inducing tube formation[139]. While EphrinB2/EphB4 deletion resulted in a general failure in angiogenic remodeling of vascular plexus, EphrinA1 may regulate post-natal angiogenesis[129]. However, much controversy remains in this new area of research, concerning the expression and function of axonal guidance cues in angiogenesis and EC maintenance.

This recycling of molecules during development suggests that these multifunctional proteins act globally in providing positional information in a variety of developmental processes such as embryonic patterning, CNS induction, and angiogenesis. In addition, many of these morphogens and axon guidance cues are also important in post-natal and adult processes that utilize and recapitulate embryonic processes such as maintenance of organotypic tissue, remodeling after repair and barriergenesis.

2.3 Neural cues control of blood-brain barrier function

Due to the close interaction of BBB-ECs with CNS processes, it is perhaps not surprising that cues typically associated with neural processes have thus been shown to impact on BBB-ECs. As mentioned previously, FGF-2 and -5 -/- mice have decreased occludin and ZO-1 expression and show defects in barrier functions[103]. BMP signaling

has also been seen to be important in astrocyte-endothelial interactions, where the loss of the receptor BMPR1a leads to an increase in leakage of Evans Blue and immunoglobulins (class G; IgG) through cortical vessels *in vivo*[140]. Stimulation of Robo4 on ECs with its ligand Slit-2 induced barrier function *in vitro*. *In vivo*, Slit-2 prevented VEGF-induced hyperpermeability and Evans Blue leakage into tissues, implying their role in vascular bed maturation[133]. Recent studies have shown that the canonical Wnt pathway is important for vascular development and for the formation of a mature BBB[141-143]. The Wnts are secreted glycoproteins that accumulate in the ECM that trigger activation of the Frizzled receptor on adjacent cells. Frizzled activation promotes β-catenin stabilization and entrance into the nucleus, where it can modulate target gene transcription. The Wnt pathway is activated in brain ECs during embryogenesis, where it is required for CNS angiogenesis and capillary bed formation[141]. Together, Wnt 7a and 7b, together, are essential for CNS vascular development as Wnt7a/b double -/- mice display severe CNS-specific hemorrhaging and enhanced vascular fragility through destabilization of inter-endothelial contacts[144]. *In vitro*, Wnt3a induces claudin-3 upregulation in mice brain vascular ECs[142] and Wnt7a elicits strong migration of mouse brain ECs across a Boyden chamber filter and induces the expression of BBB-specific transporter Glut-1[141]. Post-natally, conditional loss-of-function of B-catenin in mouse ECs has decreases claudin-3 expression and Evans Blue extravasation in the CNS-parenchyma[142]. Additional studies on CNS-related signaling molecules could potentially lead to identifying other BBB promoting factors.

3.0 THE HEDGEHOG (Hh) SIGNALING PATHWAY

The Hh pathway is a conserved signaling cascade involved in embryonic morphogenesis, pathfinding, and as recent studies show, angiogenesis. There are three vertebrae homologues of the originally identified Hh in Drosophila melanogaster: Desert Hh (Dhh), Indian Hh (Ihh) and Sonic Hh (Shh). All three activate the same signaling pathway and the presence of divergent regulatory elements cause spatial and temporal differences in their expression patterns during embryogenesis[145-149]. While Ihh and Dhh are best known for their importance in chondrocyte and gonadal differentiation respectively[146], Shh has been widely associated with morphogenic events such as posterior identity of the limb bud[150], ventral cell identity of the neural tube, and motor neuron development[151,152]. As other morphogens, Shh has also been characterized as an axonal guidance cue, capable of both stimulating commissural axon migration towards the floorplate [121,122] and repelling them after the axons have crossed the floor plate and turned rostrally[153]. Recent studies suggest that the Hh pathway may also play an important role in adult tissue homeostasis, neural progenitor proliferation and wound healing, which all draws parallels to the embryonic developmental processes [130,154].

Shh is initially translated as a ~45kDa precursor protein. Essential to Hh signaling is the internal autocatalytic cleavage of the 45kDa protein mediated by the carboxy (C)-terminal of Hh, yielding two products, the ~25kDa C-terminal fragment and the ~20kDa amino (N)-terminal signaling domain. During autoproteolysis of the precursor protein, the C-terminal fragment becomes a functionally active cholesterol transferase that covalently adds a cholesterol molecule to the C-terminus of the N-terminal fragment. A palmitoyl moiety is also added to the N-terminal of the processed signaling molecule by Skinny Hh [145,155,156]. These two lipid modifications control, in part, the ability of the Hh molecule to bind to its receptor and to signal at short- or long range. Following the post-translational addition of cholesterol and palmitoyl moieties, Shh is released from the producing cell with the help of the 12-transmembrane protein Dispatched[130]. Tethered to the cell surface or secreted, Shh binds with high affinity to the cell surface receptor Patched (Ptch-1)-1, a 12-transmembrane domain receptor. In mammals, Ptch-2 has been isolated but not much is known about its exact role in

the signaling pathway[157]. Binding of Shh to Ptch-1 alleviates repression of the signal transducer Smoothened (Smo), a serpentine transmembrane protein with a topology reminiscent of Frizzled family of Wnt receptors and other G-protein coupled receptors[158]. Although the exact mechanism in which Ptch-1 inhibits or activates Smo is unknown, it is speculated that Smo is regulated by an undiscovered small molecule, such as oxysterols or vitamin D3 that would induce conformational changes within Smo[159-161]. The interaction of Hh with Ptch-1 can be promoted by its binding to two other transmembrane proteins Boc and Cdon. Several other surface proteins can bind, mobilize and limit the range of Shh movement such as Hip (Hh interacting protein), a membrane bound glycoprotein which sequesters Hh ligands, but has no effect on Smo activity and thus attenuates the signal[162]. Once Smo is in its active conformation, activation of zinc-finger transcription factors of the Gli family (Gli-1 through -3) act at the last step of the Shh-signal-transduction pathway[145,147] (Figure ii). Activation of cytoplasmic-bound Gli components is achieved through their sequential phosphorylation by various kinases, allowing them to translocate into the nucleus and induce or repress transcription of target genes. Elegant genetic studies have exposed both the redundant and unique functions of the three Gli transcription factors. Gli-1 and Gli-2 act primarily as activators, while Gli-3 can undergo autoproteolysis to function as a transcriptional repressor. Thus, the ratio of the Gli activator to Gli repressor forms of the proteins is critical in final transcription processes[163]. In addition to the induction of specific cell fate determinants in response to the Hh signal received, proteins of the Hh cascade itself, such as Ptch-1, Hip and Gli-1 are upregulated. It is thought that this positive feedback upregulation of expression results in increased levels of Ptch-1 and Hip protein at the cell membrane, further sequestering Hh and limiting spreading of the signal [146,147,164]. Therefore, Ptch-1, Hip and more often, Gli-1 mRNA or protein upregulation assays are used as indicators of Hh pathway activation.

Given that Hh signaling is a critical developmental pathway during embryogenesis and organogenesis, mutations in pathway components result in congenital defects. The forebrain of the fetal Shh mutant fails to develop into two hemispheres, or holoprosencephaly, resulting in defects in the development of proper brain structures and functions as well as facial properties [150,165]. In human foetuses, loss of one Shh allele is enough to cause varying penetrance up to and including cyclopia (fusion of developing eyes at the midline), whereas both alleles need to be lost in the Shh -/- mouse to produce the same phenotype[166-169]. In mouse embryos, deletion

of Shh leads to cyclopia and defects in ventral neural tube, somite and foregut patterning. At later developmental stages, defects include severe distal limb malformations and failure of lung branching[150,170,171].

In addition to the vital role Hh plays in embryonic development, Hh signaling also functions as a regulator of cell proliferation, differentiation and survival in adult tissue. The importance of Hh functions, in the adult, is reflected in the loss of growth control when the pathway is damaged. Studies have shown that inappropriate reactivation of this pathway later in life can lead to the development of malignancies as Hh signaling is a molecular hallmark of several subsets of familial and sporadic tumors, including basal cell carcinoma syndrome[172], medullablastoma[146,173], pancreatic[174], breast[175], prostate[176], gastrointestinal[177] and lung cancers[178,179]. In addition, the family of Gli genes are named for their role in glioblastoma formations[180].

The highly teratogenic steroidal alkaloid toxins, cyclopamine and jervine, are extracted from corn lilies. Early gestating sheep grazing on wild corn lilies resulted in severe nervous system defects in their offspring that exhibited strong resemblances to holoprosencephaly with associated cyclopia. The toxins were later characterized to act as antagonistic compounds binding Smo and inhibiting Hh signaling[181]. Pertussis toxin (PTX) has also been seen to inhibit Smo activity through its ability to prevent G proteins from interacting with their G protein-coupled receptors, even though no known G protein has been shown to bind to G-protein-coupled receptor-like Smo[182]. High-throughput screens of chemical libraries for Hh pathway modulators have identified four compounds that potentially inhibit Shh (Smo antagonist, SANT-1 through SANT-4) and two that activate Hh signaling (Smo agonist, SAG and purmorphamine), both by binding directly to Smo (Figure ii) [183,184]. Of the antagonists, SANT-1 was found to exhibit the highest affinity for Smo and attenuated Shh stimulation to a much greater extent than the other antagonists[183].

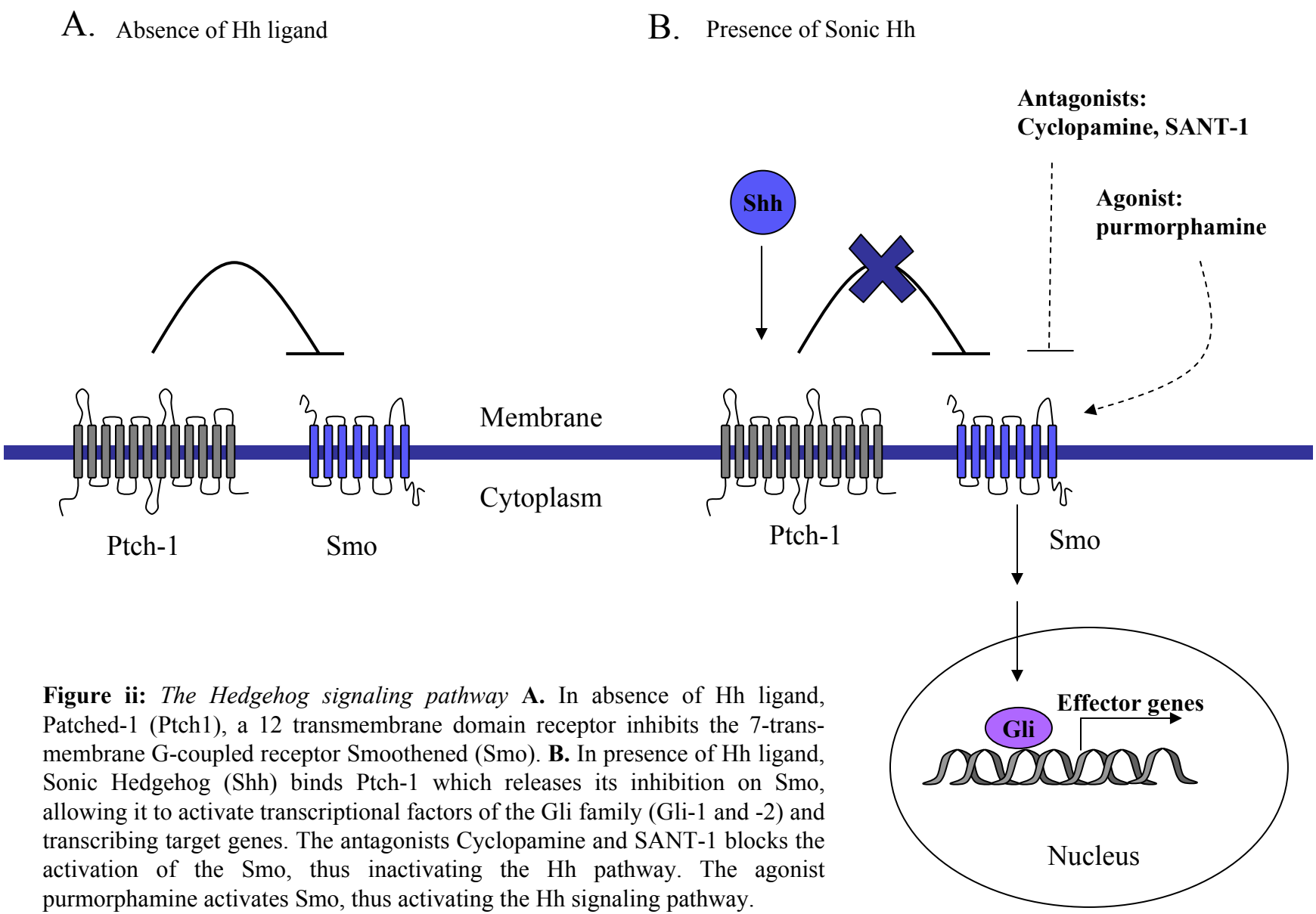


Figure ii: *The Hedgehog signaling pathway* **A.** In absence of Hh ligand, Patched-1 (Ptch1), a 12 transmembrane domain receptor inhibits the 7-trans-membrane G-coupled receptor Smoothened (Smo). **B.** In presence of Hh ligand, Sonic Hedgehog (Shh) binds Ptch-1 which releases its inhibition on Smo, allowing it to activate transcriptional factors of the Gli family (Gli-1 and -2) and transcribing target genes. The antagonists Cyclopamine and SANT-1 blocks the activation of the Smo, thus inactivating the Hh pathway. The agonist purmorphamine activates Smo, thus activating the Hh signaling pathway.

3.1 The Hh signaling pathway in vasculogenesis

As other morphogens and axonal guidance cues, the Hh pathway has been associated with the vascular system. In the developing embryo, the visceral yolk sac is the first site of blood vessel formation in the murine embryo. Ihh was shown to regulate murine yolk sac angiogenesis as the Ihh -/- mice, as well as Smo -/- or cyclopamine-treated murine yolk sacs formed smaller, flattened, disorganized vessels that easily collapsed[7,130,185]. Smo deficient embryos display a more severely abnormal yolk sac phenotype than do Ihh -/-, suggesting that Shh may compensate for the loss of Ihh, but that it is not completely redundant[185]. In zebrafish models, Shh signaling is crucial for the organization of the angioblasts into major vascular channels such as the aorta and axial vein[186,187]. Similarly, in avian and mouse embryos, Shh and Ihh act together to mediate embryonic formation of aortic and coronary vessels[4,187,188]. Vokes and colleagues[4] remarked that the first blood vessels within the embryo always formed in the mesoderm tissue in close proximity to the endoderm, and that an endodermic signal was essential for the assembly of angioblasts into tubular vessels. While VEGF treatment was not sufficient in inducing tubulogenesis in embryos lacking an endoderm, Shh was both found to be produced by the endoderm and to promote the assembly of angioblasts into vessel tubes. This elegant study showed that while VEGF was important in EPC differentiation, Shh signaling was crucial for the structural morphological formation of the vessels. Although at first, no obvious vascular defects were observed in Shh deficient mouse embryos[150], other groups have now described decreased and abnormal vascularization in tissues such as the lung, heart, pharynx, and pulmonary tissue[171,189]. Cyclopamine-treated embryos exhibited a variety of vascular abnormalities such as interrupted tubes, unassembled clusters of angioblasts and fewer vessel tubes, compared to control embryos[4]. In embryos treated with cyclopamine at embryonic stage E8.5-10.5, neural tube angiogenesis was also impaired, as perineurial vascular plexus vessel sprouting does not occur[190]. On the other hand, hypervascularization of neuroectoderm occurred in response to Shh overexpression in the dorsal neural tube of zebra fish[191] and overexpression of Shh by injection of Shh mRNA causes the formation of lumenized ectopic vessels[187].

3.2 The Hh signaling pathway in angiogenesis

During angiogenesis, remodelling of the vasculature is a process that depends on migration and reassembly of ECs. Through Smo activation, Shh can provoke cell morphology alterations together with formation of lamellipodia, suggesting a migratory response[192]. Furthermore, embryonic mouse yolk sac ECs responded to Shh treatment by activation of gene transcription involved in cytoskeletal organization, migration, and angiogenesis, as detected by GeneChip assays[193]. Wound healing is often seen as a recapitulation of angiogenesis as it requires recruitment of EPCs and endothelial tip cell chemoattraction to the target area[154,193]. Added exogenously, Shh significantly enhances wound healing and migration of embryonic ECs in wounded areas in a scratch assay, a process mediated through migration rather than cellular proliferation[193]. In an *in vivo* study of wound healing in mice, Shh treatment resulted in smaller wounds after 5 days, compared to control, a possible result of bone-marrow derived EPC recruitment, increased cellular infiltration, collagen deposition and enhanced overall vascularity in the wounded area[154]. As a morphogen, it is perhaps expected that several *in vitro* studies have shown that in addition to migration, Shh binding to Ptch-1 mediates capillary formation and morphogenesis, in mature murine brain capillary ECs, HUVECs and putative angioblasts from the bone marrow[4,154,182]. In these cells, Shh treatment increased nuclear Gli-1 localization and mediates gene transcription and protein synthesis as actinomycin D and cycloheximide, respectively, inhibit capillary morphogenesis. Cyclopamine and PTX also suppress Shh-inducing capillary morphogenesis[182]. By augmenting cytoplasmic extensions and increasing cellular contacts between cells, Shh promotes cell type specific cellular adhesion which is important in structure formation. Similar models in drosophila proposed Hh-mediated cell adhesion pathways as a mechanism of wing imaginal disc formation[194].

Shh signaling stimulates vascular remodelling and angiogenesis in various tissues at later developmental stages[195]. Decreased levels of Shh signaling as a result of treatment of mice with a Shh neutralizing antibody or with cyclopamine leads to angiogenic malformation and loss of ability of existing vessels to remodel, fuse and form branches, causing malformations and haemorrhages. In particular, Shh-supported processes such as the development of branchial region vessels and pulmonary arteries are defective in Shh null mice embryos[196]. Postnatal and adult angiogenesis can also be enhanced by Hh signaling. Ptch-1 was

also found to be normally expressed in cardiovascular tissues of juvenile and adult mice and activation of Hh signaling in the adult heart is sufficient to promote coronary neovascularization and protect from ischemia, implying an important role for Shh in maintaining cardiac homeostasis and function. Temporal and tissue-specific deletion of Smo lead to reductions in proangiogenic gene expression and loss of coronary vasculature, which resulted in tissue hypoxia, cardiomyocyte apoptosis, ventricular failure and subsequent lethality[197,198]. When Shh is administered to aged mice, new vessel growth can be observed in adult corneas and ischemic hind limbs. Indeed, Shh treatment can promote an increase in capillary density, blood flow and vessel diameter, suggesting neovascularization[199]. Similarly, implantation of Shh-containing pellets promotes neovascularization and formation of large, well-organized branched vessels in corneal angiogenesis assays in mice[199,200]. In perspective, the Hh pathway possibly aids in tumor angiogenesis. The fact that the usually high expression of Hip on ECs is diminished in cancers supports the hypothesis that Hh is enhanced in tumor tissues and contributes to tumor angiogenesis and growth[201].

Shh not only can be viewed as a potent angiogenic agent, but activation of the Hh pathway can also have an indirect role in angiogenesis, by acting upstream of angiogenic factors. One of the first publications on the Hh pathway in angiogenic processes describes the ability of Shh to upregulate angiogenic factors such as VEGF (all three isoforms of VEGF-1, -189, -165, -121) and both Ang-1 and Ang-2 in mesenchymal fibroblasts, possibly placing Hh upstream of these vascular-specific growth factors and vessel stabilization potentials[199]. However, Shh treatment did not seem to have an effect of EC migration and proliferation, implying that EC response may be dependent on their differentiation status, developmental age and organ origin[193,199]. Since, others have observed VEGF and Ang-1 upregulation in other contexts, such as in Shh-treatment of human EC lines[202], and coronary vasculature[198]. Blocking Shh with neutralising antibodies or cyclopamine inhibits VEGF upregulation and thus angiogenesis, as seen in mouse models of hind limb and cornea ischemia[199,203]. Ang-1 and its receptor Tie-2 are even downregulated in the lungs of Shh -/- embryos[204]. These studies suggest that the Hh signaling pathway is important in vascular proliferation and differentiation as well as *in vitro* capillary morphogenesis.

3.3 Evidence of the Hh signaling pathway in barrier function

Little is known for the Hh signaling pathway during the last step of vasculature maturation, barriergenesis. In the developing mouse submandibular gland, lumen formation occurs in the epithelial cell lining, leading to the establishment of apical-basal polarity and TJ formation, processes reminiscent of EC barrier formation. Shh null mice exhibit developmentally arrested submandibular gland epithelium, whereas treatment with Shh enhances epithelial lumen formation, full cell polarization and ZO-1, claudin-3 and occludin TJ protein distribution in these deficient explants[205]. In addition, laminin-5 deposition at the basal lamina region of terminal buds was accelerated in Shh-treated glands[205], suggesting a role of Shh in both junctional and extracellular cell attachment. Importantly also, in the peripheral nervous system (PNS), Dhh is expressed in myelinating Schwann cells. This Dhh signal is needed for the formation and maintenance of the perineurium, a component of the blood-nerve barrier that acts as a barrier to protect against serum molecules and cellular infiltration[206]. Comparable to the endothelial lining of blood vessels, perineurial cells are sealed by tight and gap junctions and are supported by a prominent basal lamina, thus forming an effective diffusion barrier. In Dhh -/- animals, the perineurium is looser, and the basal lamina encloses less collagen and is overall discontinuous. In addition, perineurial cells of Dhh null mice lack the gap junction protein connexion43, while expression of TJ proteins occludin and ZO-1 is disorganized. Freeze fracture electron microscopy further revealed abnormal and interrupted TJ arrays in Dhh null mice when compared to normal control perineurium TJ strands, leading to a defective nerve-tissue barrier, shown by extravasation of Evans Blue dye and immune cells invasion into the endoneurium[206]. Similarly, after a crush injury, more macrophage infiltrations is visible in both injured and non-injured nerves in Dhh -/- mice[207]. Therefore, the Hh pathway is likely to be involved in the formation of an ordered and functionally competent perineurium, seen as a later stage of perineurial sheath maturation[208]. By analogy, these studies raise the question of whether the Hh pathway plays a role in other barrier contexts, such as the BBB.

4.0 THE BASAL LAMINA

The basal lamina, found between the BBB-ECs and perivascular cells, is composed of several ECM components, including fibronectin, laminins, collagen type IV and heparin sulfate proteoglycans[209-211]. The basal lamina functions as a tissue boundary on which cells are attached to and a substrate for cellular differentiation and induction of gene expression. Astrocyte, and to a lesser degree, pericyte-derived ECMs enhanced BBB integrity, demonstrating the importance of additional glial matrix constituents[209,211,212]. For example, astrocytes can express agrin which could serve as a basal membrane factor maintaining barrier functions, as loss of agrin in human glioma accompanied a loss in TJ[8,213]. In addition, soluble factors secreted by glial cells can be captured by ECM proteins, and thus increase their local concentrations. BBB-ECs, in return, constitutively express a number of integrins that are important for EC adhesion to the basal lamina and control many signaling events critical for cell survival, growth and gene expression[210,214,215]. Integrins are heterodimers of α- and β- integrin and pairs such as $\alpha_1\beta_1$, $\alpha_3\beta_1$ and $\alpha_6\beta_1$ are commonly expressed on the cerebrovasculature and recognize the basal lamina. Alterations in expression of integrins have been shown to correlate with angiogenesis, BBB breakdown and increased barrier permeability during focal cerebral ischemia and/or MS. For instance, appearance of $\alpha_v\beta_3$ on BBB-ECs was found to be significantly upregulated after the onset of focal ischemia [210,214]. This suggests that integrin receptor function is needed for the maintenance of the BBB.

5.0 IMMUNE INTERACTIONS AT THE BBB

Under physiological conditions, a low number of immune cells, or leukocytes, continuously cross the BBB and monitor the CNS in a process called immune surveillance. The BBB-ECs are, thus, the first cells leukocytes must contact before entering the CNS and the molecular mechanisms by which leukocytes accomplish transendothelial migration is complex and involves a series of sequential and tightly controlled steps[216]. Initially, leukocyte rolling consists of a weak interaction between EC selectins, or cell adhesion molecules (CAMs), and their ligands on leukocytes. Since the CAM-ligand interaction is originally one of low-affinity, leukocytes can roll along the blood-vessel wall and sense activation factors deposited on the EC surface, such as various chemokines[217].

During inflammatory conditions, BBB-ECs can actively influence and participate in neuroinflammatory reactions by regulating cytokine/chemokine and CAM expression, both directly affecting immune cell migration into the CNS[218-221]. In addition to the ECs, CNS parenchymal cells can become activated by inflammatory cytokines to secrete chemokines in their vicinity. With over 50 identified members, chemokines constitute a large group of small cytokines (chemotactic cytokines) which are classified, according to their cystein configuration, into two main families, the CC and CXC chemokines. In response to inflammatory challenge, BBB-ECs can produce and secrete pro-inflammatory among others, monocyte chemoattractant protein (MCP)-1, also known as CC ligand (CCL)-2, MIP-1α/CCL3, RANTES/CCL5, interleukin (IL)-8/CXCL8, and IP-10/CXCL10[217,222-224]. However, MCP-1/CCL2 and IL-8/CXCL8 were seen as the major chemokines produced by BBB-ECs grown under culture conditions and upregulated upon inflammatory conditions[225]. All these chemokines bind specific G-protein coupled receptors on the leukocytes, rapidly inducing integrin conformational changes through an outside-in signaling mechanism that promote their high-affinity interactions to the cell CAMs expressed on BBB-ECs.[217]

In addition to the tight adherence, EC expression of CAMs and leukocyte integrins also mediate the ensuing diapedesis of immune cell across vascular beds. Under

normal conditions, CNS-ECs express low levels of all the appropriate CAMs, such as intercellular CAM-1 (ICAM-1) and vascular CAM-1 (VCAM-1)[226-228]. However, in the presence of inflammation, these molecules are strongly upregulated, further promoting leukocyte arrest and transmigration. Recent studies have identified additional CAMs that regulate the transmigration of lymphocytes EC barriers by the means of ALCAM (activated leukocyte cell adhesion molecule or CD166)[23] and Ninjurin-1 [229] which, for example, play important roles in invasion of particular subsets of leukocytes such as $CD4^+$ T cells and monocytes, respectively, from the blood to the CNS, especially during inflammatory contexts.

