

The Hedgehog Pathway Promotes Blood-Brain Barrier Integrity and CNS Immune Quiescence

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The blood-brain barrier (BBB) is composed of tightly bound endothelial cells (ECs) and perivascular astrocytes that regulate central nervous system (CNS) homeostasis. Here we show that astrocytes secrete Sonic hedgehog (Shh) and that BBB-ECs express Hh receptors, which together promote BBB formation and integrity during embryonic development and adulthood. Using pharmacological inhibition and genetic inactivation of the Hh signaling pathway in ECs, we also demonstrate a critical role of the Hh pathway in promoting immune quiescence of BBB-ECs by decreasing the expression of proinflammatory mediators and the adhesion and migration of leukocytes, in vivo and in vitro. Overall, the Hh pathway provides a barrier-promoting effect and an endogenous anti-inflammatory balance to CNS-directed immune attacks, as occurs in multiple sclerosis.

The blood-brain barrier (BBB) confers homeostasis to the central nervous system (CNS) and limits entry of blood-borne molecules and circulating leukocytes. The BBB is composed of specialized endothelial cells (ECs) held together by multiprotein complexes known as junctional proteins (1, 2). Astrocytes, which are closely apposed to the CNS vasculature, help maintain BBB integrity and immune quiescence through contact dependent mechanisms and by releasing soluble factors (2-5). BBB disruption is a central and early feature of multiple sclerosis (MS) that allows leukocytes to enter the CNS leading to demyelination and neuronal damage (6-8).

The Hedgehog (Hh) pathway is involved in embryonic morphogenesis, neuronal guidance and angiogenesis (9, 10). In adult tissues, it participates in vascular proliferation, differentiation and tissue repair (11-13). CNS morphogenic

events are primarily associated with Sonic Hh (Shh) signaling (14-16). Secreted Shh binds and inactivates the receptor Patched (Ptch)-1 allowing activation of Smoothed (Smo), which then induces target genes through the Gli family of transcription factors (17). Previous studies have implicated Shh signaling in MS and its animal model (18, 19). We therefore explored whether the Hh pathway contributes to the maintenance of BBB functions, including its immune quiescence.

mRNA and protein analyses demonstrated expression of Shh and its 45 kDa uncleaved precursor in human fetal astrocytes (HFAs), but not on primary cultures of BBB-ECs (fig. S1, A and B). In contrast, the highest levels of Ptch-1 and Smo expression were observed in BBB-ECs (fig. S1, A and B). Essential to Hh signaling is the autocatalytic cleavage of the 45 kDa Shh protein to yield a ~19 kDa active form, which was present in astrocyte-conditioned media (ACM) (fig. S1B). We detected Shh in human and mouse astrocytes *in vitro* and *in situ* but not in ECs or pericytes (fig. S1, C to H). Conversely, Ptch-1 and Smo were detected on cultured BBB-ECs and *in situ* in human and mouse CNS ECs, but not on astrocytes or pericytes (fig. S1, C to H). Therefore, our data suggest that the Hh pathway is used by perivascular astrocytes to communicate with BBB-ECs.

To determine whether astrocyte-secreted Shh influences BBB function, the transendothelial electrical resistance (TEER) and permeability of human BBB-ECs were evaluated under conditions stimulating or abrogating the Hh pathway. We found that human recombinant Shh (hrShh) significantly increased TEER and decreased Dextran 3kDa clearance, as well as the permeability to ¹⁴C-Sucrose and FITC-bovine serum albumin (Fig. 1, A to D). This effect was comparable to the one induced by ACM and the response of BBB-ECs to

Shh did not affect EC proliferation (fig. S2A). Activation of the Hh pathway was responsible for the barrier promoting effect of ACM as BBB-ECs treated with the Smo agonist Purmorphamine reproduced the effect of hrShh whereas Smo antagonists Cyclopamine (Fig. 1, A to D) and SANT-1 (fig. S2B) reversed the effect of ACM. These data reflect a combination of changes in TEER, paracellular permeability and inducible transport mechanisms, suggesting that Hh ligands could affect both processes, possibly through regulation of junctional proteins.

Hh activation is associated with a restrictive permeability, therefore we determined the expression of the transcription factors Gli-1 and Sex-determining region Y-box (SOX)-18, an important regulator of junctional protein expression (i.e. claudin-5) and barrier formation (20). The number of BBB-ECs expressing Gli-1 increased upon Hh activation, as compared to untreated cells. However, Cyclopamine reduced the expression of Gli-1 induced by ACM (Fig. 1, E and F). hrShh stimulation and ACM significantly upregulated Hip, Gli-1 and SOX-18 transcription (Fig. 1G and fig. S2C). SOX-18 expression was maximal 2 hours after Gli-1 activation, confirming that SOX-18 is regulated by the Hh pathway, possibly through Gli-1.