The final step of immune cell transmigration across the BBB consists of arrested leukocytes crawling along the surface of inflamed endothelium toward inter-endothelial vascular junctions. Homophilic interactions with junctional molecules such as PECAM-1 and JAMs results in junctional loosening, leukocyte extravasation and eventual basal lamina breach through gaps of low laminin expression[217,230,231]. Further proteolysis of the compact basal membrane surrounding the BBB by matrix metalloproteinases occurs to permit leukocyte entry into the parenchyma.

5.1 Multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE)

In non-pathological conditions, BBB-ECs control the CNS microenvironment and limit the entrance of immune cells by expressing low levels of integrin ligands and activation markers, to achieve a precisely regulated biochemical and immunological homeostasis essential for reliable CNS function. Immune cell infiltration and BBB breakdown is associated with many CNS diseases, including Alzheimer's disease[232], HIV-1-associated encephalomyelitis[233], stroke[234], amyotrophic lateral sclerosis[235] and especially, MS[79,223]. MS is an inflammatory and demyelinating disease of the CNS characterized by multifocal perivascular immune infiltration, leading to demyelination and astrogliosis (astroglia activation and proliferation)[236].

CD4+ T cells, also called T helper (Th) cells, are a part of the adaptive immune system which generally recognizes foreign peptides, or antigens, and subsets of Th cells

are generally distinguished by their cytokine secretion profile. Th1 associated soluble mediators, interferon (IFN)-γ and tumor necrosis factor (TNF)-α, are pro-inflammatory cytokines and MS has been considered to be initiated by auto-reactive CD4+ Th1 cells that migrate across the BBB to access the CNS[237,238]. In fact, MS is generally considered a CD4+ T cell-mediated inflammatory disease, based on the cellular composition and number of immune cells that infiltrate the brain and cerebrospinal fluid (CSF) and on data from the mouse model of MS, experimental autoimmune (allergic) encephalomyelitis (EAE). Th17 cells are another subset of Th cells which secrete IL-17 and IFN-γ and has often been implicated in autoimmune diseases as these cells are found to be in MS lesions and IL-17 deficient mice significantly suppress EAE disease scores[239,240]. Finally, Th2 cells secrete anti-inflammatory cytokines such as IL-4 and interferon (IFN)-β, thereby downregulating and suppressing the effects of pro-inflammatory Th1 and 17 effects. In fact, IFN-β is currently used as a therapy for MS due to immunomodulatory abilities[241].

Oligodendrocytes are CNS-derived glial cells responsible for the myelination of neurons. In MS, oligodendrocytes are a central target of the attacks by the host immune system[242,243]. Autoreactive CD4+ T cells have been found to recognize protein components of the myelin sheathe, such as myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP) and myelin basic protein (MBP), and initiate thus local inflammation, axonal demyelination lesions, and plaque formation[236]. Leukocytes accumulate in these lesions, most notably in the perivascular space surrounding small blood vessels. Continuous chronic inflammation can lead to recruitment of additional immune cells, furthering demyelination, axonal injury and glial scaring. In EAE, the injection of defined myelin components, together with an immune stimulating adjuvant, into naïve susceptible animals leads to a predominantly CD4+ T cell-mediated autoimmune diseases that shares similarities with MS. Whilst EAE cannot be transferred by antibodies, passively transferring encephalitogenic myelin-specific T cells in susceptible rodent and strands also leads to the diseases[244]. This demonstrates that EAE, and possibly MS, are T cell mediated autoimmune diseases. However, in certain EAE models used, the infiltration is characterized by a predominant infiltration of mostly CD4+ T cells into the spinal cord and brain stem, which are significant differences between the rodent model and the human pathology[245]. However, to induce EAE, PTX is

given, which opens the BBB[246,247]. This suggests that EAE is a combination of at least two processes, autoreactive T cells, and BBB disruption.

In MS lesioned areas, the BBB becomes compromised and leaky, allowing entry of serum proteins and cells into the CNS. Clinically, BBB breakdown can be visualized by magnetic resonance imaging using gadolinium as a tracer molecule for lesions. Gadolinium leakage into the CNS parenchyma serves as a reliable marker for disease activity as it indicates inflammatory processes, making it an essential detection and diagnosis tool[248,249]. Gadolinium leakage may even precede the appearance of new lesions, suggesting potential BBB deregulation pathologies prior to inflammatory processes[250]. Lesion development is characterized by BBB activation and disruption in TJs organization, ensuing in changes in EC permeability[42,64]. Activated ECs, containing increased number of vesicles, decreased density of mitochondria, and expressing higher levels of CAMs, are also present in MS plaques[251,252], which correlated with the accumulation of serum proteins in these areas. Nonetheless, active cell recruitment across BBB-ECs seems to be an essential step in contributing to CNS inflammation and subsequent tissue injury and BBB breakdown. Activated, myelin-specific leukocytes are primed through signaling via high levels of integrins and chemokine receptors, promoting their entry into CNS parenchyma. In fact, neutralizing antibodies (Natalizumab) against a leukocyte integrin suppress leukocyte entry into the CNS through inhibition of integrin-binding to its ligand on the BBB-EC[253].

Upon entry into the CNS, leukocytes induce molecular changes in the nervous tissue and initiate transient or even chronic inflammatory reactions. Inflammatory cytokines such as IL-1, TNF-α, and IFN-γ, increase CAM expression and chemokine secretion by BBB-ECs and further modulate their TJs expression and function, aiding in the recruitment and entrance of more leukocytes to the site. BBB-ECs thus become an important source of pro-inflammatory chemokines, cytokines[239,254] and CAMs[23,72]. This is the basis for the inflammatory cascade that leads to the focal inflammation of BBB-ECs and the recruitment and infiltration of additional immune cells. The infiltrated immune cells will subsequently secrete additional cytokines and chemokines that may further enhance CNS inflammation or promote a gradual resolution of the inflammation. CD4+ T cell-associated soluble mediators such as IFN-γ and TNF-α are found in elevated

levels in CSF, serum and lesions of MS patients[255]. TNF-α was previously shown to be a potent modulator of brain endothelium permeability and both upregulate expression of ICAM-1 and VCAM-1 on the surface of BBB-ECs and increase the expression of chemokines IL-8/CXCL8, MCP-1/CCL2 and IP-10/CXCL10 in CNS and non-CNS ECs[223]. Thus, in vitro, TNF-α and IFN-γ are often used to mimic inflammatory conditions.

Preventing leukocyte infiltration into the CNS is an important therapeutic strategy for treating patients with MS. As mentioned above, exogenously administered antibodies can block the entrance of leukocytes. Neurons and glial cells also produce a large variety of anti-inflammatory cytokines and immune modulators and the balance between pro- and anti-inflammatory cytokines is crucial to limit the inflammatory reaction in the CNS.

5.2 The Hh signaling pathway in injury and MS

Recovery from injury involves molecular and cellular events that are reminiscent of those during development as tissue repair partially recapitulates morphogenesis. After compression injuries, Shh mRNA expression is increased prominently within oligodendrocytes, Schwann cells and regenerating axons[256]. Shh expression is correlated with the expression of the neurotrophic factor brain-derived neurotrophic factor (BDNF), suggesting that Shh plays a neuroprotective and survival role for motor neurons. Indeed, on cultured Schwann cells, Shh treatment upregulates Gli-1 in addition to BDNF mRNA. Increased Shh reactivity is also reported after neural injury and Shh may play a role in neuronal repair and oligodendrocyte maturation[257].

Strong Shh expression is described on BBB endothelium, astrocytes and hypertrophic astrocytes within demyelinated brain lesions in rats with EAE[258], suggesting that the Hh signaling pathway may also play a role in astrocyte-endothelium interactions and BBB maintenance or repair. However, research on Shh and its involvement in MS and EAE has mostly focused on the fact that Shh is critical for oligodendrocyte maturation, a crucial step in their ability to myelinate axons. Mature oligodendrocytes in adult mammalian CNS are post-mitotic and unable to proliferate in response to injury[259].

Nevertheless, abundant oligodendrocyte precursor cells (OPCs) exists in the white and grey matter of normal adult CNS and could potentially contribute to remyelination. OPCs have been observed in MS lesions, but for the most part, they remain quiescent and thus represent a target for therapies to reactivate their differentiation into mature oligodendrocytes in order to enhance remyelination in MS patients[260]. Indeed, upregulation of Shh is essential for oligodendrogenesis and OPC development, including induction, survival, proliferation and migration of oligodendrocytes[261,262]. In spinal cord contusion, OPC implantation and Shh treatment improved white matter sparing[263].

In the subventricular zone (SVZ), astrocytes act as multipotent stem cells and support the neural stem cells (NSCs) niche[264]. Upon astrocytic influence, NSCs are induced to mature into neural precursor cells that will restore mature populations of astrocytes, neurons and oligodendrocytes. Wang and co-workers showed that Shh is expressed in the SVZ and constitutively expressed at low levels in the spinal cord of adult naïve mice, where it colocalizes with glial-fibrillary acidic protein (GFAP), a marker for astrocytes[265]. Non-niche astrocytes also secrete Shh, which potentially correlates to the atypical injury-induced niches that are formed by perivascular and parenchymal astroglia and ECs during EAE, facilitating NSC migration, differentiation and integration[266]. Co-cultures of astrocytes and NSCs induces the differentiation of the stem cells into neurons, oligodendrocytes and astrocytes, whereby addition of anti-Shh neutralizing antibody inhibited differentiation, suggesting that adult astrocytes can induce NSC differentiation and that this process is supported by Shh. In addition, Shh-treated NSCs accelerates further oligodendrocyte differentiation[265].

Upon stimulations such as inflammation or injury, Shh was seen to be upregulated in hypertrophic astroglia associated with GFAP expression in MS lesions compared with control. However, activation of atypical or SVZ niches does not occur in MS. Potent inflammatory cytokines, such as IFN-γ, can increase Shh in astroglia but downregulate Gli-1 expression in NSCs, thus disrupting the program of Shh-induced NSC differentiation. The downregulation of Gli-1 signal in OPCs may contribute to the impairment of OPC maturation in EAE and MS[265]. Another possible rationale for the inability of OPC activation might be the lack of Shh-cleavage within the white matter of MS patients. The white matter fraction of brains of MS patients contains the unprocessed

45kDa Shh protein, but the cleaved, active form (19kDa) of Shh was either absent or greatly reduced, compared to brains from healthy subject controls or from patients with other neurological diseases such as Alzheimer's and Huntington's[267].

IFN-β is commonly used in the treatment of demyelinating diseases such as relapse-remitting MS, but its diverse actions are not yet fully understood, but thought to impact and modulate the immune system, therefore causing an alteration of autoimmune progression. In a mouse model of spontaneously acute demyelination (ND4), Shh is upregulated during the demyelination phase, and is further upregulated after treatment of IFN-β in combination with vitamin B12. Combination therapy induces OPC maturation via Shh upregulation[268]. Finally, Niaspan is another treatment improving neurological functional recovery in EAE mice through the stimulation of the Shh pathway. *In vitro*, Niaspan treatment increases proliferation of immature OPCs. While Shh and Gli-1 mRNA and protein expression are significantly decreased in EAE spinal cord, their expression is increased in spinal cord of Niaspan treated mice. *In vivo*, Niaspan increases oligodendrocyte proliferation and neuronal regeneration[269]. However, Niaspan also caused a reduction in the number of vessels containing inflammatory cell infiltrates and a decrease of EAE scores, which is potentially associated with the beneficial effects of Shh on the BBB itself, although this subject has never been touched upon.

6.0 LIPID MEMBRANE MICRODOMAINS

This section contains excerpts from a review article entitled "Functions of lipid raft membrane microdomains at the blood-brain barrier"[270].

Due to the restrictive nature of the BBB, the movement of nutrients, molecules and cells between the blood and the CNS requires interactions with plasma membrane proteins to signal and initiate transport across the BBB. The plasma membrane of eukaryotic cells is composed of a complex mixture of membrane proteins and lipid molecules, including glycerophospholipids, sterols and sphingolipids. Glycerophospholipids tend to adopt a mobile loosely-packed fluid phase while sterols and sphingolipids possess distinct biophysical properties and readily group tightly together in a more ordered state. The differential lipid packing abilities lead to phase separation and to the organization of sphingolipid- and cholesterol-rich membrane microdomains, commonly referred to as *rafts*[271-274]. Lipid-enriched membrane rafts are dynamic plasma membrane microdomains associated with membrane compartmentalization, actin rearrangements and plasma membrane receptor-mediated signaling events[271,272,275,276]. Current data generated on lipid rafts strongly suggest that these microdomains are biologically important and form a functional unit in the plasma membranes of cells.

A specialized subset of rafts known as caveolae, defined as small cave-like surface invaginations of 50-100 nm in diameter, have been shown to mediate vesicular transport and sophisticated cell signaling. Caveolin-1, the main protein component of these structures, functions as a scaffolding protein and as a possible cholesterol sensor, regulating raft polymerization and lipid trafficking[277,278]. Unlike lipid rafts, caveolae are not present in all cell types, but are found abundantly in ECs and aid in regulating numerous endothelial functions such as transcytosis, vascular permeability and angiogenesis, and can serve as docking sites for glycolipids and GPI-linked proteins, as well as various receptors and signaling molecules[279].

Experimental studies have demonstrated that membrane clusters of glycosphingolipids and sterols were insoluble in detergents at 4°C and form detergent

resistant membranes (DRMs) that can be isolated by ultracentrifugation in a sucrose gradient. These observations, and others, have led to the *lipid raft hypothesis* that defines lipid (or membrane) rafts as 10-200 nm heterogeneous and highly dynamic structures enriched in cholesterol and sphingolipids[271,272]. While most of these lipid raft isolation protocols[280] have been performed using Triton X (TX)-100 at 4°C[281], many other detergents have also been used to prepare DRMs, including Lubrol[282], Brij-58,-96,-98[283], NP40, CHAPS and octylglucoside[280,284]. Non-detergent fractionation methods have also been used to isolate membrane rafts, and likely reproduce more accurately the *in vivo* protein oligomerizations that occur in microdomains[285,286]. In addition, due to their similar biophysical properties, caveolae represent a subset of the DRM population and can be further purified using magnetic beads coupled to antibodies against caveolin-1[287]. As a result of various DRM isolation techniques, the lipid, cholesterol and protein compositions within DRMs can vary, giving rise to biophysically diverse rafts. However, the heterogeneity of the lipid rafts, in addition to being an outcome of detergent selectivity, may also represent the underlying diversity of lipid and protein compositions.

A wide variety of proteins are targeted to lipid rafts, notably, but not exclusively, via fatty acylation (myristate and palmitate modifications) and glycosylphosphatidylinositol (GPI)-anchors. Small rafts may be stabilized to form larger platforms through protein-protein or protein-lipid interactions. The raft microdomains have thus been associated with a wide range of biological processes, including endocytosis, signal transduction[288], apoptosis, cell polarization[289], adhesion, migration, synaptic transmission and cytoskeleton tethering[275]. In addition, the existence of distinct lipid constituents within membrane microdomains is important for cellular polarization. Apically, the membrane is enriched in glycosphingolipids and sphingomyelin, favoring the targeting of GPI-anchored proteins. Conversely, proteins important for contacting neighboring cells and the underlying connective tissue are confined to the basolateral membrane, suggesting the presence, within individual cells, of functionally different membrane domains[290]. Segregation of functionally diverse microdomains is an attractive notion, allowing the clustering of molecules that serve similar purposes together in order to optimize efficient intra-cellular signaling. These observations also suggest that membrane rafts have an active role in asymmetric redistribution of membrane proteins

during cell migration. Compartmentalized lipid rafts have been described in leukocytes, and most research has focused on membrane raft function during lymphocyte migration[289,291]. In ECs, lipid membrane rafts also exist but their role and function are still not well defined.

Although lipids rafts have been extensively studied in T cell activation and migration[289,292], lipid-enriched membrane compartmentalization is an inherent property of BBB-ECs and allows for the apico-basal polarity of brain endothelium, temporal and spatial coordination of cell signaling events and actin remodeling. Junctional proteins, adhesion molecules and transporters can also physically locate into lipid raft membrane microdomains of human and mouse BBB-ECs, and their expression and localization is regulated by inflammatory and glial-derived factors. In addition, a proteomic study was done in order to identify possible novel candidate proteins or signaling pathways important in BBB functions.

PART I:

HYPOTHESIS AND OBJECTIVES

Hypothesis

In the light of the already acquired knowledge about the importance of lipid-enriched membrane microdomains in various cell types, I hypothesized that lipid rafts provide various functional platforms for effective protein interactions important in maintaining BBB properties.

Objectives:

1) To identify proteins expressed and/or enriched in human BBB-EC lipid membrane rafts and to propose a classification of endothelial membrane lipid raft based on their BBB-associated function, or at least on the function ascribed to the molecules included in such heterogeneous rafts. Data presented in Part I has been extracted from a review, on the functions of lipid membrane rafts in the BBB[270], of which I am first author.

2) To uncover novel proteins important in BBB-ECs, a liquid chromatography-mass spectrometry (LC-MSpect) proteomic analysis of rafts obtained from primary cultures of human BBB endothelium gave rise to a list of candidate proteins. My goal was to elucidate the function of novel proteins or possible signaling pathways necessary in preserving and upkeeping BBB characteristics. This set of data represents the basis of the study presented in Part II and a more detailed hypotheses and objectives specific to this aim will be described subsequently.

MATERIALS AND METHODS

1.0 Detergent-resistant membrane (DRM) isolation and analysis

BBB- EC isolation is described in Materials and methods of Part II. DRMs, or lipid membrane rafts, were isolated from confluent BBB-ECs using a previously published protocol[23,43,293]. Approximately 6 T75 flasks of BBB-ECs (per condition) were washed with ice cold PBS and scraped on ice. Following centrifugation, the pellets were resuspended in 1ml 1% of detergent in separation buffer (150mM NaCl, 25mMTris HCl pH7.4) with protease inhibitors (BD Biosciences, Franklin Lakes, NJ) and cells were solubilized for 30min on ice. Detergents used included Tween 20, Tween 80, Brij 58 and Brij98 (all from Sigma, Oakville, Ontario, Canada). The cell lysates were transferred to a glass homogenizer, dounced with 10 strokes of a loose fitting pestle and mixed with 1ml of 85% sucrose (Sigma) in separation buffer (w/v). The resulting 42.5% sucrose cell lysate was overlaid with 6ml 35% sucrose and 4ml 5% sucrose (w/v in separation buffer) and centrifuged for 24h at 39 000rpm at 4˚C in a Beckman SW41 rotor. Twelve 1ml fractions were collected at the meniscus, top to bottom, and the 4-5 fractions contained the DRMs, while the bottom fraction (10-12) were the soluble membrane fractions. Cholesterol, phospholipid and protein concentrations in each fraction were assayed using commercially available kits: Amplex red cholesterol assay kit (Molecular Probes, Eugene, OR), Phospholipids B Enzymatic colorimetric method kit (Wako, Richmond, VA) and BCA Protein assay kit (Pierce, Rockford, IL).

1.1 Western blot of lipid membrane rafts

For western blot (WB) analysis of lipid rafts, 40μg of each fraction were analyzed by standard SDS-PAGE using the following antibodies (Abs): mouse anti-JAM-A (1/500, BD Biosciences), rabbit anti-occludin (1/100, Zymed, South San Francisco, CA), rabbit anti-ZO-1 (1/100, Zymed), mouse anti-integrin α5 (1/5000), β1 (1/2500), αv (1/250) β3 (1/2500, all integrin Abs from BD Biosciences), mouse anti-ICAM-1 (1/,500, BD

Biosciences), rabbit anti-ALCAM (1/200 Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti-MCAM (1/200, Miltenyi Biotec, Auburn, CA). Lipid raft markers were also assessed by SDS-PAGE using GM-1 cholera toxin B subunit, (1/10000, Molecular Probes); goat anti-CD59 (1/200, R&D Systems), and rabbit anti-caveolin-1 (1/200, Santa Cruz Biotechnology). Specific binding was revealed with horseradish peroxidase (HRP)-conjugated anti-rabbit Ab using the ECL system (Amersham Biosciences, Piscataway, NJ).

1.2 BBB-ECs lipid membrane raft proteomic analysis

The isolated DRMs were dialyzed against denaturing buffer (50mM Tris-HCl, pH 8.5, 0.1% SDS) and precipitated with 10 volumes of ice cold acetone. The precipitated proteins were resuspended in denaturing buffer and reduced and alkylated using dithiothrietol (4mM, 10min at 95°C), followed by iodoacetamide (10mM, 20min at RT). The proteins were trypsin-digested using 5 µg of trypsin gold (12-18h at 37°C, Promega, Madison, WI) and the resulting peptides were purified on CE column (POROS® 50 HS, 50-µm particle size, 4.0mm ×15mm, Applied Biosystems, Forster City, CA) following the manufacturer's protocol. The samples were then analyzed by tandem mass spectrometry on a hybrid quadrupole time-of-flight Q-TOF™ Ultima instrument (Waters, Milford, MA) with an electrospray ionization source and an online reverse phase liquid chromatography column (LC; PepMap C18 capillary column, Dionex/LC-Packings, Sunnyvale CA). Peptides were separated on the LC column using a gradient of 5–75% acetonitrile, 0.2% formic acid in at least 90min, 350 nL/min supplied by a CapLC HPLC pump (Waters). At least one 40min blank was ran in-between samples to prevent cross-contaminations. MSpect/MSpect spectra were acquired in the auto-MSpect/MSpect mode (data-dependent scanning) on 2+, 3+, and 4+ ions. These were then submitted to a probability-based search engine, Mascot version 2.1.0 (Matrix Science Ltd., London, UK) to search against the NCBI's non-redundant human protein database. Searches were performed with a specified trypsin enzymatic cleavage with two possible missed cleavages and variable modifications for oxidation at methionine (+16 Da) and

carbamidomethyl at cysteine (+57 Da). The MSpect/MSpect spectrum of each identified peptide was manually examined and confirmed.

RESULTS

1.0 DRMs can be isolated from BBB-ECs

The role of membrane microdomains in ECs has only recently been recognized as an important part of many vascular processes. Isolation of lipid rafts from human primary cultures of BBB-ECs has been performed using different techniques and detergents[43,286]. In Figure iii, different raft isolation preparations using four distinct mild detergents are shown to express variability, especially with regard to the concentration of cholesterol, caveolin-1 and GM-1, all markers used to identify rafts. Using these protocols, BBB-EC-derived DRMs are enriched in caveolin-1 and also incorporate TJ proteins, integrins, specific transporters and CAMs. For example, in Figure iii, JAM-A, a transmembrane TJ protein associates with isolated membrane lipid rafts of BBB-ECs. Brij-58 enables a higher yield of lipid raft markers along with JAM-A expression, thus this detergent was used for the ensuing experiments. As lipid membrane rafts associate with functionally important BBB components, I thus propose to categorize BBB-EC lipid rafts based on the function of molecules found in these membrane compartments: 1) rafts associated with inter-endothelial TJ formation and EC adhesion to the basal lamina; 2) rafts involved in immune cell adhesion and recruitment across EC barriers and 3) rafts associated with transendothelial transport activity. A final category of molecules usually associated with neural process gave rise to further investigations on neural cues in the BBB (see Part II).

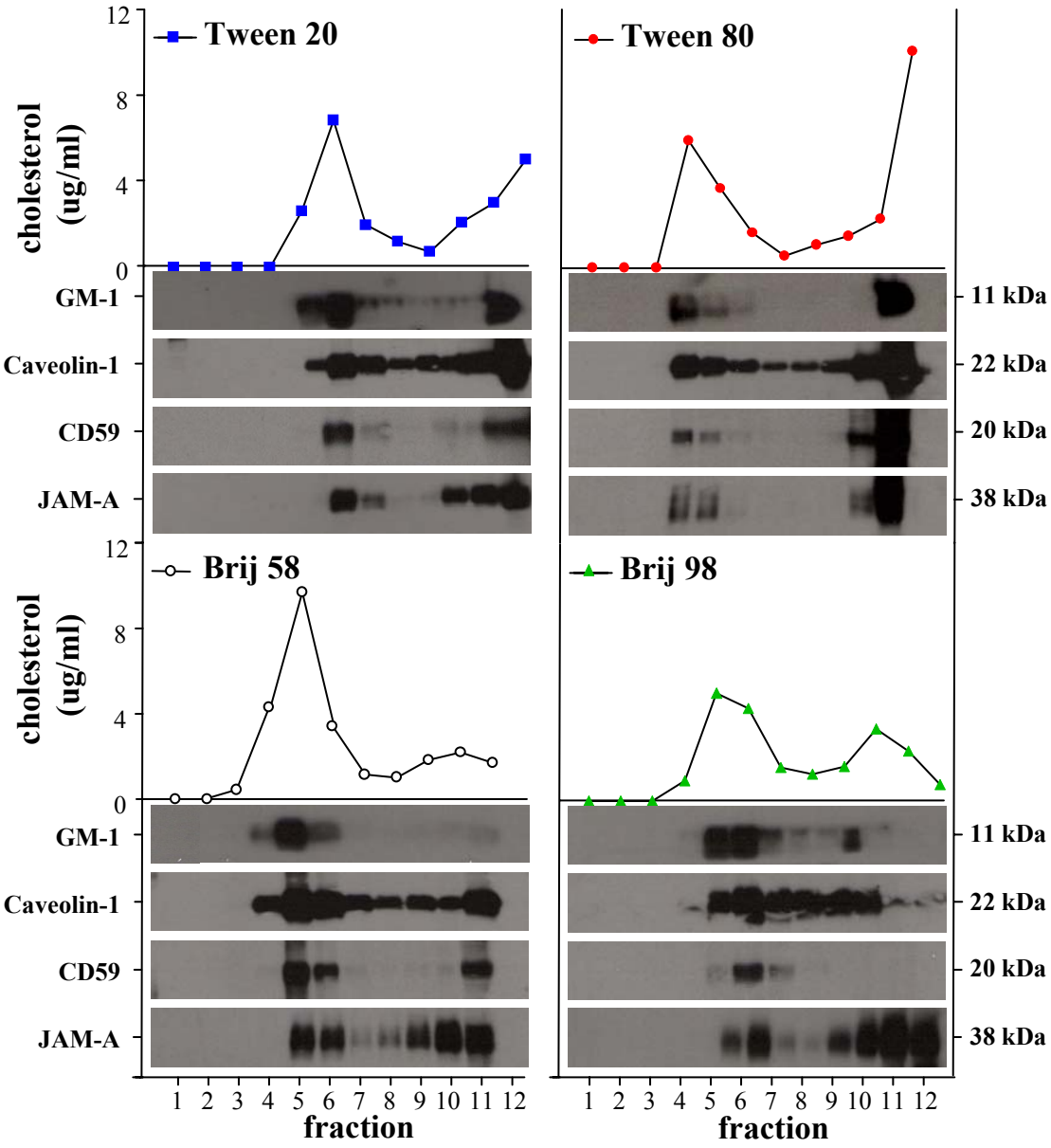


Figure iii: *Use of different detergents to isolate cholesterol-enriched microdomains from human primary cultures of BBB-ECs.* Four detergents were used to optimize cholesterol-enriched DRM isolation from primary cultures of human BBB-ECs. Following treatment of the cells with the detergent, membrane microdomains were separated by sucrose-density centrifugation. Cholesterol and phospholipid concentrations, as well as GM-1, caveolin-1, CD59 were used to identify raft fractions by Western blot. JAM-A is shown as an example of a TJ protein expressed in the raft fraction and its modulation according to the detergent used.

1.1 BBB-EC lipid membrane rafts contain junctional and integrin proteins

In order to maintain a stable and tight functional barrier, lipid membrane rafts could potentially serve to regulate integrin-mediated attachment to the basal lamina, as well as TJ- and AJ-mediated cell-cell association[8,210,214]. Both AJs and TJs are important for the regulation of paracellular permeability of ions, solutes and nutrients and both junctional complexes localize, at least in part to membrane rafts[293]. Figure ivA shows examples of TJ proteins found in membrane rafts of human primary cultures of BBB-ECs, isolated using Brij-58 and sucrose gradient centrifugation. The integral proteins occludin and JAM-A and the accessory protein ZO-1 were all found in DRMs, alongside caveolin-1, suggesting that lipid rafts can accommodate interacting transmembrane and cytosolic proteins.

The endothelial basement membrane formed by BBB-ECs and perivascular astrocyte end feet are composed of several ECM components, including fibronectin, different laminin isoforms, collagen type IV and heparan sulfate proteoglycans[209-211]. Its dense and compact nature provides the ability to function as a substrate for soluble protein immobilization and cell attachment. BBB-ECs constitutively express a number of integrins important for adhesion to the endothelial basal lamina, such as $\alpha_1\beta_1$, $\alpha_3\beta_1$ and $\alpha_6\beta_1$. In that respect, although the significance of lipid raft associated integrins remains to be established, my data presented in Figure ivB suggest that Brij-58-derived lipid rafts from human BBB-ECs also contain integrin proteins, such as α_5, α_v and β_1, but not β_3 integrins, suggesting integrin specificity. This confirms that distinct BBB-EC membrane rafts are involved in the clustering of TJ, AJ and specific integrins, which collectively mediate attachment of ECs to adjacent cells and to the vessel wall.

1.2 BBB-EC lipid membrane rafts contain adhesion molecules

Under physiological conditions, only low number of immune cells continuously cross the BBB and enter the CNS perivascular space in a process called immune surveillance. This process is highly regulated in both leukocytes and ECs and lipid membrane rafts have been associated with leukocyte recruitment across the endothelium[218,219].

Leukocyte rolling is dependent on CAMs and their respective carbohydrate ligands. In fact, CAM association with lipid membrane rafts is important for CAM-dependent intracellular signaling[216,294] and lipid rafts can influence leukocyte-ECs interactions. Lymphocyte and myeloid cell diapedesis across CNS endothelium involves prototypic BBB CAMs such as ICAM-1 and ICAM-2 (for lymphocytes[295] and VCAM-1 (for myeloid cells)[296,297] which are expressed on the cell surface and/or at intercellular junctions. These CAMs have been shown to associate with membrane microdomains[217,226,228]. In Figure ivC, I provide evidence that various CAMs can be expressed in lipid rafts. A large portion of total ICAM-1 can be found in BBB-EC DRMs. Novel CAMs recently associated with leukocyte transmigration, ALCAM and melanoma CAM (MCAM) were also found to be expressed in DRMs, suggesting that lipid membrane rafts are functionally important in regrouping CAMs for BBB-related purposes.

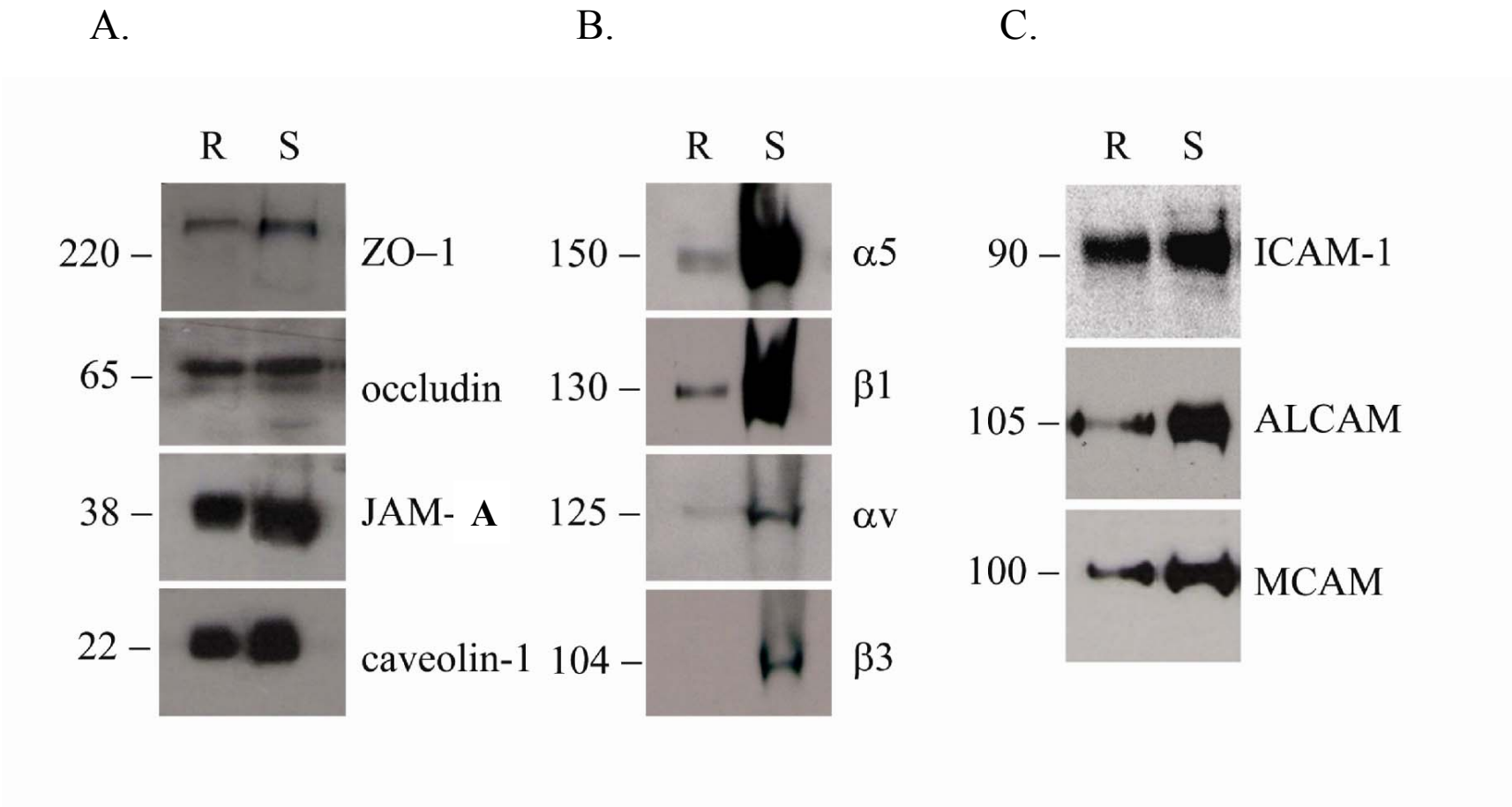


Figure iv: *Representative Western blots of isolated lipid rafts probed for scaffolding, TJ proteins and adhesion molecules.* Lipid raft were generated with treatment of primary cultures of human BBB-ECs with Brij 58 detergent followed by sucrose gradient centrifugation. Representative raft fraction (R) and detergent soluble fraction (S) were used to assess the presence of TJ, integrins and adhesion molecules. **A.** The TJ proteins ZO-1, occludin and JAM-A are present in the lipid raft fraction, in addition to caveolin-1. **B.** Integrins α5 and β1 are readily detected in BBB-EC lipid rafts. Integrin αv is seen at lower levels and integrin β3 does not seem to be associated with lipid raft under physiological conditions. **C.** The cell adhesion molecules ICAM-1, ALCAM and MCAM can be detected in lipid raft fractions of BBB-ECs under normal culture conditions.

1.3 BBB-EC lipid membrane rafts contain transporter proteins

Transport of nutrients to the CNS is regulated by the expression of specific transporters in BBB-ECs. Expression of active transport systems for glucose, amino acids, nucleosides, fatty acids, minerals, vitamins, peptides, proteins and ions define the prototypic nature and function of the BBB. In our LC-Mspect analysis of rafts obtained from primary cultures of human BBB-ECs, several members of the transporter system were identified. Due to the expected high number of false-negative data generated by Mspect, this list can only be considered partial or incomplete, but includes many different transporter systems such as ion channels and lipid and fatty acid transporters (Table i). These findings indicate that rafts might also play an active role in trans-BBB trafficking of numerous lipid soluble and insoluble molecules.

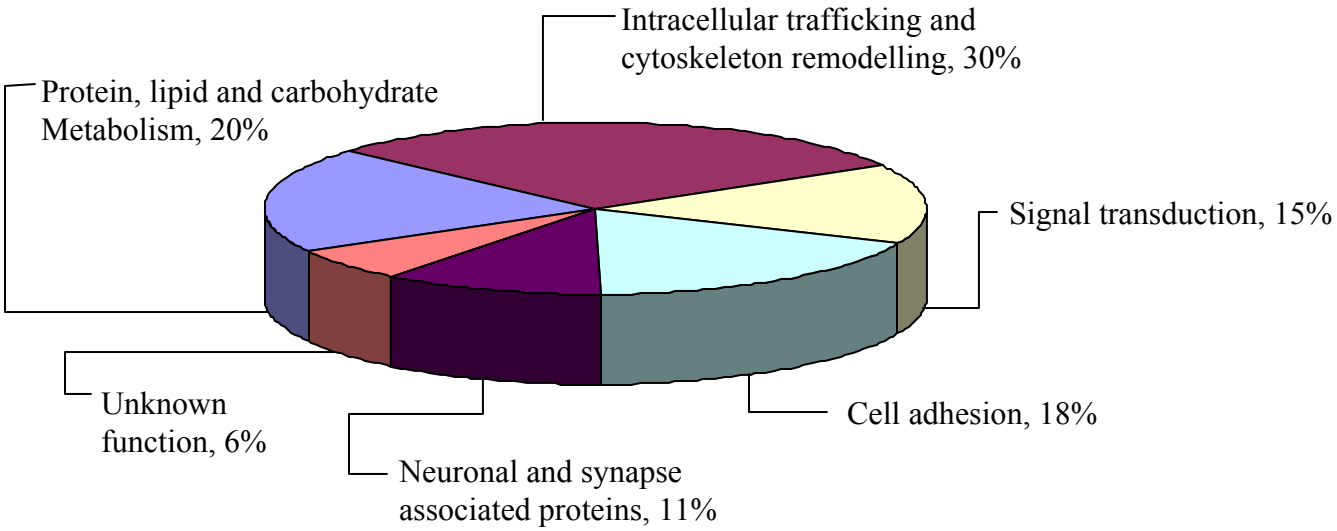
Transport System	Lipid raft associated transporter
Anion/cation transporters	*VDAC1, VDAC3*
Glutamate receptor	*GRIN2B*
Non voltage-gated sodium channel	*SCNN1A*
Voltage dependant calcium channel	*CACNA2D1*
Calcium transporting ATPase	*ATP2B4*
Potassium/chloride solute carrier family	*SLC12A6, SLC4A3, SLC35E1, SLC41A2, HCN2*
ABC transporters	*ABCA6, ABCB7 and ABCA1*
Carbohydrate transporters	*SLC2A6, SLC45A4, SLC2A14*
Lipid and fatty acid transporters	*ApoB, ABCA6, PITPNM2, ABCA1*
Glycosyl, acetyl, methyl or nucleoside transferases	*GGTLA1, MGAT2, UGT2B11, B4GalNac-T3, THUMPD2, MAT1A*

Table i: *Membrane raft microdomain associated transporters identified by proteomic analysis* Human primary cultures of BBB-ECs were grown to confluence, lysed in Brij58 detergent and cholesterol enriched membrane microdomains were isolated by sucrose gradient flotation. The raft fraction was subjected to trypsin digestion, separated by liquid chromatography and analyzed by LC-MSpect.

1.4 BBB-EC lipid membrane rafts contain proteins typically associated with the nervous system

In the last set of experiment and in an attempt to identify new proteins expressed in BBB lipid membrane rafts, lipid membrane rafts were isolated from monolayers of human BBB-ECs using the detergent Brij-58 and were sent for proteomic analysis by nanoflow LC-tandem Mspect (as described previously). This analysis confirmed the presence of proteins involved in cell-cell adhesion processes (18%), intracellular trafficking and cytoskeleton remodeling (30%) as BBB-EC lipid rafts are highly involved in cell adherence and transport properties. According to the nature of the BBB, proteins involved in lipid, protein and carbohydrate metabolism (20%) as well as signal transduction proteins (15%) and proteins of unknown function (6%) were also found (Figure v). Surprisingly, amongst the candidates found in the proteomic screen were proteins well known for their role in nervous system development and neuronal synapse function (11%). For example, slit-1 is a ligand for its receptor Roundabout (Robo) and is primarily known to act as a neural guidance cue for commissural neurons crossing the floor plate during embryonic development and recent reports have associated this pathway to angiogenic processes[120,125]. FAT tumor suppressor and Dachsous are atypical cadherins that mediate planar cell polarity and tissue morphogenesis[298]. Similarly, Hedgehog-interacting protein (Hip) is part of the Hedgehog (Hh) signaling pathway, a pathway important in tissue morphogenesis, neural development, axon guidance, and angiogenesis[147,155]. Nerve growth factor (NGF) receptor is critical for survival and maintenance of neurons[299]. As for the rest of proteins included in the candidate list, the stomatins, RIM-binding protein-2, GABA transporter, contactin 4 (also know as axonal CAM, or AXCAM), 43kDa receptor associated protein of the synapse and NMDA receptor precursor, all are expressed pre or post-synaptically[300-303]. Of these novel candidates and their associated pathways, I selected to study the importance of the Hh signaling pathway at the level of the BBB for Part II of my project.

A.



B.

Neuronal and synapse associated proteins, 11%
• stomatin-like 3
• Nerve growth factor receptor precursor
• RIM-binding protein 2
• slit homolog 1
• dachsous 1 precursor
• FAT tumor suppressor 2 precursor
• GABA/noradrenaline transporter
• hedgehog-interacting protein
• 43kD receptor-associated protein of the synapse isoform 1
• contactin 4 isoform a precursor
• N-methyl-D-aspartate receptor subunit 2B precursor

Figure v: *BBB-EC DRM isolation and proteomic analysis* **A.** Pie chart classification of the DRM-associated proteins identified by nanoflow LC-tandem MSpect. BBB-EC DRMs are mainly composed of signal transduction molecules (15%) with some associated cytoskeleton proteins (30%) and proteins involved in protein, lipid and carbohydrate metabolism (20%). Additional proteins identified are cell-cell adhesion molecules (18%) and neuronal and synaptic associated proteins (11%). **B.** Representative list of BBB-EC lipid DRM-associated proteins identified by nanoLC-MSpect which are typically found associated with nervous system processes such as in neural development or pre and/or post-synaptic termini.

PART II:

HYPOTHESIS AND OBJECTIVES

Preamble:

As for now, the influence of astrocytes has been reported on BBB permeability and activation during immune reactions occurring within the CNS. However, the identity of the specific factors is still under investigation. The Hh pathway has been implicated in vasculogenesis and angiogenesis, but no function has yet been ascribed to the BBB directly.

Hypothesis:

I hypothesize that astrocyte-derived Shh contributes to the maintenance of BBB properties and functions. My aim is to identify the functional significance of the Hh pathway at the human BBB. To address this hypothesis, I propose the following specific aims:

Objectives:

1) To characterize the expression and cellular localization of Hh pathway proteins at the level of human astrocytes and BBB-ECs, both *in vitro*, in primary cultures of human astrocytes and BBB-ECs, and *in situ*, using normal human brain tissue sections.

2) To study the role of the Hh pathway on the permeability of human BBB-ECs to soluble molecules *in vitro* and *in vivo* using available pharmalogical tools to stimulate or block the Hh pathway.

3) To determine the contribution of the Hh pathway in the generation and maintenance of TJ expression in BBB-ECs *in vitro*, using pharmacological tools and *in vivo*, using embryonic CNS tissue from Shh -/- mice.

4) To identify the immune responses of the BBB-ECs upon Hh activation by investigating chemokine release, adhesion molecule surface expression, and BBB immune permeability *in vitro*.

5) To examine the expression of Hh pathway components in relation to inflammation *in vitro*, in the context of the inflammatory cytokines TNF-α and IFN-γ, and in *situ* with immunostainings of human brain tissue sections from MS patients. To assess differences in expression of Hh pathway proteins, lesions and normal appearing white matter will be compared.

ORIGINAL ARTICLE

SONIC HEDGEHOG PROMOTES BLOOD-BRAIN BARRIER INTEGRITY AND IMMUNE-QUIESCENCE

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Abstract

During embryogenesis, vascular development is characterized by sequential steps of vasculogenesis, angiogenesis, and in some organs, barriergenesis. In the CNS, the blood-brain barrier (BBB) is composed of tightly bound endothelial cells (ECs) which regulate the entry of blood-borne molecules and immune cells into the CNS. Perivascular astrocytes are known to regulate BBB permeability and quiescence by secreting essential factors, the identity of which remains unclear. Our study shows that human astrocytes express and secrete Sonic Hh (Shh) and conversely, that human BBB-ECs bear the Hh receptor Patched-1 (Ptch-1), the signal transducer Smoothened (Smo) as well as transcription factors of the Gli family. Furthermore, we show that activation of the Hh pathway in BBB-ECs restricts the passage of soluble tracers *in vitro* and *in vivo*. Blocking Hh signaling *in vitro* and using Shh knock-out (-/-) embryonic mice, we demonstrate a reduced expression of TJ molecules claudin-5, occludin and ZO-1. Hh activation also decreases the surface expression of cell adhesion molecules ICAM-1 and VCAM-1, and decreases BBB-ECs secretion of pro-inflammatory cytokines IL-8/CXCL8 and monocytes chemoattractant protein 1 MCP-1/CCL2, resulting in a reduction of migrating $CD4^+$ lymphocytes across human BBB-EC monolayers. *In vitro* treatment with inflammatory cytokines TNF-α and IFN-γ, upregulates the production of astrocytic Shh and the BBB-EC surface expression of Ptch-1 and Smo. In active Multiple Sclerosis (MS) lesions, in which the BBB is disrupted, Shh expression is drastically upregulated in hypertrophic astrocytes, while Ptch-1 and Smo expression is down-regulated or left unchanged, suggesting that a deregulation in the Hh signaling pathway may prevent the barrier stabilizing properties of Hh. Our data demonstrate an anti-inflammatory and BBB-promoting effect of astrocyte-secreted Hh and suggest that a pro-inflammatory environment disrupt the BBB by impacting, at least in part, on Hh signaling in brain ECs.

Key words: Blood-brain barrier, tight junction, Sonic Hedgehog, astrocyte, endothelial, endothelium, permeability, chemokine, cell adhesion molecule, inflammation, Multiple Sclerosis

Introduction

The blood-brain barrier (BBB) protects the central nervous system (CNS) from blood-borne molecules and circulating immune cells. The BBB is composed of specialized endothelial cells (ECs) held together through multiprotein complexes known as tight junctions (TJ)[11,27,270]. Astrocytes, which are in close apposition to the cerebral vasculature, help maintain BBB integrity and immune quiescence through contact dependant support mechanisms and the release of essential soluble factors[11,24,304]. In the course of multiple sclerosis (MS), a breakdown in the BBB occurs, allowing auto-reactive immune cells to enter the brain parenchyma causing demyelination and neuronal damage[21,79,223]. The current view is that astrocytes are key regulators of BBB development, maintenance and regulation and that understanding the complex astrocyte-EC interactions under physiological and pathological conditions may lead to the development of novel therapeutic strategies.

The hedgehog (Hh) pathway is a conserved signaling cascade involved in embryonic morphogenesis, pathfinding, and as recent studies show, angiogenesis[130,147]. In adult tissues, it plays an important role in vascular proliferation, differentiation and tissue repair[7,182,193]. Hh signaling is initiated by one of three secreted homologues of the Drosophila Hh; Indian Hh (Ihh), Desert Hh (Dhh) and Sonic Hh (Shh). Shh is the most studied of these ligands has been widely associated with morphogenic events such as posterior identity of the limb bud[150], ventral cell identity of the neural tube, and motor neuron development[151,152]. Essential to Hh signaling is the internal autocatalytic cleavage of the 45kDa protein to yield the ~19kDa N-terminal signaling domain. Following the post-translational addition of cholesterol and palmitoyl moieties, Shh is released from the producing cell and binds with high affinity to the cell surface receptor Patched (Ptch-1)-1, a 12-transmembrane domain receptor. Engagement of Ptch-1 alleviates repression of the signal transducer Smoothened (Smo), a serpentine protein with a topology reminiscent of Frizzled family of Wnt receptors and other G-protein coupled receptors[146,158]. Once Smo is in its active state, activation of zinc-finger transcription factors of the Gli family (Gli1-3) act at the last step of the Shh-signal-transduction pathway. In addition to the influence

of specific factors on the response of a cell to the Hh signal received, proteins of the Hh cascade itself, such as Ptch-1 and Gli-1 are upregulated[149].

Members of the Wnt, Hedgehog (Hh), fibroblast growth factor (FGF) and bone morphogenic protein (BMP) families were shown to act as classical morphogens in a number of different contexts[116,117]. FGF[103], BMP[140] and Wnt[141,142,144] signaling pathways have been reported to act on cerebral vascular development and on the formation of a mature BBB. Prior studies have demonstrated strong Shh expression on BBB endothelium, astrocytes and hypertrophic astrocytes within demyelinated and remyelinating brain lesions in rats with experimental autoimmune encephalomyelitis (EAE)[258], suggesting that the Hh signaling pathway may play a role in astrocyte-endothelium interactions and BBB maintenance or repair. However, research on Shh and its involvement in MS and EAE has mostly focused on the fact that Shh is critical for oligodendrocyte maturation[263], a crucial step in their ability to myelinate axons. Wang and coworkers described astrocyte upregulation during MS inflammation, where Gli-1 was paradoxically decreased in oligodendrocyte progenitor cells in chronic inflammatory lesions, proposing that failure to remyelinate may be due compromised Hh pathway in myelinating oligodendrocytes[265].

In the peripheral nervous system (PNS), Dhh is needed for the formation and maintenance of the perineurium, a component of the blood-nerve barrier that acts as a barrier against blood borne molecules and cellular infiltration[206]. Therefore, the Hh pathway seems to be involved in the formation of and ordered and functionally competent perineurium, seen as a later stage of perineurial sheath maturation. By analogy, these studies raise the question of whether the Hh pathway plays a role in other barrier contexts, such as the BBB. In the current study, we demonstrate that astrocyte secreted Shh impact on BBB-ECs and that the activation of the Hh signaling cascade contributes to the maintenance of BBB functions.

Results

Astrocyte and blood-brain barrier endothelial cell expression of hedgehog pathway components

Reverse transcription - polymerase chain reaction (RT-PCR) was performed using primers specific for human Sonic Hedgehog (Shh), Pactched-1 (Ptch-1) and Smoothened (Smo). As seen in Figure 1A, two separate preparations of primary cultures of human fetal astrocytes (HFA) express Shh mRNA. Human adult blood-brain barrier (BBB) endothelial cells (ECs) did not express Shh (30 PCR cycles). Expression of Ptch-1, and Smo mRNA was observed at low levels in HFAs, and higher levels were detected in BBB-ECs. Assessment of protein production by Western Blot (WB) revealed that whole cell lysates of primary cultures of HFAs contain the uncleaved precursor form of Shh protein at 45kDa (Figure 1B). However, to ascertain that HFAs have the capacity to secrete the active cleaved form of Shh (at 19KDa, 170108 antibody recognizes both cleaved and uncleaved forms), astrocyte conditioned media (ACM) was precipitated and blotted for Shh. ACM collected after one week of conditioning was found to contain the cleaved form of Shh (19kDa, Figure 1B, right panel), and it was detected at the same molecular weight as the human recombinant Shh (hrShh) peptide. While human adult BBB-ECs did not express Shh, Ptch-1 expression on these cells was confirmed (Figure 1B, left panel). Immunocytofluorescence analysis further confirmed intracellular astrocytic Shh immunosignal (Figure 1C). As shown, HFAs positive for Shh expressed GFAP, confirming that the source of Shh in these cultures was indeed the astrocytes and not possible contaminating neurons. Membrane-bound Ptch-1 and Smo were also detected on BBB-ECs (Figure 1C). Immunohistochemistry analysis of these Hh components was further performed on frozen control human adult brain sections. Determined by their morphology and location, perivascular astrocytes were seen to be most immunopositive for Shh (arrowheads, Figure 1D), with the strongest staining seen at the boundary of the blood vessels. Higher magnification of perivascular astrocyte (arrowhead) demonstrates Shh expression in the cell body and endfeet (arrows) opposing parenchymal vessel (asterisk). This confirms previous work that suggested Shh immunoreactivity in astrocytes[258,265]. Ptch-1 and Smo were strongly expressed in BBB-

ECs (Figure 1D). Thus, human astrocytes express and secrete Shh, *in vitro* and *in situ* and brain endothelium, on the other hand, expresses Ptch-1 and Smo, supporting the possibility that the Hh pathway is used for communication between perivascular astrocytes and BBB-ECs.

Astrocyte-secreted sonic hedgehog decreases permeability *in vitro*

Shh was previously shown to induce *in vitro* capillary morphogenesis in mouse brain-derived EC lines, bEnd.3 and IBE[4,182]. Primary cultures of confluent human BBB-EC monolayers *in vitro* form a semi-permeable barrier which restricts diffusion of soluble tracers. We and others have previously demonstrated that astrocyte-derived soluble factors present in the ACM decrease BBB-EC permeability to soluble tracers[43,68]. In a modified Boyden chamber permeability assay using human BBB-ECs, 40% ACM (v/v) induced an important decrease in permeability to the large molecular weight tracer Fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) (Figure 2A). This effect was long lasting, as evidenced by the important difference in tracer diffusion up to 72h (n=8 independent experiments, in duplicate, *$p<0.05$). Addition of hrShh (0.1μg/ml) to BBB-ECs 24h prior to tracer addition reproduced the effect of ACM (Figure 2A, n=8, *$p<0.05$). The concentration used reflects the lowest concentration required by endothelial precursor cells to induce migration and vascular-endothelial growth factor (VEGF) production[305]. In our experiment, BBB-ECs responded to hrShh in a dose-dependent fashion, as permeability decreased with increasing hrShh concentrations, and did not affect EC proliferation (Supplementary Figure 1). To assess whether activation of the Hh pathway was responsible for the BBB-EC barrier promoting effects of ACM, BBB-ECs were treated with Smo non-peptide receptor agonist purmorphamine (100nM) or antagonist cyclopamine (30μM). Treatment with the Smo agonist (purmorphamine) reproduced the effect of hrShh on BBB-ECs (Figure 2B, n=8, *$p<0.05$). However, Smo antagonist (cyclopamine) treatment of BBB-ECs grown in ACM reversed the barrier promoting effects of the ACM, resulting in an increase of the permeability of BBB-ECs that compared to the levels of untreated ECs (Figure 2C, n=6). Another Smo antagonist, SANT-1, also abrogated the effect of ACM on permeability (data not shown), suggesting that Smo is important in the EC barrier properties. Treatment with Smo antagonists alone

had no effect on the permeability or proliferation of the BBB-ECs as compared to control treatments (data not shown). In separate experiments, similar decreases in permeability, using hrShh and Smo agonist, were obtained with the small molecular weight tracer ^{14}C-sucrose (Supplementary Figure 1). Thus, our data demonstrates that astrocyte-produced Shh promotes barrier properties in human BBB-ECs through activation of the Hh signal transducer Smo.

Cyclopamine induces BBB disruption *in vivo*

To confirm the role of the Hh pathway in BBB maintenance, we studied the extent of BBB leakage in 7-8 week old c57Bl/6 mice injected with 10mg/kg of cyclopamine or equal volume of vehicle (Hydroxypropyl-β-cyclodextrin, HPBCD). The extent of BBB disruption was determined by extravasation of the 70kDa exogenous soluble tracer Dextran-FITC, as well as by the endogenous plasma protein fibrinogen, a molecule that does not cross the BBB in normal conditions. Using confocal microscopy and fluorescent quantification imaging techniques, we found that cyclopamine-injected mice displayed a significant increase in the area and peak fluorescence intensity of Dextran-FITC and fibrinogen ($4.49x10^5 \pm 1.38x10^5$ and $1.36x10^6 \pm 4.19x10^5$, respectively; n=12 vessels) in both small and large vessels, as compared to counterparts from vehicle treated mice ($3.27x10^4 \pm 1.02x10^4$ and $1.98x10^5 \pm 8.25x10^4$, n=9 vessels; *$p<0.05$) (Figure 2D). Representative images taken of Dextran-FITC and fibrinogen immunostaining demonstrate leakage (arrowheads) 6h after cyclopamine treatment, while in vehicle treated mice, these tracers remained in the lumen of the vessels (Figure 2E). Our results demonstrate that in mice treated with the Smo antagonist cyclopamine, BBB leakage occurs with molecules of molecular sizes of 70 and 340 kDa, leaking from the blood into the parenchyma. Thus, the Hh pathway is important in the maintenance of adult mouse BBB function and disruption of this pathway leads to, at least, acute BBB breakdown *in vivo*.

Sonic hedgehog promote and maintain tight junction protein expression

Parmentier and colleagues have shown that formation of the blood-nerve barrier made by the perineurium and the nerve bundle endothelial cells[206,306], depends on

Schwann cell-derived Dhh signal. We tested whether expression of endothelial TJs in human BBB-ECs depended on astrocyte-derived Shh signal. Quantitative real-time PCR of human BBB-ECs shows an upregulation of Ptch-1 mRNA expression when cells are grown with ACM or treated with hrShh (0.1μg/ml, 24h, Figure 3A), confirming Hh activation in either case. In addition, occludin and claudin-5 mRNA expressions by human BBB-ECs were upregulated with ACM and hrShh (Figure 3A). WB analysis of protein expression demonstrates that ACM increases claudin-5, occludin, and JAM-A expression in whole cell lysates of BBB-ECs (Figure 3B). However, when the Hh pathway is antagonized by cyclopamine treatment, expression of claudin-5, occludin and JAM-A expression decreases to 21.3%, 77.9%, and 31.3%, respectively, compared to ACM-grown astrocytes (Figure 3B).

The generation of Shh deficient mice has been previously described[150]. Shh knock-out (-/-) mice have an overall reduction in size from their wildtype (WT) littermates, and because of early embryonic lethality, embryos need to be harvested between E11 and E13.5. The development of the BBB occurs gradually, as disappearance of fenestration and appearance of TJs in the endothelium occurs between embryonic stages E11 and E13[307] such that by E12, the CNS has already become impermeable to trypan blue and HRP tracers[308]. Brain sections from E13.5 WT and Shh-/- mice were thus immunostained for TJ protein claudin-5, occludin, and ZO-1. FITC-conjugated *Lycopersicon Esculentum* (Tomato) lectin was used to provide a frame of reference for the localization of the blood vessels. Although distinct TJ strands were visible in brain capillaries of both WT and Shh -/- brains (Figure 3C), the brains from Shh-/- had significantly fewer ECs immunopositive for specific TJ proteins (Figure 3C and corner insets displaying lectin-positive ECs). In WT brain, a relatively high percentage of lectin-binding ECs were immunopositive for claudin-5 (79.2 ± 3.1), occludin (64.7 ± 6.4), and ZO-1 (87.8 ± 2.3). In contrast, the lectin positive cells of Shh -/- animals demonstrated a significant reduction in immunopositivity for claudin-5, (37.0 ± 2.6, ***$p<0.0001$) and ZO-1 (48.1 ± 6.0, ***$p<0.0001$), while occludin expression was nearly absent (8.1 ± 3.8, ***$p<0.0001$) (Figure 3D). Peak fluorescence intensity was also measured, and the TJ strands of Shh-/- were significantly less intense than TJs from WT brains (data not shown). Therefore, the data suggest that Shh secreted by astrocytes is important in the

induction and maintenance of TJ expression, and that loss of Shh signaling may contribute to loss of barrier function.

Hedgehog activation influences cell adhesion molecule expression, cytokine secretion and lymphocyte migration

ECs, in general, and BBB-ECs in particular, actively participate in the inflammatory reactions and can directly influence immune cell migration by regulating cell adhesion molecules (CAMs) expression as well as the secretion of cytokines and chemokines[218,219,309]. Under basal conditions, cultures of human adult primary BBB-ECs express intracellular cell adhesion molecule (ICAM)-1 and to lesser extent, vascular cell adhesion molecule (VCAM)-1 (73.9% and 3.4%, respectively, Figure 4A). When grown with 40% ACM (v/v), the levels of ICAM-1 and VCAM-1 decrease (63.9% and 1.7%), suggesting that astrocytic factors contribute to the immuno-quiescence of the barrier. On the other hand, when Hh pathway activation was impeded by the use of cyclopamine treatment (30μM, 24h), ICAM-1 and VCAM-1 levels remained at basal levels (72.7% and 3.6%). BBB-ECs treated with hrShh (0.1μg/ml, 24h) and purmorphamine (1uM, 24h) had decreased levels of ICAM-1 and VCAM-1 (66.6% and 2.8% for hrShh and 59.3% and 2.2% for purmorphamine), demonstrating that Shh is a potent anti-inflammatory signal decreasing the expression of CAMs at the surface of BBB-ECs. These findings were confirmed by calculating the mean fluorescent intensities (MFI) for ICAM-1 (n=3, *$p<0.05$, Figure 4B). Cytokine/chemokine secretion from BBB-ECs was also assessed, by ELISA, to further evaluate the role of Shh in BBB quiescence. Upon treatment with hrShh or purmorphamine, the secretion of both IL-8/CXCL8 and MCP-1/CCL2 significantly decreased (Figure 4C, n=4, **$p<0.005$ and ***$p<0.0005$ respectively). Smo inactivation with cyclopamine, even in the presence of hrShh treatment, abrogates this effect. These observations suggest that Shh negatively influences the ability of BBB-ECs to chemoattract and recruit leukocytes. To address this possibility, the ability of leukocytes to transmigrate through Hh activated BBB-ECs was evaluated using the modified Boyden chamber assay. $CD4^+$ T cell migration was significantly reduced in BBB-ECs grown with ACM, as well as BBB-ECs treated with hrShh and purmorphamine

(80%, 75% and 65% reduction in $CD4^+$ lymphocyte transmigration compared to resting BBB-ECs, *p<0.05, **p<0.005, ***p<0.0005, respectively, Figure 4D). Furthermore, the transmigration of $CD4^+$ lymphocyte across cyclopamine treated BBB-ECs did not differ from the migration of untreated BBB-ECs (Figure 4D). Collectively, these findings point to an endogenous, anti-inflammatory role of the Hh pathways at the level of the BBB, as Smo engagement reduces CAM expression, chemokine secretion and lymphocyte migration.

Transcription factor upregulation upon hedgehog activation

Upregulation of Gli-1 expression is generally used as an indicator of Hh activation[146,147]. Flow cytometry analysis of intracellular staining for Gli-1 was performed to determine the level of Hh activation in BBB-ECs. BBB-ECs treated with hrShh (24h, 0.1μg/ml) or with purmorphamine (24h, 1μM) showed increased expression of Gli-1 (45.3% and 65.4%, respectively), compared to untreated control, (26.7%, Figure 5A). Similarly, BBB-ECs cultured in ACM increased the expression of Gli-1 (43.1%) compared to untreated control (26.7%). However, blocking the Hh cascade with cyclopamine reduced the expression of Gli-1 induced by ACM (34.8% compared to 43.1% in ACM-grown BBB-ECs, Figure 5A). Mean fluorescent intensities for Gli-1 were also evaluated and confirmed the pattern of effect previously described (n=2, *p<0.05, **p<0.01, Figure 5B).

Quantitative real-time PCR was used to determine the kinetics of BBB-EC Gli-1 mRNA upregulation upon hrShh stimulation and to study the activation of transcription factors involved with BBB function. Sex-determining region Y-box (SOX)-18 is an endothelial-specific transcription factor that is important in regulating claudin-5 gene expression and EC barrier formation[310]. Upregulation of Gli-1 mRNA in BBB-ECs occurs rapidly following hrShh stimulation (n=2, ***p<0.001 at 1h, **p<0.01 at 3h, Figure 5C). However, the expression of SOX-18 occurs 3h after hrShh stimulation (n=2, ***p<0.001 at 3h, **p<0.0.1 at 6h, Figure 5C), suggesting not only that SOX-18 is regulated by the Hh pathway, but that Gli-1 may regulate SOX-18 expression in BBB-ECs.

Pro-inflammatory cytokines modulates the hedgehog signaling pathway *in vitro*

As neuroinflammatory conditions are associated with a BBB breakdown, we further evaluated the *in vitro* effects of the pro-inflammatory cytokines TNF-α and IFN-γ on Hh component expression. In primary cultures of HFAs, extracellular staining for major histocompatibility complex I (MHC I or HLA-ABC) and intracellular staining for GFAP differentiated the population of astrocytes from potentially contaminating neurons, which are both MHC I and GFAP negative (Figure 6A). In the astrocyte population (gated for MHC I^+/$GFAP^+$), expression of Shh substantially increases (63.4%) after treatment with both IFN-γ and TNF-α (100 U/ml each, 24h) compared to untreated control (44.5%, Figure 6A). MHC I is drastically increased upon inflammatory stimulation and was used as a marker for astrocytes, as it is rarely expressed by contaminating neurons[311]. Surface immunostaining for Ptch-1 and Smo on BBB-ECs grown in ACM (40%) revealed that treatment with inflammatory cytokines TNF-α and IFN-γ (100U/ml each, 24h) increased expression levels of both Ptch-1 (59.3%) and Smo (54.4%), compared to control levels (30.0% and 27.6% respectively; Figure 6B). These data suggest that upon inflammatory stimulation, astrocytes and BBB-ECs react by upregulating the anti-inflammatory Hh pathway members.

Modulation of the hedgehog signaling pathway *in situ* in Multiple sclerosis

Plaque formation and inflammatory cell infiltrations into brain tissue are hallmark signs of Multiple Sclerosis (MS). In control brain tissue and normal appearing white matter (NAWM), we observed pronounced Shh immunostaining in astrocytes (Figure 7A). Interestingly, astrocyte processes and endfeet surrounding parenchymal vessels display Shh immunoreactivity (arrowheads; Figure 7B) that is strikingly enhanced in hypertrophic astrocyte cell bodies and processes throughout active demyelinating MS lesions (arrowheads; Figure 7C, n=6 lesions). Double immunofluorescent staining confirmed expression of Shh (green) in GFAP (red) positive perivascular astrocytes (arrowheads), as seen in the overlay pictures (Figure7D).

Whereas *in vitro* cultures of BBB-ECs, derived from essentially healthy brain tissues, upregulated both Ptch-1 and Smo under inflammatory conditions (Figure 6), MS

brain BBB-ECs exhibited a different pattern of expression. In control NAWM tissue, Ptch-1 expression was mainly restricted to blood vessels (arrowheads; Figure 8A). In active MS lesions, while Ptch-1 and Smo immunostaining were predominantly observed in macrophages (arrows; Figure 8A and B), their level expression in brain ECs remained unchanged (arrowheads; Figure 8A and B). Double fluorescent staining revealed colocalization of Ptch-1 with Gli-1 in blood vessels of NAWM and active MS tissue (Figure 8C). No difference in expression levels of Ptch-1 and Gli-1 were observed in brain ECs, although Gli-1 immunoreactivity was detected in infiltrating leukocytes in the MS lesion. The expression pattern of Shh, Smo and Patched-1 in NAWM was similar to that observed in control brain tissue.

Discussion

In this study, we demonstrated that astrocyte-derived Shh acts on human BBB-ECs to decrease permeability of the BBB to soluble factors *in vitro* and *in vivo*, a process that is Smo-dependent. This effect is driven by the ability of Shh to induce and maintain TJ protein expression through activation of Gli-1 and SOX-18. We further demonstrate that Shh regulates BBB-EC activation by lowering their expression of surface CAMs and secretion of pro-inflammatory chemokines. Consequently, a decrease in transmigrating lymphocytes across the BBB-EC monolayer was observed, suggesting that Shh acts as a direct negative regulator of CNS inflammation. In inflammatory contexts, an increase in Shh was observed when human astrocytes were cultured in the presence of inflammatory cytokines TNF-α and IFN-γ. Surface expression of both Ptch-1 and Smo were also increased in BBB-ECs treated with the same inflammatory cytokines. However, in MS plaques, while hypertrophic astrocytes upregulated Shh expression, ECs were not found to correspondingly increase the expression of the receptors, Ptch-1 and Smo.

The anatomical proximity of the astrocytic endfeet to the CNS-ECs has lead to the notion that astrocytes impact on BBB properties. Astrocyte-derived soluble factors have been shown essential for the development and maintenance of an impermeable BBB as ACM induces junction formation and strong transendothelial electrical resistance in

either CNS or non-CNS-derived ECs, *in vitro*[24,312,313]. Morphogens have been linked to cooperate in astrocyte-endothelial interactions. Proteins of the FGF family were found to decrease the permeability of the BBB *in vitro*[103]. Similarly, conditioned media containing Wnt3a induces TJ strand formation, junctional protein expression and BBB-EC morphology[142]. Although human and mouse astrocytes have previously been shown to express Shh[258,265], its impact on BBB function has not been studied. Our data clearly identifies the Hh signaling pathway as an important determinant of BBB function and maintenance.

Shh has been involved in a multitude of developmental processes, ranging from embryonic tissue patterning, ventralization of neural tube, and establishment of posterior identity in the developing limb bud[152,314]. Others have found that Shh can induce capillary morphogenesis[182], a process which requires promoting assembly via cell-cell contact. Evidence that Shh may be involved in maturation of endothelium can be seen in developing mouse models. In the developing mouse submandibular gland, lumen formation and TJ assembly occurs in the epithelial cell lining, processes dependent on Shh. Shh null mice had developmentally arrested submandibular gland epithelium, and treatment with Shh recovered and enhanced epithelial lumen formation, full cell polarization as well as epithelial TJ protein distribution[205]. In the PNS, Dhh is expressed in myelinating Schwann cell and is needed for the formation and maintenance of the perineurium, a component of the blood-nerve barrier. Dhh -/- animals have a less compact perineurium, resulting in abnormal and interrupted TJ strands. Consequently, a defective nerve-tissue barrier is formed and extravasation of Evans Blue dye and immune cells invasion into the endoneurium occurs[206]. Therefore, our study further investigated the potential of the Hh pathway in the formation of a mature, ordered and functionally competent barrier. We demonstrate that astrocyte-derived Shh decreases permeability of soluble tracers in the modified Boyden chamber assay, an *in vitro* model of the BBB. Cyclopamine-injected mice endure acute BBB disruption as FITC-Dextran and endogenous serum proteins enter the brain parenchyma. We further demonstrate that Hh activation is essential for TJ expression, as cyclopamine induces a downregulation of junctional proteins in BBB-EC. Shh -/- mice exhibited a significantly lower expression of

claudin-5, occludin and ZO-1 on BBB-ECs *in vivo*, confirming the validity of the *in vitro* observation.

The activation of the Hh pathway involves activation of the downstream transcription factor Gli-1 which act as an activator to mediate and/or amplify the Hh signal as it is transcriptionally induced by Hh signaling in nearly all contexts examined[315]. Our data using flow cytometry demonstrates that in Hh activation in BBB-ECs induces an intracellular upregulation of Gli-1, which is cyclopamine-sensitive. However, further mechanisms in promoting BBB properties were sought. SOX-18 was found to be an endothelial specific transcription factor as *in silico* analysis of the claudin-5 promoter revealed an evolutionary conserved SOX consensus binding site. In HUVECs, SOX-18 overexpression increases claudin-5 mRNA and protein expression while SOX-18 silencing reduces claudin-5 transcription and transendothelial resistance[310]. Our results suggest that Gli-1 activation proceeds SOX-18 upregulation. Whether or not Gli-1 directly upregulates SOX-18 is still not known but in different contexts, the Hh pathway has already been seen to regulate other members of the SOX family. Shh was seen to induce changes in families of transcription factors such as SOX, further altering the expression of downstream genes[316]. Expression of SOX-14 in spinal cords was found to be regulated by Shh in a dose-dependent manner[317]. In neuroepithelial cells Gli-2 binds to an enhancer region vital for SOX-2 expression necessary for NSC proliferation and differentiation[318]. In this context, we postulate that Smo activation modulates barrier properties by impacting on TJ protein expression via Gli-1 and SOX-18.

The BBB is an important entity in keeping the CNS an immunologically privileged site, a region where immune cells access in infrequent[319]. Astrocytes are key regulators of BBB functions and have been shown to secrete a variety of factors that help promote and maintain BBB properties[11,24,312,313]. However, less often are the factors shown to regulate the immune quiescence of BBB-ECs. We present evidence suggesting a novel and unique role of Shh in the regulation of CNS immunity. During CNS inflammation such as MS, its animal model EAE, and following various cerebral insults, upregulation of chemokines and CAMs in BBB-ECs contribute to the recruitment of immune cells. We selected to study chemokines MCP-1/CCL2 and IL-8/CXCL8 for they

were found to be important chemokines produced by BBB-ECs[238,320]. BBB-ECs treated with hrShh or with purmorphamine significantly reduce the secretion of both MCP-1/CCL2 and IL-8/CXCL8, further demonstrating anti-inflammatory action. In fact, MCP-1/CCL2 significantly contributes to lymphocyte migration, as blocking MCP-1/CCL2 reduces the severity of EAE and significantly inhibits migration of MS lymphocytes across BBB-EC monolayers[320]. We further show that BBB-ECs grown with ACM or treated with rhShh or a Smo agonist, purmorphamine, all activators of the Hh pathway, reduce the expression of ICAM-1 and VCAM-1, demonstrating anti-inflammatory potential. The fact that both adhesion molecules expression and chemokine secretion decreased suggested that BBB-ECs were less effective in recruiting lymphocytes to the site of inflammation, an act that was indeed observed *in vitro.*

In MS, BBB breach concurs alongside with EC activation and lymphocyte infiltration. Since the Hh pathway seems capable of promoting barrier properties as well as reducing EC activation and lymphocyte recruitment, we observed the effect of inflammation on Hh components. We showed that human astrocytes exposed to TNF-α and IFN-γ upregulate Shh production. This was paralleled *in situ* by the increase in Shh immunoreactivity around blood-vessels in active MS lesions, when compared to NAWM of the same patient. As Shh has been observed to be upregulated in various cells after acute injury and wound healing, implicating an important role in cellular and tissue repair[154,256], we postulate that inflammatory cytokines activate Shh production in astrocytes in order to promote repair of the BBB. Shh thus may serve a protective function in acute immune-mediated injury. Indeed, a stable form of Shh was previously shown to positively impact on nerve injury[321]. In addition, due to its potential anti-inflammatory activity shown herein, Shh upregulation could be also be beneficial in regulating and containing the inflammation.

In contrast, while BBB-ECs treated with TNF-α and IFN-γ upregulated both Ptch-1 and Smo on their surface, the same could not be seen *in vivo*, in active MS lesions. This demonstrates a potential dysregulation of the pathway at the level of the BBB, and provides a plausible explanation to BBB compromise. Recent studies have observed that Gli-1 was upregulated in active MS lesions, but was significantly downregulated in

chronic active and inactive lesions[265], suggesting that long term inflammation impairs Gli-1 signal in chronic inflammatory conditions.

In chronic gastrointestinal inflammation, Shh is also upregulated in epithelium of areas of inflammation such as in gastritis, Crohn's disease and ulcerative colitis, an effect that was linked to the ability of injury-related cytokines to induce Hh production[322]. However, no increase in Ptch-1 mRNA and protein expression were observed in inflamed colonic epithelium, similarly to MS endothelium[323]. In a hypothetical model, it was suggested that injured epithelium, via the release of Shh, may communicate damage to both neighboring cells and to the immune system such as $CD4^+$ T lymphocytes and macrophages that express Ptch-1 and thus capable of processing a Shh signal, a process that could further aid in repairing damaged epithelium [322-324].

Identifying factors that are required for BBB integrity and maintenance is necessary for our understanding of neuroinflammation and specifically MS. These experiments allowed us to determine that the Hh pathway is an important astrocytic contributor to BBB integrity. These data also suggest that Shh acts as a direct negative regulator of CNS inflammation through its regulatory effect on BBB-EC activation. Our study will shed some light on a novel role of the Hh pathway in the CNS and help in the development of strategies to regulate the migration of immune cells, the delivery of drugs across the BBB and to design new therapies in the treatment of neuroinflammatory diseases such as MS.

Materials and methods

Primary cell isolation & culture

Human adult CNS tissue was obtained from temporal lobe resection paths of patients undergoing surgery for intractable epilepsy, as previously described[223,325]. Informed consent and ethic approval were given prior to surgery (ethic approval number HD04.046). BBB-ECs were isolated and grown in medium composed of M199 (Invitrogen, Carlsbad, CA) supplemented with 10% feral bovine serum (FBS), 20% normal human serum (HS), endothelial cell growth supplement (5mg/ml) and insulin-selenium transferring premix on 0.5% gelatin-coated tissue culture flasks (reagents from Sigma, Oakville, ON, Canada). Expression of EC markers von Willebrand Factor VIII and *Ulex europaeus agglutinin* I binding sites until passage (P) 7-8 were confirmed. No immunoreactivity for α-myosin or glial fibrillary acidic protein (GFAP) could be detected, confirming the absence of contaminating smooth muscle cells and astrocytes. Human fetal astrocyte isolation has previously been described[326,327]. Briefly, fetal CNS tissue was obtained at 16-22 weeks of gestation following CIHR-approved guidelines. Astrocytes were cultured in complete Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FBS, glutamax, penicillin and streptomycin (Sigma). Cultures were determined to be >90% pure, as determined by GFAP immunostainings, and used between P3-6. Once a week, ACM was harvested from confluent flasks, filtered and added to BBB-EC culture media when specified[43].

Immunocytofluorescence

Primary cultures of BBB-ECs and astrocytes were grown to confluency in 8 chamber slides and fixed with 4% paraformaldehyde (PFA) or methanol (10min, RT). Slides were subsequently permeabilized with 0.3% triton X-100 (5min, RT) and blocked by incubation in 10% goat serum (1h, RT, Sigma) for 1h at RT. Primary antibody (Ab) incubation with rat anti-Shh (1/50, R&D Systems, Minneapolis, MN), rabbit anti-Ptch-1 (1/50, Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-Smo (1/200, MBL, Woburn, MA) were done overnight at 4˚C. Following washes, signals were amplified using biotin-conjugated anti-rat and anti-rabbit Abs followed by Cy3- or FITC-labeled

streptavidin (1/400 and 1/1000 respectively, 30min, RT; DakoCytomation, Glostrup, Denmark). GFAP staining was revealed with Cy3-conjugated anti-mouse Ab (1/400, 30min, RT; Jackson ImmunoResearch Laboratories, West Grove, PA). Nuclei were stained with TO-PRO3 (1/300 in PBS, 30min, RT; Molecular Probes, Eugene, OR). In all cases, control stainings were performed using isotype controls and no immunopositive cells could be detected. Staining was visualized using the Leica SP5 confocal microscope and analyzed using the Leica LAS AF software.

Tissue and immunohistochemistry

Brain tissue from 13 patients with clinically diagnosed and neuropathologically confirmed MS was obtained at rapid autopsy and immediately frozen in liquid nitrogen. Three cases without neurological disease were selected as controls. Tissue samples control cases were taken from the subcortical white matter, corpus callosum and cortex. White matter MS tissue samples were selected on the basis of post-mortem MRI and lesions were classified according to standard histopathological criteria[328,329]. Based on these findings, 6 lesions sampled in this study were classified as active with abundant phagocytic perivascular and parenchymal macrophages containing myelin degradation products, 7 lesions as chronic active with a hypocellular demyelinated center and a hypercellular rim containing numerous macrophages and 6 lesions as chronic inactive with only a few leukocytes and extensive cell loss. All patients and controls, or their next of kin, had given informed consent for autopsy and use of their brain tissue for research purposes.

The generation of Shh deficient mice has been described previously[150]. Mouse embryos (either Shh-/-, Shh+/- or WT) were extracted and snap frozen. Similarly, the C57Bl/6 adult mice, used for the *in vivo* experiments, were perfused with 5ml of PBS and brain, spleen and liver were removed and quickly snapped frozen.

For immunohistochemical and fluorescent staining, 5-8 μm cryosections were cut, air dried and fixed in acetone for 10min. Sections were preincubated for 30min at RT with 20% animal serum, the source of which was determined by the specific secondary Ab used. Afterwards, sections were incubated overnight at 4˚C with primary Abs for Proteolipid protein (1/500, Seorotec Ltd, Oxford, UK, MHC II (1/100, DakoCytomation),

Shh (1/200, R&D systems), Ptch-1 (1/300), Smo (1/200, Santa Cruz Biotechnology), fibrinogen (1/1000, Innovative Research, Novi, MI), occludin, claudin-5, or ZO-1 (1/200, Zymed, South San Francisco, CA). Subsequently, for immunohistochemistry, sections were incubated with appropriate secondary biotin-labeled Abs for 1h at RT and with ABC (DakoCytomation) according to the manufacturer's description. Diaminobenzidine (DAB) was used as chromogen. Between the incubation steps, sections were thoroughly washed with PBS. After a short rinse in tap water, preparations were incubated with hematoxylin (Sigma) for 1min and extensively washed in tap water for 10min. Finally, sections were dehydrated with ethanol, followed by xylol and mounted with Entellan mounting media (Canemco & Marivac, Lakefield, Quebec, Canada). For immunohistofluorescence, sections were incubated with appropriate secondary Cy3-labeled Abs (1/400, DakoCytomation) for 1h at RT. FITC-conjugated *Lycopersicon Esculentum* (Tomato) lectin (1/200, 1h, RT, Vector Laboratories, Burlingame, CA) was used to provide a frame of reference for the localization of blood vessels and nuclei were stained with Hoechst 33258 dye (1/5000, 1min, RT, Invitrogen) and/or with TOPRO-3 (1/300, 30min, RT, Molecular Probes). In all cases, Abs were diluted in PBS containing 0.1% blocking serum, which also served as a negative control. Stainings were visualized using either a Leica DM6000 microscope with OpenLab software or the Leica SP5 confocal microscope.

Reverse-transcription and real-time quantitative polymerase reaction

Cellular RNA was isolated using the TRizol reagent (Invitrogen) and Qiagen RNeasy Mini extraction kit (Mississauga, Ontario), according to the manufacturers' instructions. RNA concentration and purity were determined on a spectrophotometer Ultrospec 2100 pro at 260 nm. Samples were then treated with DNAse I (Amersham Biosciences, Piscataway, NJ) for 10min at 37˚C followed by 10min at 75˚C. Complementary DNA (cDNA) was obtained by mixing 3 µg of RNA with 3.3µM random hexamer primers, 3mM dNTPs, RNase-OUT recombinant ribonuclease inhibitor, 3nM DTT and 400U Maloney murine leukemia virus (MMLV)-reverse transcriptase for 1h at 42˚C and 10min at 75˚C (Invitrogen). For polymerase reactions (PCRs) to be run on agarose gel, 2 µl of

cDNA were amplified using 5U Taq DNA polymerase, 1.5mM $MgCl_2$, 0.2mM dNTPs and 50pmol of forward (F) and reverse (R) primers. Primers used were: Shh; F: 5'-AAAAGCTGACCCCTTTAGCC-3'; R: 5'-CTCTGAGTGGTGGCCATCTT-3'; Ptch-1; F: 5'-GTCGCACAGAACTCCACTCA-3'; R: 5'-AAGAGCGAGAAATGGCAAAA-3'; Smo; F: 5'-CAACCTGTTTGCCATGTTTG-3'; R: 5'-ACATCAGCTGAGGGCTCATT-3'; Gli-1 F: 5'-CGGGGTCTCAAACTGCCCAGCTT-3'; R: 5'-GGCTGGGTCACTGGCCCTC-3'[330]; GAPDH; F: 5'-CAAAGTTGTCATGGATGACC-3'; R: 5'-CCATGGAGAAGGCTGGGG-3' (all primers ordered from Invitrogen). The reaction mixtures were placed in an Eppendorf 'mastercycler' thermal cycler for 10min at 94˚C, followed by 30-35 cycles of 94˚C for 1 min, 45sec at 60˚C and 1min at 72˚C. After amplification, 15µl of each sample was resolved on a 1.5% agarose gel with ethidium bromide. Real-time quantitative PCR was performed for four genes: ribosomal 18S RNA (as an internal amplification control), Gli-1, SOX-18, and claudin-5 (primers from Applied Biosystems, Foster City, CA). The amplification was performed in the presence of AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP, Passive Reference (ROX), and optimized buffer components according to manufacturer's instructions. For 18S amplification, 200 nM of internal probe conjugated to VIC dye and 50 nM of the forward and reverse primers were added. For the target genes, 12.5 nM FAM-labeled probe and 45 nM of the forward and reverse primers were added (Applied Biosystems). Each sample was run in a 96-PCR plate incubated in 7900 Fast Real Time PCR System (Applied Biosystems) for 2min at 50°C, 95°C for 10min, followed by 40 cycles of PCR (95°C for 15 sec and subsequent 60°C for 1min). The relative expression of each target gene was compared to ribosomal 18S and ΔC_T ($C_{T\ (target)}$ - $C_{T\ (reference)}$) was calculated.

Western blots

For western blot analysis (WB) of whole cell lysates, astrocytes and BBB-ECs were lysed in denaturing buffer (50mM Tris-HCl pH 8.5 and 0.1% SDS) supplemented with protease inhibitors, sonicated and centrifuged at 10,000g for 10min at 4˚C. Electrophoresis of 35µg of the supernatant was performed on 6-10% SDS-polyacrylamide gels under

reducing conditions. For WBs of supernatants, 100µg of chloroform-precipitated ACM and control DMEM (supplemented ± with 0.1µg/ml of hrShh) were used. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Biorad, Hercules, CA) and blocked for 1h at RT in 5% milk. Membranes were then incubated overnight with rat anti-human Shh (1/500, R&D systems), rabbit anti-human Ptch-1 Ab (1/250, Santa Cruz Biotechnology), rabbit anti-claudin-5, rabbit anti-occludin (1/150, Zymed), mouse anti-JAM-A (1/500, BD Biosciences), or mouse anti-β-actin (as loading control, Sigma) followed by 1h incubation with species-specific horseradish peroxidase (HRP)-conjugated Abs (DakoCytomation). All signals were revealed using ECL reagents (Amersham Biosciences).

Permeability and migration assays

Primary cultures of BBB-ECs were used to generate an *in vitro* model of the human BBB, as previously reported[238,320,331]. BBB-ECs were plated on gelatin-coated 3µm pore size Boyden chambers (BD Biosciences, Franklin Lakes, NJ) at a density of $2x10^4$ cells per well in EC culture media, supplemented with 40% (v/v) ACM when applicable, and were allowed to grow for 72h to reach confluency. At 48h, cells were treated with hrShh (0.1µg/ml, Sigma), Smo agonist purmorphamine (1µM), or Smo antagonist SANT-1 (0.1µM, Calbiochem Biochemicals, Darmstadt, Germany) or cyclopamine (30µM, Sigma). At 72 hours, FITC-labelled bovine serum albumin (BSA; 50µg/ml; Invitrogen), ^{14}C-sucrose (250nCi/ml; MP Biomedicals, Aurora, OH) or $1x10^6$ freshly purified human $CD4^+$ lymphocytes sorted by magnetic cell sorting (MACS) isolation columns (Miltenyi Biotec, Auburn, CA) were added to the upper chambers. Tracer diffusion across the EC monolayer was assessed by sampling upper and lower chambers at specific time points. Fluorescence intensity in the media was monitored using a FL600 microplate fluorescent reader (Biotek, Winooski, VT). Molecular tracer diffusion representing BBB permeability is calculated using the formula [tracer lower chamber] X 100 / [tracer upper chamber]. The radioactive isotype ^{14}C was read using A2100 Packard Instruments liquid scintillation analyzer (Downers Grove, IL). Immune cell migration was assessed 24h following lymphocyte addition by recovering the migrated cells in the lower chamber

with 0.1M EDTA, stained with trypan blue, and counted using a hemacytometer under blinded conditions. Dead cells were excluded from total counts of migrated cells (1% of cells).

In vivo permeability

To establish BBB disruption *in vivo*, 7-8 week old C57Bl/6 (Charles Rivers, Wilmington, MA) were injected intra-peritoneally (i.p.) with a single dose of cyclopamine at a concentration of 10mg/kg or with equal volume of its vehicle (45% Hydroxypropyl-β-cyclodextrin, HPBCD, Sigma)[332]. Following a waiting period of 6h, 0.1ml of 70kDa FITC-conjugated Dextran (1mg/ml) was injected intravenously (i.v.) into the tail vein of each animal, prior to 0.1ml pentobarbital i.p. injection. Microscopic visualization of the extravasation of Dextran-FITC, as well as the plasma protein fibrinogen was done on brain cryosections and images several blood vessels from vehicle and cyclopamine-injected animals were taken using identical camera settings to allow comparison of extravasation. The area (number of pixels) and fluorescent intensity of the signal (mean intensity of pixels) of both the Dextran-FITC and fibrinogen stainings surrounding vessels were measured using ImageJ. The relative extent of fluorescent extravasation and BBB disruption was then calculated by multiplying the area of pixels with the mean intensity of pixels.

Flow cytometry and enzyme-linked immunosorbent assay

Confluent BBB-ECs were treated for 24h with fresh media supplemented with 40% ACM, purmorphamine (1μm, 24h), SANT-1 (0.1μm, 24h), cyclopamine (30μm, 24h), hrShh (0.1μg/ml, 24h, Sigma), or with IFN-γ and TNF-α (R&D Systems). Quantitative changes in cytokine production were assessed from culture supernatants by enzyme-linked immunosorbent assay (ELISA) for IL-8/CXCL8 and MCP-1/CCL2 (BD Biosciences), according to the manufacturer's protocol. Treatments with ACM could not be done for the possible source of IL-8/CXCL8 or MCP-1/CCL2 found in the ACM, resulting in non-conclusive analysis. Plates were read using a Bio-Tek EL800 96-well

plate reader at a 450nm wavelength and analyzed using KC Junior program (Bio-Tek, Mississauga, ON, Canada).

From the same experiments, extracellular stainings of confluent BBB-ECs were performed after trypsin recovery. The cells were then incubation in 10% fetal bovine serum with 20µg/ml of rabbit anti-Ptch-1 (Santa Cruz Biotechnology) or rabbit anti-Smo (MBL) for 30min at 4°C. Following a wash, the cells were reincubated in 10% FBS with 2ul of FITC-labeled goat anti-rabbit and 5ul of anti-CD54 (ICAM-1) and anti-CD106 (VCAM-1; both from BD Biosciences) for 30min at 4°C. Cell staining was acquired on a LSRII (BD Biosciences) and analyzed using FACSDiva software.

For intracellular cytokine staining (ICS), primary cultures of astrocytes were stained for HLA-ABC coupled to Pacific Blue and were then fixed and permeabilized in 4% (w/v) PFA with 0.1% (w/v) saponin in Hank's Balanced Salt Solution (10min at RT). Intracellular staining was performed by incubating cells with Abs against Shh or Gli-1, (5µg/ml, R&D systems) for 30 min on ice in PBS buffer containing 0.1% (w/v) saponin, 1% FBS, 0.1% (w/v) NaN3, followed by a wash and another reincubation with 2ul of APC-labeled donkey anti-rat (eBioscience, San Diego, CA) resuspended in FACS buffer [1% (v/v) FBS, 0.1% (w/v) NaN3 in PBS]. Cells were acquired on a BD LSRII and analyzed using BD FACSDiva software.

Statistical analysis

Data were analyzed using GraphPad Prism software (San Diego, CA, USA). Results are shown as mean and standard error of the mean (SEM) and statistical analyses included paired student's t-test.

Figure legends

Figure 1: *Human astrocytes express and secrete Sonic Hedgehog and human blood-brain barrier endothelial cells express receptor, Patched-1 and signal transducer Smoothened.* **A.** Polymerase chain reaction (PCR) of primary cultures of human fetal astrocytes (HFA; two preparations are shown: 1 and 2) and human adult blood-brain barrier (BBB) endothelial cells (ECs; two preparations are shown: 3-4). **B**. Protein expression of Hedgehog (Hh) pathway components by astrocytes and BBB-ECs revealed by western blots (WBs). Whole cell lysates from BBB-ECs and HFAs were solubilized in denaturing buffer, resolved on 8% SDS-PAGE gels and immunoblotted using anti-human antibodies (left panels). Precipitated astrocyte-conditioned media (ACM) contains the cleaved form of Sonic Hh (Shh) at 19kDa. Human recombinant Shh (hrShh) protein was used as a positive control (right panel). Gels shown are representative of 3 independent experiments (n=3). **C.** Protein localization of Hh pathway components in HFAs and BBB-ECs detected by immunocytofluorescence. Primary cultures of HFAs and BBB-ECs were plated in chamber slides, fixed with paraformaldehyde 4% or methanol 100% and stained using anti-human antibodies. Intracellular staining of Shh (green) was found in GFAP immunopositive (red) HFAs. BBB-ECs showed surface expression for the receptor Patched-1 (Ptch-1), and the co-receptor, Smoothened (Smo). Merged overlay are shown (right panels). Hoechst-stained nuclei are blue. Scale bars represent 50μm. No staining was detected in controls lacking primary antibodies. **D.** In adult human CNS tissue, perivascular astrocytes express Shh as determined by the location and morphology of the cells. Higher magnification of perivascular astrocyte (arrowhead) shows the cell body and endfeet (arrows) opposing parenchymal vessel (asterisk). Ptch-1and Smo expression in human CNS tissue was detected in human brain vascular endothelial cells. Nuclei were counterstained with haematoxylin. Scale bar represents 50μm. All stainings were performed on 5-8 μm frozen sections of control non-pathological human brain tissue (n=10).

Figure 2: *Astrocyte secreted Shh decreases BBB-EC permeability via Smo signaling.* **A-C.** In the *in vitro* assay for permeability, BBB-ECs are plated in the top chamber and

grown to confluency. After treatment, Fluorescein Isothiocyanate-bovine serum albumin (FITC- BSA) is added to the top chamber and the time course of its diffusion is measured. **A.** When BBB-ECs are grown in ACM, permeability of BSA-FITC decreases by 40%. (■, n= 8, *p <0.05) when compared to cells grown under basal conditions (○, untreated). Similar effects were obtained when BBB-ECs monolayers were treated with hrShh (▼, 0.1 μg/ml; 24h, n=8, *p<0.05) or **B.** purmorphamine (▼, 1μM; 24h, n=8, *p<0.05). **C.** The effect of ACM on the permeability of BBB-ECs was prevented by treating the cells with the antagonist cyclopamine (▲, 30μM; 24h, n=6, p<0.05) or SANT-1 (not shown), suggesting that Shh ligand is responsible, at least in part, for the barrier promoting effects of astrocytes. **D.** The relative extent of BBB leakage was studied in the CNS parenchymal tissue of mice intraperitoneally injected with cyclopamine (10mg/kg in 45% Hydroxypropyl-β-cyclo-dextrin, HPβCD, 6h). BBB disruption was determined with a 70 kDa Dextran-FITC injected in the tail vein 5min prior to sacrifice and by measuring exogenous fibrinogen. Quantitative analysis of Dextran-FITC and fibrinogen extravasation was significantly higher in cyclopamine-injected animals, compared to vehicle-injected mice (*p<0.05 for both markers). **E.** Parenchymal vessels (asterisk) showed various points of BBB leakage (arrowheads) in the cyclopamine-treated mice. Both FITC-Dextran and fibrinogen leakage into the surrounding parenchymal tissue was detected at the breaking points (arrowheads). In the vehicle treated mice (HPβCD), Dextran-FITC and fibrinogen staining were found solely in the lumen of the vessels. Hoechst stained nuclei are blue. Scale bars represent 50μm.

Figure 3: *Shh is important in tight junction maintenance.* **A.** Quantitative, real-time PCR reveals that BBB- ECs grown with ACM or with hrShh (0.1μg/ml, 24h) upregulates Ptch-1 expression. Claudin-5 and occludin were also upregulation upon Hh activation. **B.** WB (left panels) resolved on 8% SDS-PAGE gels and immunostained for tight junction (TJ) proteins claudin-5, occludin and JAM-A on BBB-EC whole cell lysates, showed downregulation of all three proteins after ACM-grown BBB-ECs were treated with cyclopamine (30μM, 24h; 35 μg of protein/well), compared to ACM-grown BBB-ECs. Semi-quantitative graph shows claudin-5 downregulation of 21.3%, occludin of 77.9%, and ZO-1 of 32.4%, compared to ACM control. **C.** Shh knock-out (-/-) embryonic mouse

brains exhibited reduced levels of claudin-5, occludin, and ZO-1 on lectin$^+$ BBB-ECs, compared to WT animals. Overlays are shown (bottom panels) with TOPRO-3 nuclei stained in blue. Left corner insets demonstrates lectin$^+$ ECs positive for claudin-5, occludin, or ZO-1 in WT mice, compared to loss of TJ positivity in Shh -/- mice. Scale bars in fluorescent pictures represent 50μm. **D.** Quantification plot of the number of lectin$^+$ ECs immunopositive for the TJ proteins claudin-5, occludin, or ZO-1. Compared to WT BBB-ECs, Shh -/- had significantly less lectin$^+$/claudin-5$^+$ (n=10, ***p<0.0001), lectin$^+$/occludin$^+$ (n=5, ***p<0.0001), and lectin$^+$/ZO-1$^+$ (n=10, ***p<0.0001) cells.

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Figure 4: *Modulating Hh activity influences BBB-EC expression of cell adhesion molecules, chemokine secretion and leukocyte ability to transmigrate.* **A.** Flow cytometry analysis of the cell adhesion molecules (CAMs), intercellular-CAM-1 (ICAM-1) and vascular-CAM-1 (VCAM-1), was determined on BBB-ECs. When grown in ACM, both surface VCAM-1 and ICAM-1 levels decreased to 1.7% and 63.9% from control (untreated) conditions of 3.4% and 73.9%, respectively. This reduction could be inhibited if cyclopamine (30μM, 24h) was added to the ACM. When treated with exogenous hrShh (0.1μg/ml, 24h) or purmorphamine (1μM, 24h), VCAM-1 and ICAM-1 surface expression on BBB-ECs reduced to levels of 2.2-2.8% and 59.3-66.6%, respectively. **B.** Mean fluorescent intensity was measured for ICAM-1 in BBB-ECs. Activation of the Hh pathway in ACM-grown BBB-ECs as well hrShh and purmorphamine treatments decreased the intensity of ICAM-1 signal on BBB-ECs (n=3, *p<0.05), an effect that could be blocked by cyclopamine treatment. **C.** BBB-EC chemokine secretion was detected by enzyme-linked immunosorbent assay (ELISA). Treating primary cultures of human BBB-ECs with hrShh (0.1μg/ml, 24h) or purmorphamine (1μM, 24h) lead to a decrease in secretion for both IL-8/CXCL8 and MCP-1/CCL2 chemokines (n=4, **p<0.005, ***p<0.0005, respectively). Addition of cyclopamine (30μm, 24h) to hrShh (0.1μg/ml, 24h)-treated BBB-ECs inhibited the decrease in chemokine secretion. **D.** CD4$^+$ T lymphocyte migration was measured using the modified Boyden chamber assay. 2x10^6 human CD4$^+$ lymphocytes (n=4 donors) were allowed to migrate across untreated BBB-ECs or ACM-grown BBB-ECs. Data demonstrates that ACM promotes the BBB capacity to restrict immune cell migration. BBB-ECs treated with hrShh (0.1μg/ml, 24h)

or with purmorphamine (1μM, 24h) restricted the migration of $CD4^+$ leukocytes across the monolayer. ACM-grown BBB-ECs treated with cyclopamine (30μM, 24h) permitted the migration of lymphocytes to the same degree as the untreated control (*$p < 0.05$, **$p<0.005$, ***$p < 0.0005$, n=8, in duplicate).

Figure 5: *In vitro expression pattern of transcription factors in BBB-ECs.* **A.** Flow cytometry analysis of the transcription factor Gli-1 was determined intracellularly in BBB- ECs. In resting conditions, Gli-1 was expressed in 26.7% of the cells (compared to normal rat isotype control, outline shown in black). Gli-1 expression increases in ACM-grown-BBB-ECs (dark grey) to 43.1%, compared to untreated control (light grey). The effect of cyclopamine (light grey, 30μM, 24h) reversed the effect of the ACM upregulation of Gli-1 down to 38.8%, suggesting that ACM drives Gli-1 upregulation via Smo. BBB-ECs treated with hrShh (dark grey, 0.1μg/ml, 24h) and purmorphamine (dark grey, 1μM, 24h) increased levels of Gli-1 to 45.3% and 65.4% respectively. **B.** Mean fluorescent intensity was measured for Gli-1 in BBB-ECs. Activation of the Hh pathway in ACM-grown BBB-ECs as well hrShh and purmorphamine treatments increased the intensity of Gli-1 signal in BBB-ECs (n=2, *$p<0.05$, **$p<0.01$), an effect partially blocked by cyclopamine treatment. **C.** Quantitative real-time PCR experiments demonstrated the time course of Gli-1 and SOX-18 transcription factor upregulation upon hrShh treatment. Rapid induction of Gli-1 mRNA upregulation occurs 1h after Shh treatment (0.1μg/ml). Following Gli-1 upregulation, SOX-18 mRNA levels increase after 3h following hrShh treatment (n=2, **$p<0.01$, ***$p<0.001$).

Figure 6: *Pro-inflammatory cytokines upregulate Hh components in astrocytes and BBB-ECs in vitro.* **A.** Flow cytometry analysis of Shh ligand was determined intracellularly in primary cultures of human astrocytes. Triple staining with GFAP and MHC-I identified the astrocytes ($GFAP^+/MHC\text{-}I^+$) from the potentially contaminating neurons ($GFAP^-/MHC\text{-}I^-$, approximately 5% of population). Forward scatter (FSC, y-axis) and side scatter (SSC, x-axis) differentiates both cell volume and granulocity respectively. In resting astrocytes, Shh was expressed in 35.4% of the cells when compared to normal rat isotype control. Treatment with pro-inflammatory cytokines TNF-α and IFN-γ (100U,

24h) increased levels of Shh to 63.4%. **B.** Flow cytometry analysis of the Hh receptors Ptch-1 and Smo was determined on BBB-ECs when grown in ACM. 30.0% and 27.6% of ACM-grown BBB-ECs express surface Ptch-1 and Smo respectively (compared to normal rabbit isotype control, outline shown in black). When treated with pro-inflammatory cytokines TNF-α and IFN-γ (100U, 24h), the expression of BBB-ECs express Ptch-1 and Smo nearly doubles to levels of 59.3% and 54.4%, respectively.

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Figure 7: *Expression of Shh in MS tissue.* Formalin-fixed MS brain material from autopsy contained normal appearing white matter (NAWM) as well as acute MS lesions with heavy perivascular mononuclear cell infiltrates and marked demyelination. **A.** Shh expression in human MS tissue is increased in active MS lesions compared to nearby NAWM (delineated by black line). **B.** Shh expression was observed in perivascular and parenchymal astrocytes (arrowheads) in the NAWM. **C.** In active MS lesions, hypertrophic astrocytes and astrocyte processes are strongly Shh immunopositive (arrowheads). **D.** Double fluorescent staining shows higher expression of Shh (green) in perivascular and hypertrophic endfeet (arrowheads) colocalizes with GFAP (red), as seen in overlay. Nuclear counterstaining with Hoechst (blue).

Figure 8: Expression of Hh components in MS tissue. Formalin-fixed MS brain material contained Hh components Ptch-1, Smo and Gli-1. **A.** Immunostainings of NAWM of MS brain material showing expression of Ptch-1 on endothelial cells (arrowheads). In active demyelinating lesions Ptch-1 is expressed by macrophages (arrow), but absent in the vasculature (arrowheads). **B.** Smo is expressed by microglia (arrowhead) and in the cerebrovasculature (arrow) in the NAWM. Macrophages (arrowhead) and brain endothelial cells (arrow) are Smo immunopositive in active MS lesions. **C.** Double fluorescent staining shows that expression of Ptch-1 (red) colocalizes with Gli-1 (red) in endothelial cells in NAWM, as seen in overlay. No considerable change in expression of Ptch-1 and Gli-1 are observed in active MS lesions. Gli-1 staining can be distinguished in infiltrating cells. Nuclear counterstaining with Hoechst (blue).

Supplemental Figure Legend

Supplemental Figure 1: *Activation of Hh pathway is dose-dependent and affects permeability of small molecule tracer ^{14}C-sucrose, without affecting proliferation.* **A.** hrShh decreases permeability of BBB-EC monolayers to BSA-FITC in a dose dependent fashion, with higher concentrations of hrShh leading to lower the diffusion rates of BSA-FITC. **B.** Due to the progressive halving of carboxyfluorescein succinimidyl ester (CFSE) fluorescence within daughter cells following each cell division, cell proliferation can be compared between treatments. Compared to untreated control BBB-ECs, hrShh (0.1μg/ml, 24h) does not induce an increase in cell proliferation. **C.** In the *in vitro* assay for permeability, BBB-ECs are plated in the top chamber and grown to confluency. After treatment, small molecule tracer ^{14}C-sucrose is added to the top chamber and a 6 hour time course of its diffusion is measured. When BBB-ECs are grown in ACM (▲, 40%) or treated with hrShh (▼, 0.1 μg/ml; 24h) or purmorphamine (◆, 1μM; 24h, n=8, *$p<0.05$), permeability of ^{14}C-sucrose decreases, compared to cells grown under basal conditions (○, untreated). The effect of ACM on the permeability of BBB-ECs was prevented by treating the cells with the antagonist cyclopamine (▲, 30μM; 24h, n=6, $p<0.05$).

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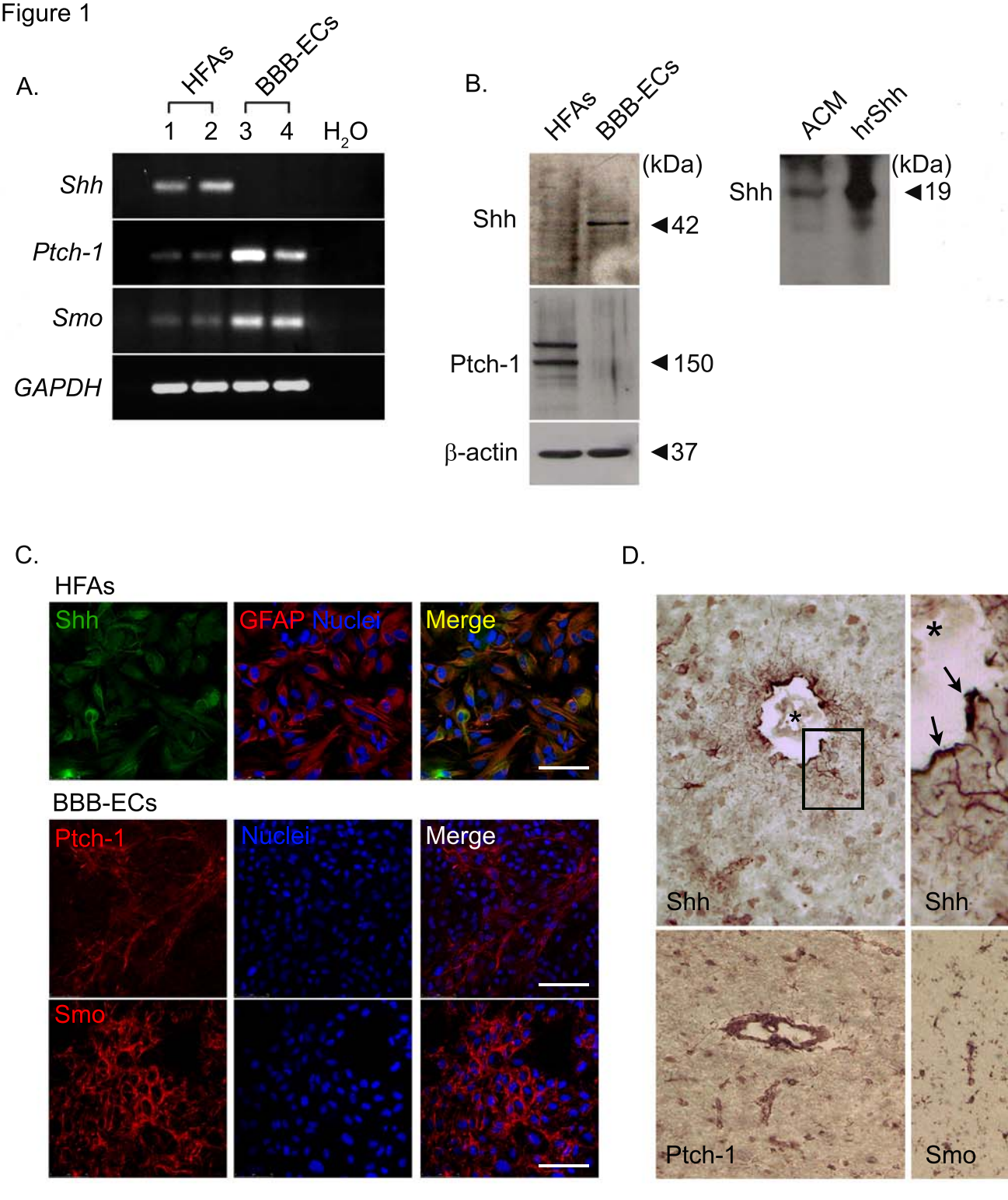
Figure 1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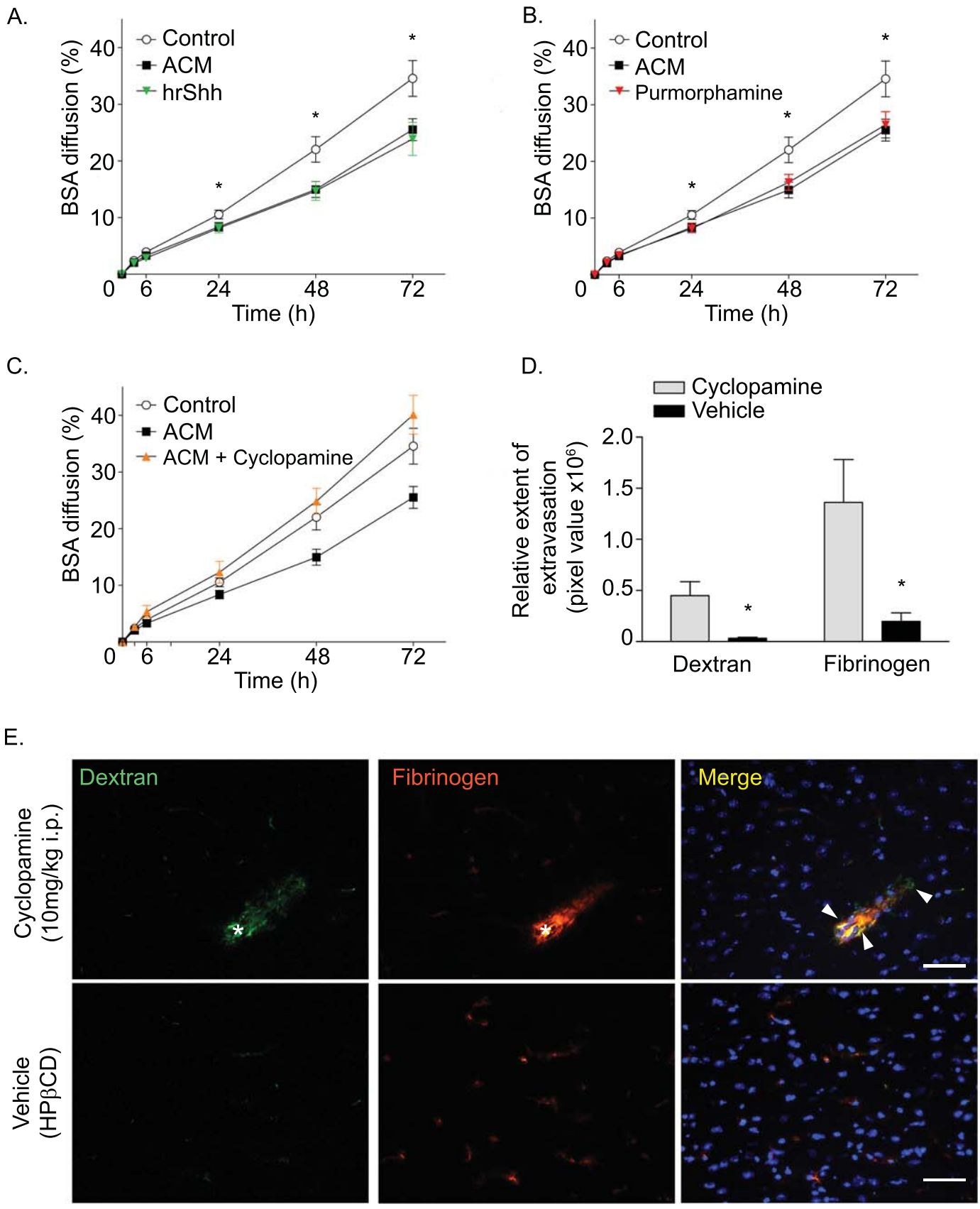
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ACM
hrShh
BSA diffusion (%)
Time (h)
B.
Control
ACM
Purmorphamine
BSA diffusion (%)
Time (h)
C.
Control
ACM
ACM + Cyclopamine
BSA diffusion (%)
Time (h)
D.
Cyclopamine
Vehicle
Relative extent of extravasation (pixel value x10⁶)
Dextran
Fibrinogen
E.
Dextran
Fibrinogen
Merge
Cyclopamine (10mg/kg i.p.)
Vehicle (HPβCD)

Figure 3

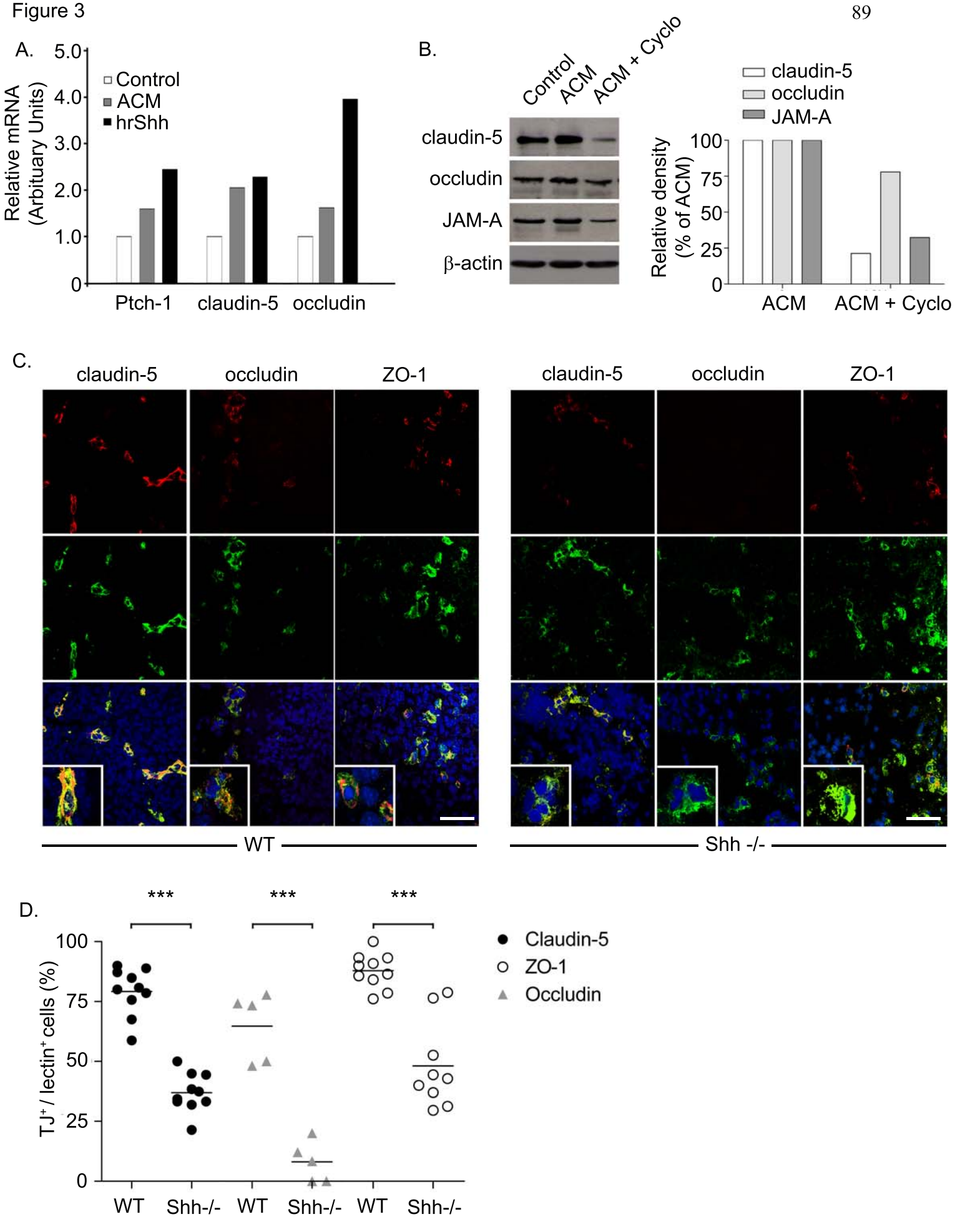

A.
Relative mRNA (Arbituary Units)
5.0
4.0
3.0
2.0
1.0
0
Control
ACM
hrShh
Ptch-1
claudin-5
occludin
B.
Control
ACM
ACM + Cyclo
claudin-5
occludin
JAM-A
β-actin
claudin-5
occludin
JAM-A
Relative density (% of ACM)
100
75
50
25
0
ACM
ACM + Cyclo
C.
claudin-5
occludin
ZO-1
claudin-5
occludin
ZO-1
WT
Shh -/-
D.
***
***
***
TJ+ / lectin+ cells (%)
100
75
50
25
0
WT
Shh-/-
WT
Shh-/-
WT
Shh-/-
Claudin-5
ZO-1
Occludin

Figure 4

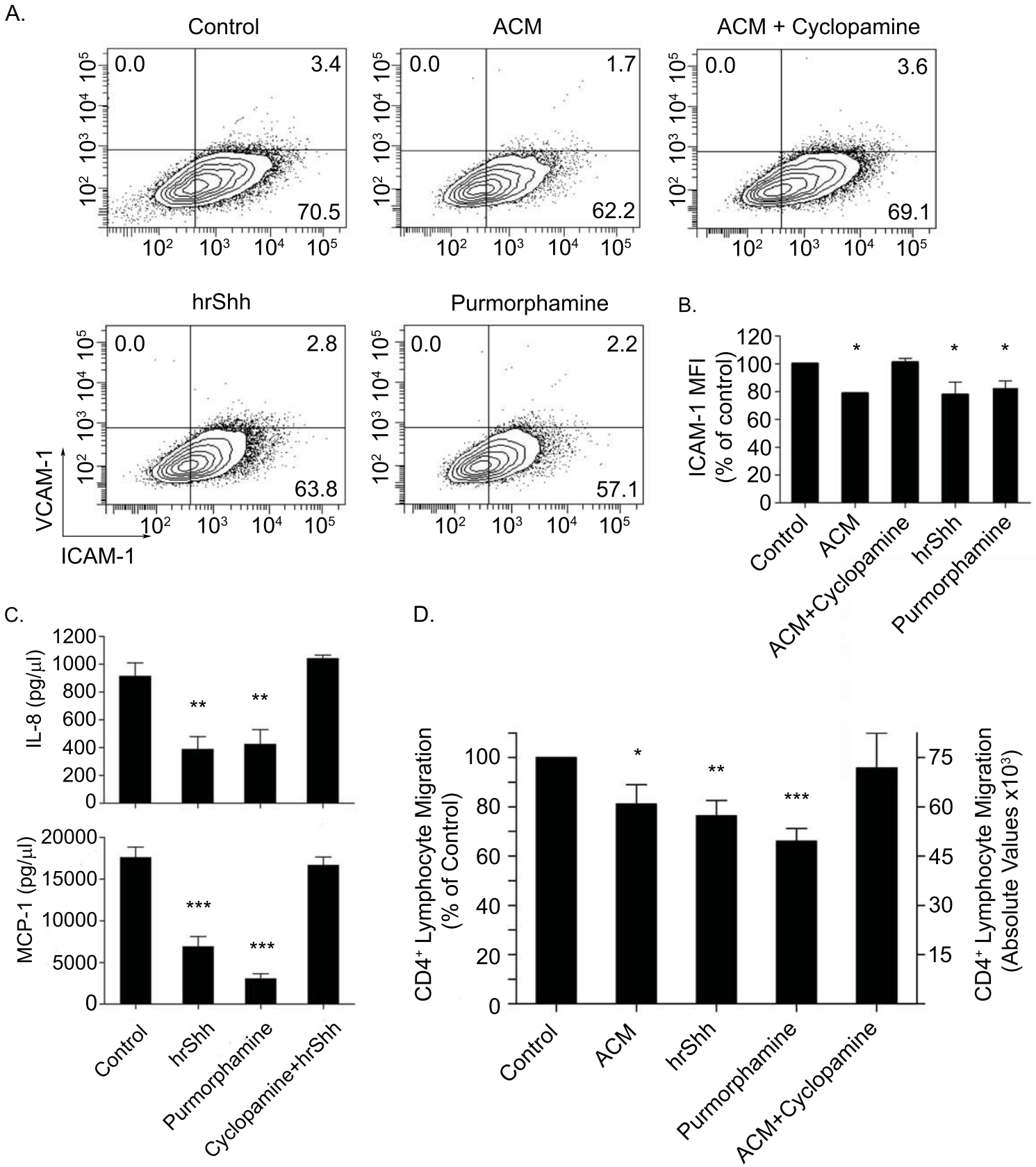
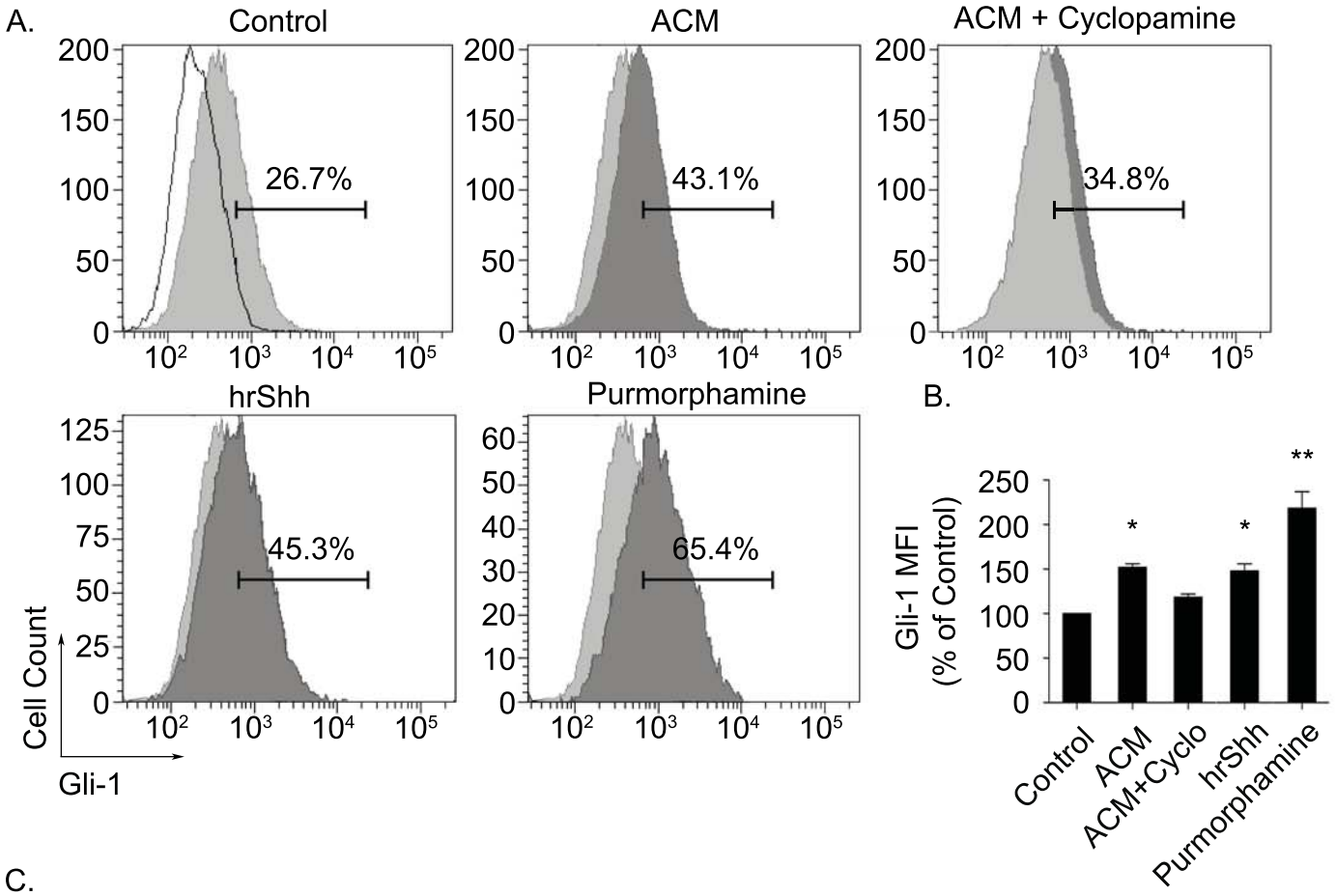

A.
Control
ACM
ACM + Cyclopamine
hrShh
Purmorphamine
0.0
3.4
70.5
1.7
62.2
3.6
69.1
2.8
63.8
2.2
57.1
VCAM-1
ICAM-1
B.
ICAM-1 MFI (% of control)
Control
ACM
ACM+Cyclopamine
hrShh
Purmorphamine
C.
IL-8 (pg/μl)
MCP-1 (pg/μl)
Control
hrShh
Purmorphamine
Cyclopamine+hrShh
D.
CD4+ Lymphocyte Migration (% of Control)
CD4+ Lymphocyte Migration (Absolute Values x10³)
Control
ACM
hrShh
Purmorphamine
ACM+Cyclopamine

Figure 5


A.
Control
ACM
ACM + Cyclopamine
26.7%
43.1%
34.8%
hrShh
Purmorphamine
45.3%
65.4%
Cell Count
Gli-1
B.
Gli-1 MFI (% of Control)
Control
ACM
ACM+Cyclo
hrShh
Purmorphamine

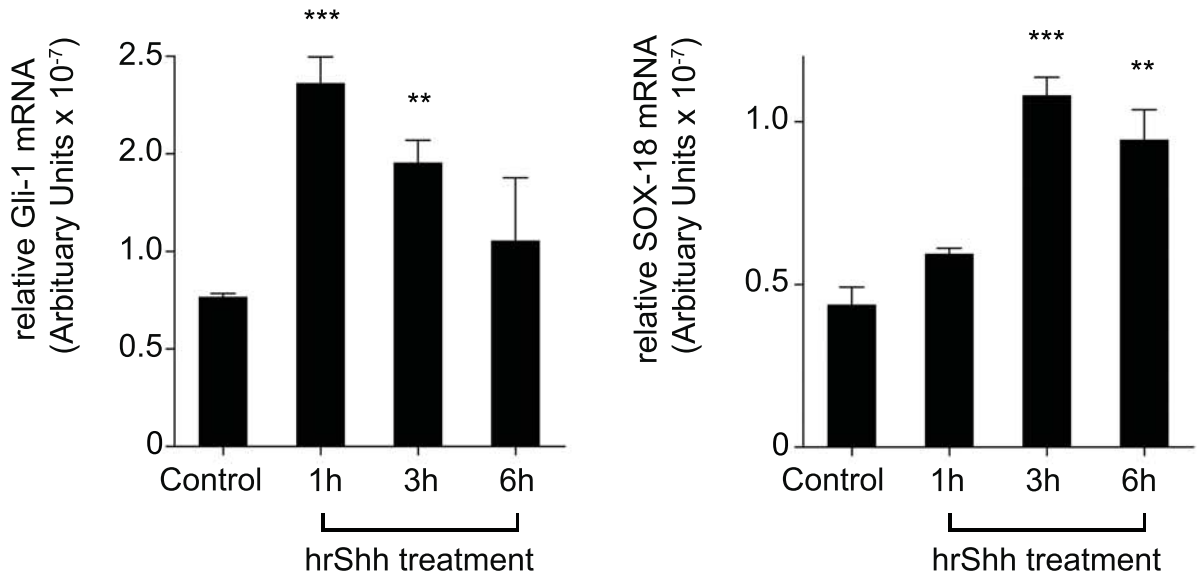
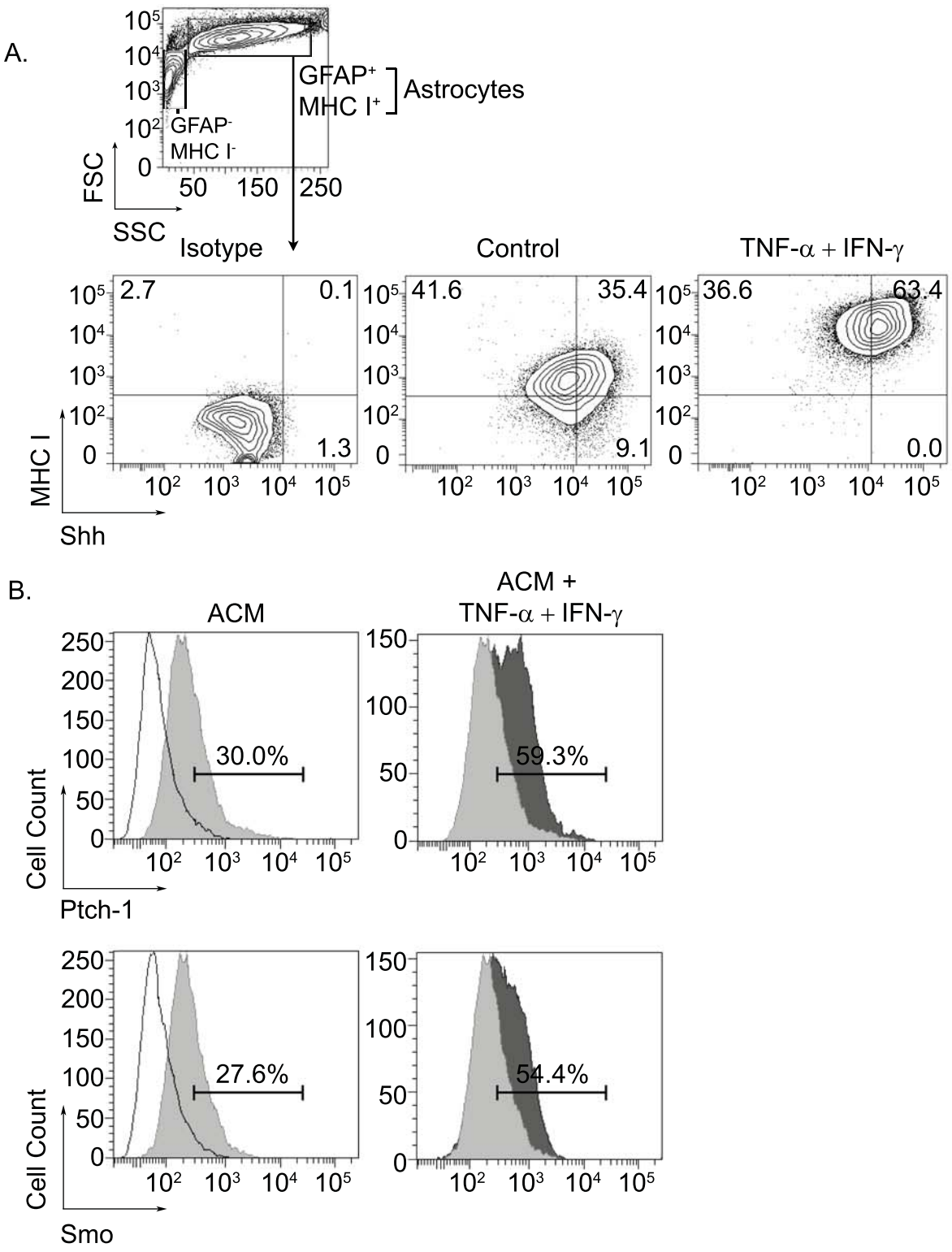
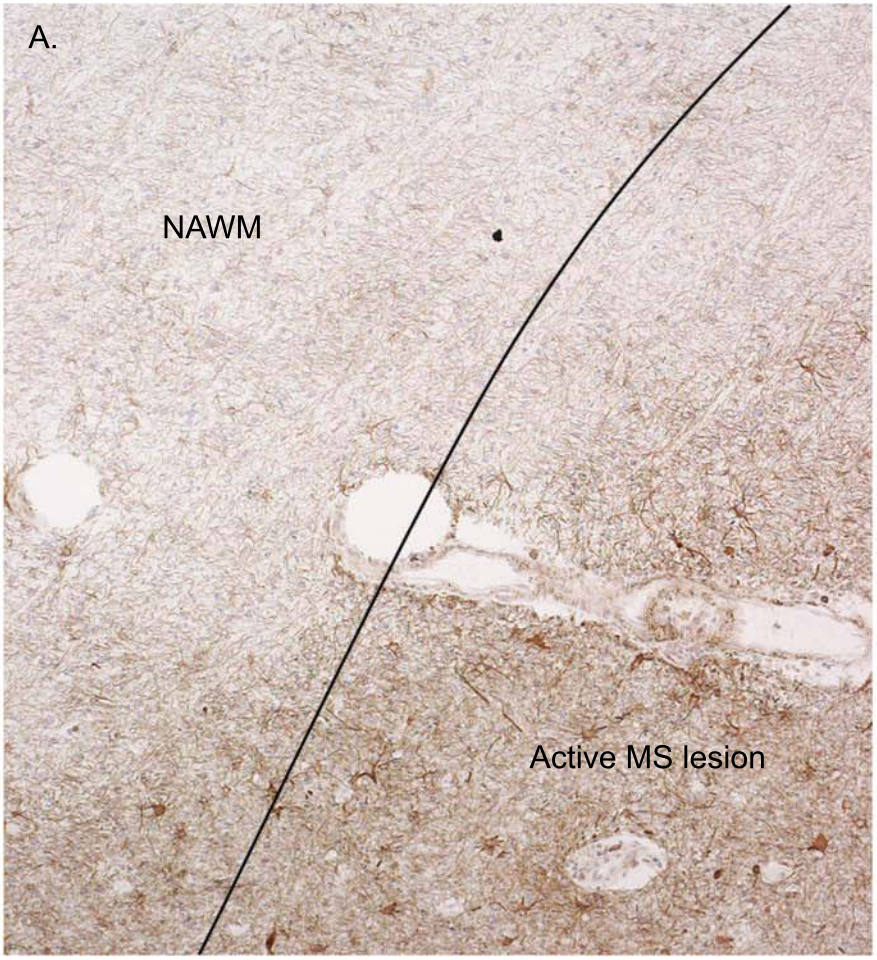
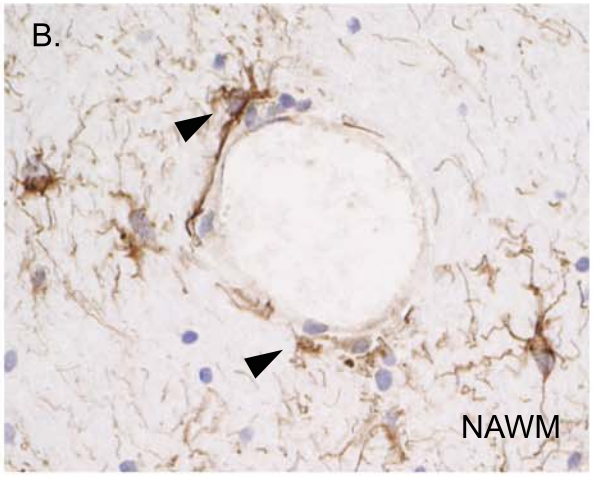

C.
relative Gli-1 mRNA (Arbituary Units x 10-7)
Control
1h
3h
6h
hrShh treatment
relative SOX-18 mRNA (Arbituary Units x 10-7)
Control
1h
3h
6h
hrShh treatment

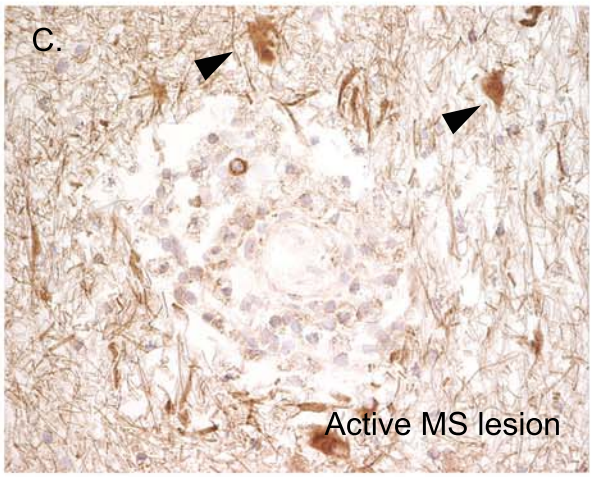
Figure 6


A.
GFAP+
MHC I+
Astrocytes
GFAP-
MHC I-
FSC
SSC
Isotype
Control
TNF-α + IFN-γ
2.7
0.1
1.3
41.6
35.4
9.1
36.6
63.4
0.0
MHC I
Shh
B.
ACM
ACM +
TNF-α + IFN-γ
30.0%
59.3%
Cell Count
Ptch-1
27.6%
54.4%
Smo

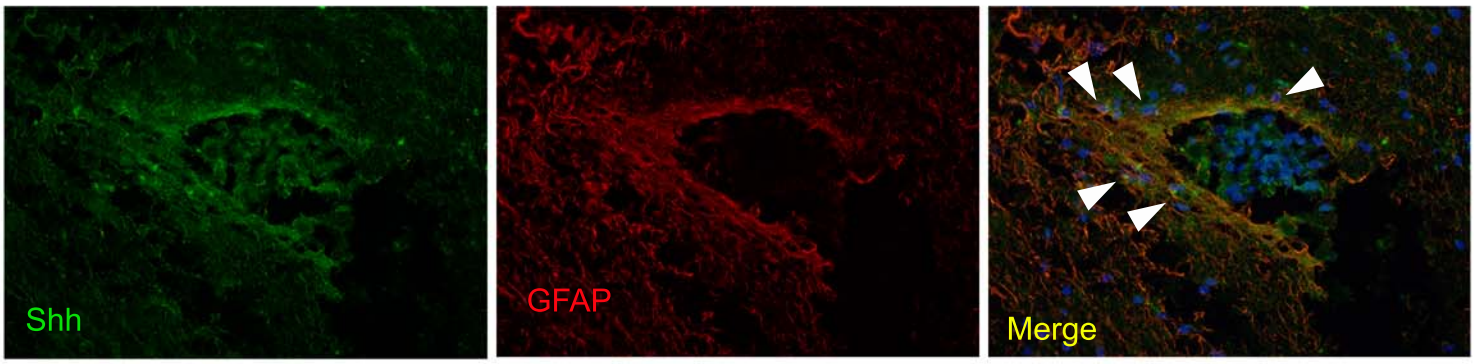
Shh

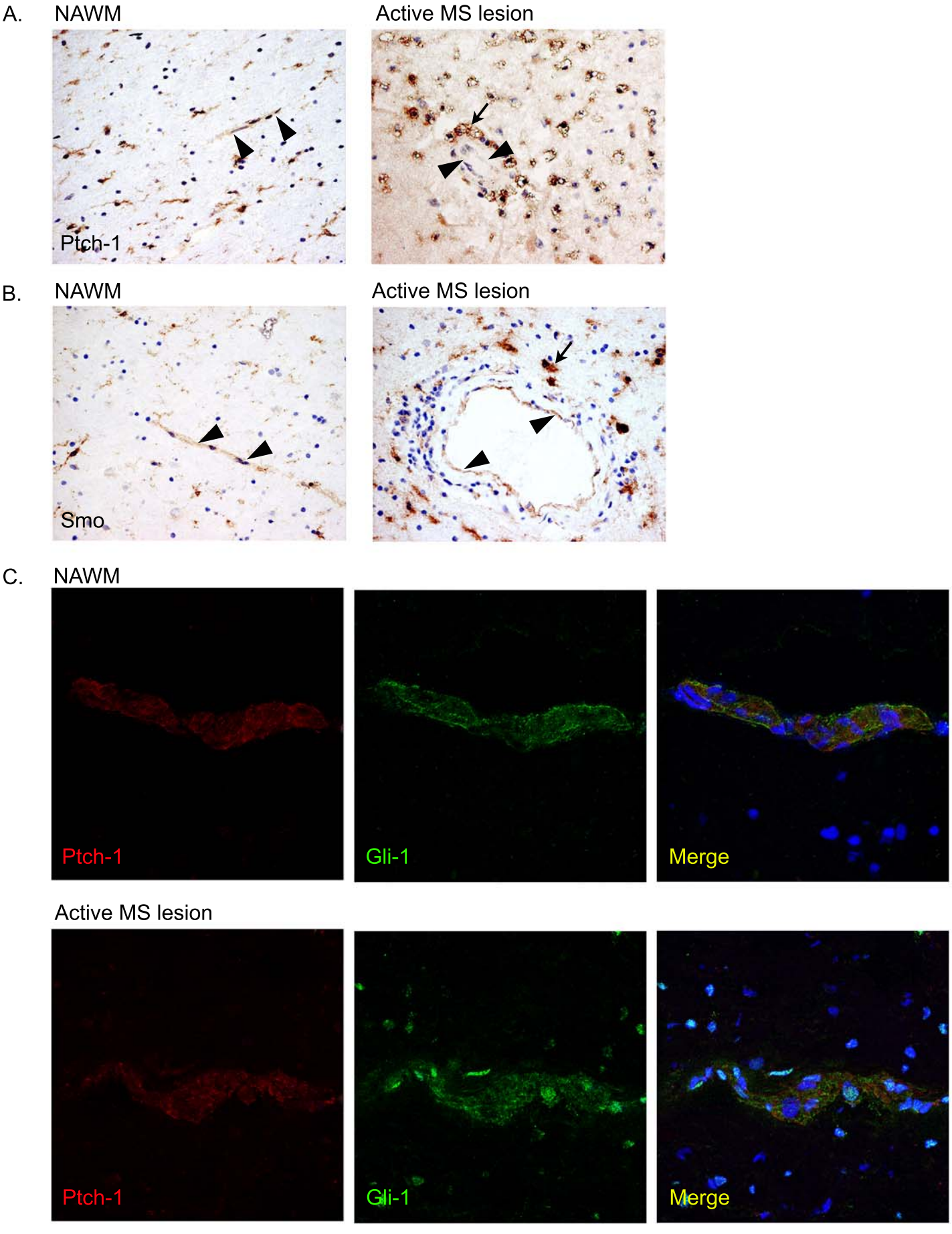




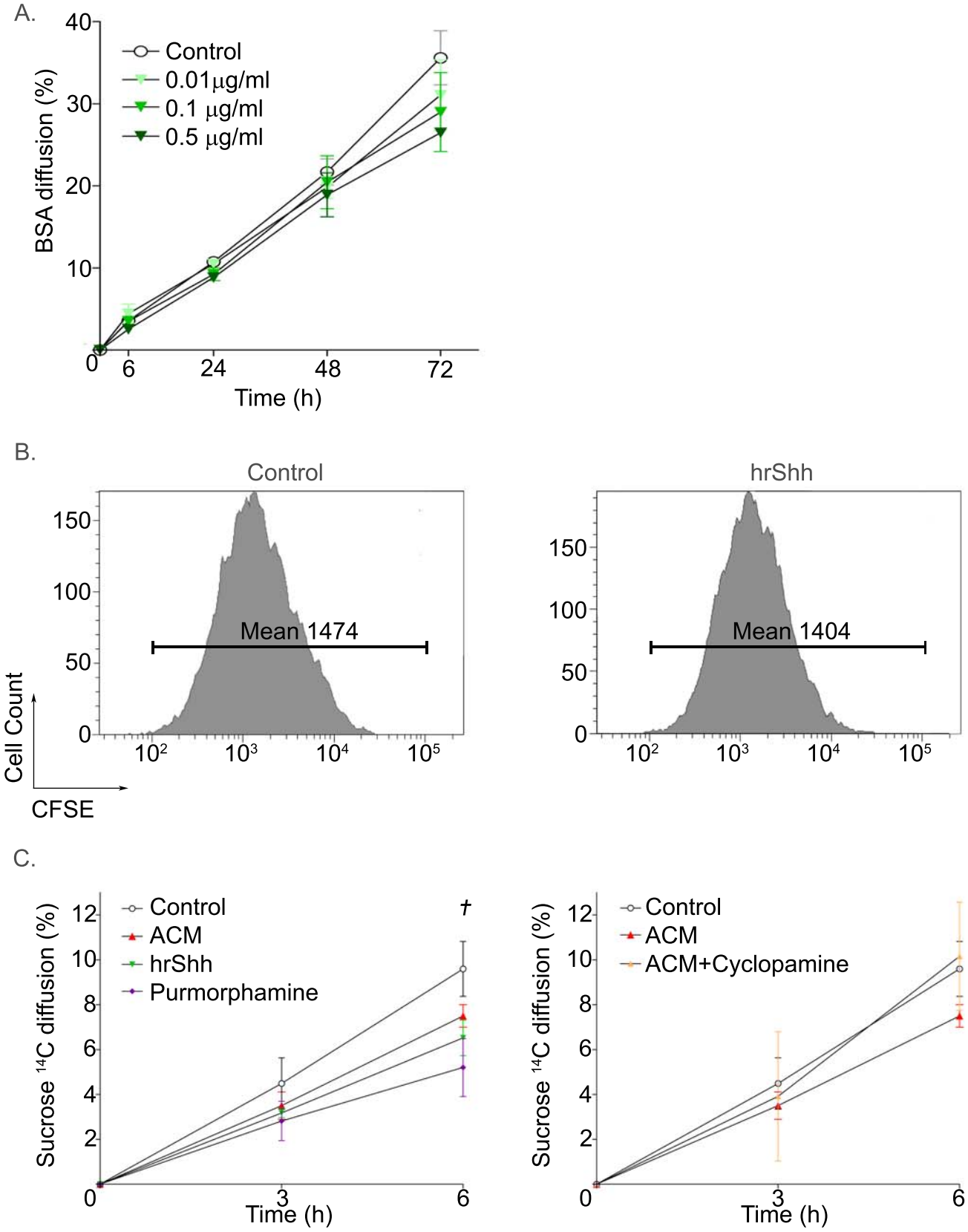


D. Active MS lesion




A.
NAWM
Active MS lesion
Ptch-1
B.
NAWM
Active MS lesion
Smo
C.
NAWM
Ptch-1
Gli-1
Merge
Active MS lesion
Ptch-1
Gli-1
Merge

Supplementary Figure 1



DISCUSSION

1.0 LIPID MEMBRANE RAFTS IN BBB

Membrane lipid rafts are cholesterol and sphingolipid-enriched platforms which compartmentalize specialized cellular processes. In BBB-ECs, they serve as organization centers for various endothelial-specific TJ, ECM-scaffolding, adhesion and transporter molecules. Mspect analysis of DRMs isolated from human primary cultures of BBB-ECs has confirmed the presence of proteins involved in such BBB-related activities, suggesting a crucial role of lipid membrane rafts in maintaining segregated and highly organized regions within the plasma membrane.

1.1 Role of membrane lipid rafts in junctional protein complexes

TJs consist of at least three known types of transmembrane proteins, including occludin, claudins and the junctional adhesion molecules (JAMs)[8,17,25,26]. Overall, the expression levels of TJ proteins have been found to decrease in many neurological disorders that are characterized by loss of BBB permeability integrity, such as in MS[15,43,45,46]. AJs of BBB-ECs are primarily composed of vascular endothelial cadherin (VE-cadherin), which interact with intracellular proteins, including the different catenin isoforms (α-, β-, γ-catenin) and ZO-1 to create the association with the actin cytoskeleton[54,59-62]. Nusrat and coworkers first proposed that TJ molecules of epithelial cells were enriched in lipid raft membrane microdomains. Using the T84 intestinal epithelial cell line and TX-100 raft isolation procedures, occludin and ZO-1 were found to be significantly increased in the insoluble fractions compared to the non-raft soluble fractions[293]. This functional membrane raft idea was adopted by others and recent publications confirmed the close association of membrane rafts with various TJ and AJ proteins in both endothelial and epithelial cells[43,286,333]. In Part I of the current thesis, I demonstrate that TJ proteins occludin, JAM-A and ZO-1 are localized in BBB-EC lipid membrane rafts, alongside caveolin-1 (Figure ivA) .

Distribution of TJ proteins to lipid rafts can occur via specific post-translational fatty acid modifications. Inhibition of palmitoylation, required for optimal claudin function, restricts claudin enrichment into DRM domains and leads to a decrease in TJ

barrier function[334]. Using a novel, detergent-free OptiPrep density-gradient method to fractionate rat cerebral microvessels, McCaffrey and co-workers observed TJ proteins in isolated raft fractions and found that the molecular weights of claudin-5, ZO-1 and occludin contained in rafts were twice as large as the predicted weights. This suggests that raft microdomains could be an environment in which TJ oligomerization occurs[285,286,335]. Other studies show that alterations in caveolin-1 expression induces alterations of the TJ and AJ complexes[277,336] and caveolin-1 -/- mice suffer from microvascular hyperpermeability[337]. In addition, caveolin-1 knock-down in BBB-ECs induced a shift in occludin, VE-cadherin and β-catenin subcellular distribution from the DRM to the soluble fraction along with an increase in endothelial permeability[277]. Taken together, these data demonstrate that specialized rafts contribute to barrier permeability through clustering of TJ and AJ proteins.

Several chemicals, described to affect cholesterol synthesis or availability, are known to modulate raft integrity. Modulation of cell membrane cholesterol by treating cells with the cholesterol chelator methyl-β-cyclodextrin (MβCD) induces an initial rise and subsequent fall in TEER measures, associated with actin redistribution and reduced membrane expression of occludin and ZO-1[338,339]. Moreover, it has been demonstrated that MβCD treatment leads to specific displacement of occludin, claudin-3, -4, -7 and JAM-A out of the Lubrol WX-resistant lipid rafts and coincides with a decrease in TJ integrity[340]. Cholesterol lowering agents, such as HMG-CoA reductase inhibitors (i.e. statins) induced the mobilization of occludin outside of the raft compartment in epithelial cells[293]. In the human *in vitro* system however, statins were shown to decrease BBB permeability without altering subcellular localization of occludin, JAM-A, VE-cadherin, ZO-1 or ZO-2[254]. This suggests that protein localization into rafts is not uniquely regulated by cholesterol concentration, and that additional effects of statins, unrelated to their cholesterol lowering effects might account for these discrepancies. In that sense, we and others have also shown that the barrier promoting effect of statins was dependent on their effect on geranylation, and independent on the cholesterol concentration[254,341].

TJ enrichment within membrane rafts is positively influenced by astrocyte-secreted angiotensin metabolites and is negatively regulated by pro-inflammatory cytokines[43]. Similarly, additional proteins secreted by astrocytes can modulate the BBB-

EC TJ microdomains, such as the chemokine MCP-1/CCL2 acting via its receptor CCR2[342]. MCP-1 was reported to induce changes in caveolin-1 expression and to impact on ZO-1 and occludin localization in membrane microdomains, suggesting that during inflammatory conditions, BBB destabilization can occur through inflammatory cytokine-induced reorganization of TJ proteins on the BBB-EC surface[343].

In addition to junctional proteins, there are several reports associating integrins with caveolae and lipid rafts[344]. Membrane rafts in ECs are enriched in $\alpha_5\beta_1$ integrin, which binds to ECM components and mediates EC attachment to the basal lamina[345,346]. I showed that these integrin subunits, in addition to α5 can be identified in BBB-EC lipid membrane rafts (Figure ivC), suggesting that lipid membrane rafts can be a crucial component to preserving integrin polarity within the cell.

1.2 Role of lipid membrane rafts in adhesion molecules

The association of CAMs with cholesterol-enriched membrane microdomains is important for optimal signal transduction events during both transendothelial and transjunctional migration. ICAM-1 and novel adhesion molecules ALCAM and MCAM have been found to localize partially in DRMs (Figure ivB), suggesting a potential functional role of lipid rafts in cell adhesion processes. Accordingly, knock-down of lipid raft-associated protein caveolin-1 in ECs was shown to inhibit transendothelial leukocyte migration[347]. Recent studies using non-CNS derived endothelium and epithelial cultures have identified novel molecules that coordinate leukocyte transmigration, including JAMs, CD44, CD47, CD73[348], CD90[349], CD137[350], CD81[351] and CD166/ALCAM[23]. Most of these adhesion molecules are enriched in lipid rafts derived from primary cultures of human BBB-ECs[23]. PECAM-1 is also known to act on leukocyte migration, but has rather been documented to halt leukocyte emigration, as PECAM-1 -/- animals have enhanced immune cell infiltration into the CNS[352]. ICAM-1 and PECAM-1 are enriched in lipid membrane rafts and signal through lipid raft platforms[231,333,353].

During the final phase of transmigration, complex intracellular signaling events take place in both the leukocyte and the EC to prepare the cells for

transmigration/diapedesis. These events are dependent on adhesion molecule signaling and include cytoskeleton remodeling, TJ and AJ disassembly and reassembly and cell body retraction. Recent data from Greenwood and colleagues support the notion that VE-cadherin re-localization in BBB-ECs during diapedesis is dependent on ICAM-1 signaling events mediated by membrane raft signaling platforms. ICAM-1 signaling lead to VE-cadherin phosphorylation and allow paracellular leukocyte migration[333]. As recently described, a key feature of transendothelial migration (also called emperiopolesis) is the formation of an EC cup-like structure surrounding the migrating leukocytes[218,219,354-357]. Endothelial membranes positive for caveolin-1 are associated with the transmigrating leukocyte in the transmigration cup, which is dependent on the presence of lipid rafts and their recruitment of CAMs[347]. In this context, lipid rafts are believed to coordinate outside-in signaling in ECs and regulation of actin remodeling by lipid raft associated small GTPases, such as Rho[40,358,359] and Rac[360,361], myosin dependent contractions[362] and junctional protein regulation[43,363]. These transendothelial migration events are highly organized around CAM-mediated signaling within lipid membrane raft structures and support the concept of functionally distinct adhesion-specific endothelial membrane rafts.

1.3 Proteomic analysis of BBB-EC lipid membrane rafts reveal proteins typically associated with the nervous system

Lipid membrane raft isolated from human BBB-ECs and LC-MSpect identified proteins in BBB-related functions, such as junctional, adhesion and transporter molecules. Proteins involved in intracellular trafficking, cytoskeleton remodeling and signal transduction were also found. In addition, further analysis of specialized cellular functions revealed candidate molecules usually involved in nervous system functions, notably in synaptic transmission, axon guidance, morphogenesis, and planar cell polarity (Figure v). A recent report described similar findings[287]. Purified epithelial cell TJ complexes were subjected to MSpect and fingerprint analyses, and bioinformatic results identified unexpected clusters of synaptic and signaling proteins. For example, ion

channels and neurotransmitter transporters P/Q-type calcium channel subunit CPα1A, voltage-gated potassium channel Kv2.1, NMDA glutamate receptor zeta subunit, AMPA glutamate receptor subunit GluR and scaffold proteins including presynaptic piccolo as well as postsynaptic GRIP and Homer were found in the TJ microdomain. To make sense of the results, the author describes noteworthy functional and structural features shared between the TJ and the neuronal synapse[287]. As for neuronal and synapse associated proteins may function in association with the TJ proteins, additional functions are plausible. Due to no known studies on the possible use of the Hh pathway in BBB functions, this subject was selected to be studied.

2.0 THE Hh SIGNALING PATHWAY

2.1 The Hh signaling pathway in lipid membrane rafts

Although I have not extensively studied the importance of the Hh pathway in BBB-EC membrane rafts per se, lipid rafts are essential in maintaining a functioning Hh cascade. Lipid, cholesterol and sterols have been linked to the Hh signaling pathway since it was first shown that the Hh morphogen is covalently attached to lipid moieties[364]. In fact, diseases caused by defects in the final stages of cholesterol synthesis, such as Smith-Lemli-Opitz syndrome, desmosterolosis, and lathosteolosis, have been ascribed to defective Hh signaling[365]. Furthermore, embryonically disrupted cholesterol metabolism can lead to aberrant neural phenotypes which strikingly resemble Hh-deficient malformations, cyclopia and holoprosencephaly, demonstrating the close connection between cholesterol and Hh signaling[168].

Cholesterol prefers to associate with sphingolipids rather than phospholipids, due in part to the higher degree of saturation of the fatty acids in sphingolipids, which allows for a more favorable packing of the planar rings of cholesterol. Since Hh ligands are covalently linked to cholesterol, Hh should be able to associate with sphingolipids, and to, in this manner, confer affinity for lipid membrane rafts. Numerous reports have additionally identified Hh components in membrane lipid rafts. In Drosophila, Triton-insoluble DRMs isolated from a density gradient have identified the Hh ligand, along with GPI-anchored proteins, to be specifically associated with DRMs[366]. This suggests that the sterol linkage may be an efficient raft-targeting signal. Indeed, Hh that is not modified by cholesterol is capable of signaling, but addition of a cholesterol moiety increases dramatically its potency in signal transduction[367], raising the possibility that the efficiency of Hh signaling may depend on its localization to membrane lipid rafts. Similarly, in a rat hepatic stellate cell line, Shh interacts with caveolin-1 to form a protein complex that associates with lipid membrane rafts[368]. In adult rat brains, Shh was also found to partition with the raft component ganglioside GM-1 during Triton X-100 solubilization and sucrose density gradient centrifugation. Disruption of Shh association with the plasma membrane leads to a reduction in Shh secretion[369,370], further suggesting that lipid raft microdomains are important in Shh signaling.

Due to the high affinity of the Hh ligand to the membrane component, mature Hh proteins can remain tethered to the cell surface of the producing cell. Association of Hh with DRMs has been proposed important for Hh packing into high order aggregates[370-372]. Release of the soluble Hh is an active and controlled process which requires the action of Dispatched, a protein containing sterol-sensing domain[365]. In fact, Dispatched was recognized to package Hh ligands into different soluble forms such as micellar multimers or lipoprotein particles[371]. While Hh multimers consists of a multimerization of the Hh protein via its lipophilic tails, Hh ligands can also get solubilized and integrated into lipoproteins particles[371]. In cell cultures, mammalian Hh was indeed found to from highly diffusive, biologically active aggregates of a high molecular weight[373]. Lipoproteins are assemblies of lipids and proteins and high and low density lipoproteins (HDL and LDL, respectively) allow intercellular transport of water-soluble lipids, cholesterol and signaling metabolites throughout aqueous circulation[365,372]. Although no Hh ligands have yet been identified in HDLs, Hh has been found to associate with LDL particles[372].

Interestingly, HDLs have anti-inflammatory properties maintain EC integrity and reduces the expression of adhesion molecules in ECs, inhibiting blood cell adhesion to vascular endothelium[374,375]. Vitamin B3 and Niaspan treatments raise levels of protective HDL and are proposed to prevent demyelinating diseases and promote health of myelin sheaths[269,376]. In particular, Niaspan has been found to increase Shh levels[269], suggesting that the anti-inflammatory properties of HDL could plausibly be due to the transport of Shh in these HDL, thus increasing the signaling abilities of Shh.

Membrane lipid rafts can also include other components of the Hh signal transduction pathway. Many receptors and G-proteins coupled receptors are found to be concentrated in lipid rafts in order to facilitate protein-protein interactions and signaling events. Similarly to our own DRM isolation and proteomic analysis, which identified the presence of Hip in lipid membrane raft microdomains of ECs of the BBB, Hip was found in raft-like microdomains of Human embryonic kidney (HEK) cells[369]. Caveolin-1 may be integral for sequestering the Hh receptor complex in these lipid-enriched microdomains, which act as a scaffold for the interactions with the Hh protein. Both Smo and Patched-1 are also found in caveolin-1/cholesterol-rich membrane microdomains[377]. While ptch-1 associates and interacts directly with caveolin-1, as the two proteins

coprecipitate and colococalize, Smo does not[377]. However, lipid raft microdomains appear to play a role in assembling Hh receptors on the target cell. Cyclopamine, a sterol-related protein, is capable of exogenously modifying Smo activity, possibly through its targeting to Hh-receptor enriched lipid membrane rafts. Recent finding have suggested that Ptch-1 endogenously regulates the secretion of small lipophilic molecules, such as vitamin D3 or other oxysterols, in order to modulate Smo activity, a process that could easily occur in lipid raft signaling platforms[159,161].

Although our candidate list of proteins found in lipid rafts of BBB-ECs did not contain proteins of the Wnt signaling pathway, it is not impossible that these signaling platforms also act in regards to other morphogens important in BBB function. Comparable to Hh ligands, Wnt proteins are doubly N-glycosylated and bear palmitate and palmitoylate groups[372,378]. The physical properties of these lipid anchors can likely promote ligand localization to particular membrane rafts. *In vivo*, both Wnt and Hh ligands bind to the cell surface and can associate and partition with DRMs, suggesting their presence in membrane lipid rafts[366].

2.1 The Hh signaling pathway in the BBB

In Part II, I demonstrate that astrocyte-derived Shh acts on human BBB-ECs to decrease permeability of the BBB to soluble factors *in vitro* and *in vivo*, a process that is Smo-dependent. This effect is driven by the ability of Shh to induce and maintain TJ protein expression. I further demonstrate that Shh regulates BBB-EC activation by lowering their expression of surface CAMs and secretion of pro-inflammatory chemokines. Consequently, a decrease in transmigrating lymphocytes across the BBB-EC monolayer is observed, suggesting that Shh acts as a direct negative regulator of CNS inflammation. Downstream transcription factors Gli-1 and SOX-18 are upregulated upon Hh activation, implying a mechanism of action. In inflammatory contexts, an increase in Shh was observed when human astrocytes were cultured in the presence of inflammatory cytokines TNF-α and IFN-γ. Surface expression of both Ptch-1 and Smo were also increased in BBB-ECs treated with the same cytokines. However, in MS plaques, while

hypertrophic astrocytes upregulated Shh expression, ECs were not found to correspondingly increase the expression of the receptors, Ptch-1 and Smo.

The anatomical proximity of the astrocytic endfeet to the CNS-ECs has lead to the notion that astrocytes impact on BBB properties. Astrocyte-derived soluble factors have been shown essential for the development and maintenance of an impermeable BBB as ACM induces junction formation and strong transendothelial electrical resistance in either CNS or non-CNS-derived ECs, *in vitro*[24,312,313]. Other morphogens and neural cues have previously been linked to cooperate in astrocyte-endothelial interactions. Proteins of the FGF family were found to decrease the permeability of the BBB *in vitro*[103]. Similarly, conditioned media containing Wnt3a induces TJ strand formation, junctional protein expression and BBB-EC morphology[142]. Although human and mouse astrocytes have previously been shown to express Shh[258,265], its impact on BBB-ECs has not yet been studied.

Shh has been involved in a multitude of developmental processes, ranging from embryonic tissue patterning, ventralization of neural tube, and establishment of posterior identity in the developing limb bud [152,314]. Others have found that Shh can induce capillary morphogenesis[182], a process which requires promoting assembly via cell-cell contact, a process not distant from barrier morphogenesis, where this cell-cell contact is further enforced by junctional proteins. Therefore, as a morphogen, Shh dictates positional information to individual or groups of cells and how these should assemble to form a functional tissue or unit. It is perhaps not striking that Shh can impact on CNS-EC to guide them to behave and function as barrier cells. Evidence that Shh may be involved in maturation of endothelium can be seen in developing mouse models. In the developing mouse submandibular gland, lumen formation and TJ assembly occurs in the epithelial cell lining, processes dependent on Shh. Shh null mice had developmental arrested submandibular gland epithelium, and treatment with Shh recovered and enhanced epithelial lumen formation, cell polarization as well as ZO-1, claudin-3 and occludin TJ protein distribution[205]. As a mature vascular bed, ECs do not behave independently of one another; rather they form a monolayer that prevents movement of protein, fluid and cells from the endothelial lumen into the surrounding tissue. This can be seen upon Hh activation in the PNS where Dhh, expressed in myelinating Schwann cell, is needed for

the formation and maintenance of the perineurium, a component of the blood-nerve barrier. Dhh -/- animals have a less compact perineurium, resulting in abnormal and interrupted TJ strands. Consequently, a defective nerve-tissue barrier is formed and extravasation of Evans Blue dye and immune cells invasion into the endoneurium occurs[206]. Therefore, my data further demonstrates the potential of the Hh pathway in the formation of a mature, ordered and functionally competent barrier. I demonstrate that astrocyte-derived Shh decreases permeability of soluble tracers in the modified Boyden chamber assay, an *in vitro* model of the BBB (Figure 2A). Cyclopamine-injected mice endure acute BBB disruption as FITC-Dextran and endogenous serum proteins enter the brain parenchyma (Figure 2D-E). Indeed, Hh activation is essential for TJ expression, as cyclopamine induces a downregulation of junctional proteins in BBB-EC (Figure 3B). Shh -/- mice exhibited a significantly lower expression of claudin-5, occludin and Z0-1 on BBB-ECs *in vivo* (Figure 3C-D), confirming the validity of the *in vitro* observation.

The activation of the Hh pathway involves activation of the downstream transcription factor Gli-1 which act as an activator to mediate and/or amplify the Hh signal as it is transcriptionally induced by Hh signaling in nearly all contexts examined[315]. My data using flow cytometry demonstrates that Hh activation in BBB-ECs induces an intracellular upregulation of Gli-1, which is cyclopamine-sensitive (Figure 5A). However, further mechanisms in promoting BBB properties were sought. SOX-18 was found to be an endothelial specific transcription factor as *in silico* analysis of the claudin-5 promoter revealed an evolutionary conserved SOX consensus binding site. In HUVECs, SOX-18 overexpression increases claudin-5 mRNA and protein expression while SOX-18 silencing reduces claudin-5 transcription and transendothelial resistance[310]. My results suggest that Gli-1 activation proceeds SOX-18 upregulation (Figure 5C). Whether or not Gli-1 directly upregulates SOX-18 is still not known but in different contexts, the Hh pathway has already been seen to regulate other members of the SOX family. Shh was seen to induce changes in families of transcription factors such as SOX, further altering the expression of downstream genes[316]. Expression of SOX-14 in spinal cords was found to be regulated by Shh in a dose-dependent manner[317]. In neuroepithelial cells Gli-2 binds to an enhancer region vital for SOX-2 expression necessary for NSC proliferation and differentiation[318].

In addition to Gli-1 activation, other non-canonical Hh signaling pathways have been described in migrating cells. Although Gli-1 activation was studied in BBB formation, other pathways may be involved. In fibroblast cell lines, Shh signals via Smo to induce alterations in cell morphology, cytoskeleton rearrangement and lamellipodia formation, a process which is transcription/translational-independent. However, rather than Gli-1 activation, Shh-induced migration employed the metabolism of arachidonic acid through the 5-lipoxygensase pathway[192]. In axon guiding, Shh stimulates the activity of Src family kinases members, also in a Smo-dependent manner[379]. Furthermore, additional receptors at the surface of the target cell are known to transduce Hh signals. Boc and Cdo are coreceptors and positively transduce the Hh signal via Gli-1 or Src family kinases and can regulate membrane proteins and cytoskeleton remodeling[162,379]. Coupling of these coreceptors with Ptch-1 may also integrate the Hh pathway with other signaling cascades as extracellular domains of Cdo and Boc mediate their heterodimerization and are able to associate with cadherins and netrins[380]. Although Boc and Cdon have yet to be described in ECs, these receptors could provide an elegant method for Hh to execute morphological changes and transcriptional programs, in addition to integrating responses with other signaling cascades[162].

2.2 The Hh signaling pathway in MS

The BBB is an important entity in keeping the CNS an immunologically privileged site, a region where immune cells access is infrequent, as opposed to most organs[319]. Astrocytes are key regulators of BBB functions and have been shown to secrete a variety of different factors that help promote and maintain BBB properties[11,24,312,313]. However, less often are the factors shown to regulate the immune-quiescence properties of BBB-ECs. Although extensively studied in embryogenesis, Shh has been shown to be expressed in adult tissues and to be involved in tissue morphogenesis and repair after injury[154,321,381,382]. I present evidence suggesting a novel and unique role of Shh in the regulation of CNS immunity. During CNS inflammation such as MS, its animal model EAE, and following various cerebral insults, surface CAMs involved in immune cell diapedesis, ICAM-1 and VCAM-1, are dramatically upregulated on BBB-ECs[217,325]. Similarly, *in vitro* treatment of BBB-ECs with pro-inflammatory

cytokines such as TNF-α and IFN-γ have also been shown to strongly enhance endothelial CAM expression[23]. The anti-inflammatory cytokines IFN-β as well as glucocorticoids, lead to a significant reduction in CAM expression, leading to a subsequent reduction in leukocyte migration[241,383-385]. My results show that BBB-ECs grown with ACM or treated with rhShh or a Smo agonist, purmorphamine, all activate the Hh pathway and lessen the expression of ICAM-1 and VCAM-1 (Figure 4A-B), demonstrating anti-inflammatory potential.

BBB-ECs are also potent local producers of chemokines that can influence the recruitment, activation and migration of immune cells into CNS tissue[224,225,320]. During MS, various chemokines and chemokine receptors have received special attention as key regulators of cellular infiltration. MCP-1/CCL2 and IP-10/CXCL10 have been detected within active MS plaques, while MCP-1/CCL2 and IL-8/CXCL8 have been found in the CSF of MS patients[386-388]. MCP-1 is a C-C chemokine that has preferential affinity for the chemokine receptor CCR1, 2 and 4 and has been shown to increase transendothelial migration of $CD4^+$ lymphocytes in chemotaxis assays[389]. In fact, blocking MCP-1 with an anti-MCP-1 antibody reduces the severity of EAE and significantly inhibits by 60% the rate of migration of MS lymphocytes across BBB-EC monolayers[320]. I chose to focus our attention on the chemokines MCP-1/CCL2 and IL-8/CXCL8 for they were found to be important chemokines produced by BBB-ECs grown under culture conditions [238,320]. BBB-ECs treated with hrShh or with purmorphamine significantly reduce the secretion of both MCP-1/CCL2 and IL-8/CXCL8 (Figure 4C), further demonstrating another mode of anti-inflammatory action. The fact that both adhesion molecules expression and chemokine secretion decreased tends to signify that BBB-ECs are less effective in recruiting lymphocytes to the site of inflammation, an act that was indeed observed *in vitro*. BBB-ECs are also known to be able to produce anti-inflammatory cytokines themselves, such as IFN-β and TGF-β[390], and it would be of interest to determine if activation of the Hh pathway could in turn upregulate the expression of additional potent anti-inflammatory signals. Interestingly, TGF-β can induce activation of Gli-1 and Gli-2 in various cancer cell lines, independent of Hh receptor activation[391], suggesting a possible anti-inflammatory feedback loop. Therefore, the next logical step in exploring the anti-inflammatory effect of the Hh pathway in BBB-ECs would be to assess whether

Hh activation could prevent or lessen the expression of adhesion molecules and chemokine secretion produced by an inflammatory insult.

In MS, BBB breach concurs alongside with EC activation and lymphocyte infiltration. Since the Hh pathway seems capable of promoting barrier properties as well as reducing EC activation and lymphocyte recruitment, I observed the effect of inflammation on Hh components. I showed that human astrocytes exposed to TNF-α and IFN-γ upregulate Shh production (6A). This was paralleled *in situ* by the increase in Shh immunoreactivity around blood-vessels in active MS lesions, when compared to NAWM of the same patient (Figure 7A-C). As Shh has often been observed to be upregulated in various cells after acute injury and wound healing, implicating an important role in cellular and tissue repair[154,256], I postulate that inflammatory cytokines activate Shh production in astrocytes in order to possibly aid in repairing BBB damage. Shh thus may serve a protective function in acute injury, such as in the liver[322], endothelium[382] and spinal cord injury[381]. Indeed, a stable form of Shh was injected to treat nerve injury[321]. In addition, due to its potential anti-inflammatory activity, Shh upregulation could also be beneficial in regulating and containing the inflammation.

In contrast, while BBB-ECs treated with TNF-α and IFN-γ upregulated both Ptch-1 and Smo on their surface (Figure 6B), the same could not be seen *in vivo*, in active MS lesions (Figure 8A-B). This demonstrates a potential dysregulation of the pathway at the level of the BBB, and provides a plausible explanation to BBB compromise. Further investigation of MS material, as well as examination of other CNS inflammatory and non-inflammatory diseases, is necessary to fully comprehend the expression pattern. Recent studies have observed that Gli-1 was upregulated in active MS lesions, but was significantly downregulated in chronic active and inactive lesions[265], suggesting that long term inflammation impairs Gli-1 signal in chronic inflammatory conditions.

Other studies have, in fact, observed Shh upregulation after inflammation. IFN-γ expression linked to the GFAP promoter in perinatal CNS greatly upregulates Shh expression in the developing cerebellum[392,392,393]. In chronic gastrointestinal inflammation, Shh is also upregulated in epithelium of areas of inflammation such as in gastritis, Crohn's disease and ulcerative colitis, an effect that was linked to the ability of injury-related cytokines to induce Hh production[322]. Ptch-1 mRNA and protein

expression were absent in inflamed colonic epithelium, similarly to MS endothelium[323]. In a hypothetical model, it was suggested that injured epithelium, via the release of Shh, may communicate damage to both neighboring cells and to the immune system such as $CD4^+$ T lymphocytes and macrophages that express Ptch-1 and thus capable of processing a Shh signal, a process that could further aid in repairing damaged epithelium [322-324].

In a spontaneous acute EAE mouse model, vitamin B12 together with IFN-β treatment upregulated Shh and reduced immune cell infiltration and general EAE scores, compared to single treatments[268]. The authors explained the beneficial effect of this combinational therapy as the ability of Shh to enhance oligodendrocyte maturation and thus, promoting oligodendrocyte remyelination[268]. However, a reduction of clinical score is a rapid process that involves immune and/or barrier modulation. Thus, the upregulation of Shh could lead to immunoregulation and barrier stabilization effects which would explain the decrease in infiltration and EAE scores seen. In fact, in a chronic EAE model, an identical combination therapy was capable of reducing the frequency of multiorgan inflammatory lesions (heart, liver, lungs and salivary glands - all organs which express Shh), suggesting a general immunomodulatory effect, possibly via Shh[268].

Shh has been classified as a classical stem molecule[394] and embryonic stem cells are known to promote immunoprivilege sites[395,396], possibly implying a role of Shh in immunomodulation and decreasing immune reactions. However, little is known on the relationship between the immune system and the Hh signaling pathway. T-cell development is regulated, in part, by Shh, and studies have shown the importance of the Hh pathway in maintaining immature immune cell populations[163,397]. In fact, Shh influences very early lymphocyte development as Shh -/- thymi at E13.5 were smaller than that of littermates, but contained a reduced amount of cells of haematopoietic origin[398], suggesting that Shh is necessary for their development prior or during entry of thymocyte progenitors to the thymus. Later in development, Shh protects germinal-centre B cells from apoptosis[399]. While addition of rhShh to $CD4^+$ T cell populations *in vitro* has shown to enhance induction of T cell activation and proliferation[400], other studies have seen that constitutive Gli-2 activity in T cells inhibited T cell activation and proliferation[401].

Although studies have examined the expression of Hh components in EAE, the effects of modulating pharmacologically the Hh pathway, with use of Smo agonists and antagonists, on the disease evolution have not yet been verified. Keeping in line with these current results, I expect that Hh activation would prove to have beneficial and neuroprotective effects by stabilizing the BBB. PTX is widely used for the induction of EAE by increasing BBB permeability[246,247]. While the exact mechanism of action is unknown, PTX blocks the interaction of G proteins with their G protein coupled receptors. Smo, the signal transducer of the Hh pathway is a protein related to a G protein coupled receptor[158]. PTX has been shown to affect and inhibit Smo. Thus, it would be of interest to investigate if cyclopamine can efficiently replace PTX to induce BBB permeability. The Hh pathway acts also in reducing inflammation and activation statuses of the BBB and potentially of the immune cells. Cyclopamine could thus aggrevate the disease, while purmorphamine could prevent or delay disease susceptibility. Both MOG-induced and PLP-induced EAE could be performed to evaluate the acute and chronic models, respectively. Neuropathological studies on the CNS of such mice should be performed to evaluate the extent of demyelination, immune cell infiltration, as well as BBB dysfunction (serum protein extravasation, junctional proteins expression and EC death/apoptosis).

Identifying factors that are required for BBB integrity is necessary in our understanding of neuroinflammation and specifically MS. These experiments allowed us to determine that the Hh pathway is an important astrocytic contributor to BBB maintenance and function. These data also suggest that Shh acts as a direct negative regulator of CNS inflammation through its regulatory effect on BBB-EC activation. This work will help in the development of strategies to regulate the migration of immune cells, the delivery of drugs across the BBB and to design new therapies in the treatment of neuroinflammatory diseases such as MS.

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