To further confirm the role of the Hh pathway in maintaining BBB properties *in vivo*, we injected mice with Cyclopamine and studied the extent of BBB leakage. Cyclopamine induced acute BBB disruption (6h post-injection), as demonstrated by the increased extravasation of exogenous Dextran and endogenous fibrinogen (Fig. 1, H and I). Furthermore, Cyclopamine treatment also induced extravasation of perivascular blood-derived leukocytes (Fig. 1H and fig. S2D), without inducing hemorrhage. Thus, the Hh pathway is important in the maintenance of adult BBB function and disruption of Smo-dependent signaling leads to acute BBB breakdown.

Because an active Hh pathway promotes BBB properties, we addressed whether expression of junctional proteins in human BBB-ECs is dependent on the astrocyte-derived Shh signal. mRNA encoding occludin and claudin-5 were upregulated in response to Hh signaling (Fig. 2A). Protein analyses from BBB-ECs treated with ACM revealed a moderate increase in the expression of occludin, junctional adhesion molecule (JAM)-A, VE-cadherin, claudin-3 and claudin-5 (Fig. 2B); while Cyclopamine and SANT-1 decreased the expression of these proteins (Fig. 2B and fig. S2E). The effect of Shh on junctional protein expression was also studied in Shh-deficient mice (*Shh*^{-/-}). These animals exhibit embryonic lethality and embryos were harvested between E11 and E13.5 (14), at the time of gradual BBB development (21). Junctional strands were visible in brain capillaries from E13.5 WT mice (fig. S3A); however, the expression of junctional proteins per vessel was significantly

lower in *Shh*^{-/-} mice (fig. S3,A and B) and did not affect the total number of blood vessels in the developing brain (fig. S3C).

To specifically assess the role of the Hh pathway in ECs, we selectively deleted a conditional allele of the signal transducer Smo from ECs using a Tie2-Cre driver (Tie2-Cre; Smo^{ec/c}) (fig. S4,A to D). Deletion of *Smo* in ECs was associated with a significant increase in BBB permeability to the endogenous serum proteins fibrinogen, immunoglobulins and apolipoprotein B at E14 and P19 (Fig. 2C and figs. S4E and S5A) and to the exogenous tracers in 8 wk old adult mice (figs. S5, B to D, and S6A). The plasma protein leakage into the CNS correlated with a significant decrease in the expression of occludin, claudin-3, claudin-5, ZO1 and p120 (Fig. 2C and figs. S4E and S6B) and with a compromised basement membrane as laminin fragmentation and lower laminin expression were detected (figs. S5A and S6A) in the absence of hemorrhage (fig S6, C and D). In WT developing brains, the lack of serum protein extravasation in parenchymal vessels confirmed the BBB restrictiveness previously reported during embryogenesis (22, 23). Moreover, astrocyte association with the BBB was considerably reduced at P19 (Fig. 2C), and resulted in perivascular gliosis in adult mice (fig. S5D). Thus, the genetic neutralization of the Hh pathway in CNS endothelium resulted in lower expression of junctional proteins and accumulation of plasma proteins in the CNS, hallmarks of a perturbed and compromised BBB.

Under normal conditions, the restrictiveness of the CNS to immune responses is in part attributable to the BBB. To determine the role of Shh in this process, we studied its effect on the expression of chemoattractants and cell adhesion molecules (CAMs). Hh activation in human BBB-ECs drastically decreased the secretion of the chemokines CXCL8/IL-8 and CCL2/MCP-1, whereas Cyclopamine abrogated this effect (Fig. 3A). Under basal conditions, primary cultures of BBB-ECs expressed ICAM-1 (Fig. 3B). Stimulating Hh signaling significantly reduced the percentage and levels of ICAM-1 expression in these cells (Fig. 3B and fig. S7A), while Cyclopamine reverted this effect (Fig. 3B). In contrast, neither Shh nor ACM affected the expression of α 4 β 1 integrin ligands VCAM-1 or CS-1 (fig. S7, B and C). We next assessed CD4⁺ T cell adhesion to, and migration across Hh-activated BBB-ECs. Adhesion of unstimulated (*ex vivo*) human CD4⁺ T cells was significantly reduced after 1h and 2h in Hh activated BBB-ECs (Fig 3C and fig. S7D, respectively) and was increased by 45% with Cyclopamine treatment (Fig 3C). Likewise, ACM, hrShh and Purmorphamine significantly reduced CD4⁺ T cell transmigration across BBB-ECs grown in Hh stimulating conditions (Fig. 3D) without affecting T cell proliferation (fig. S7E).

To further characterize the role of the Hh pathway in inducing CNS immune quiescence, we evaluated the effect of Hh activation on T helper (Th) 1 and Th17 cells, which are known to participate in neuroinflammatory processes such as MS (24, 25). *Ex vivo* monocytes and T cells can respond to Shh as they express Ptch-1, Smo and Gli-1 (fig. S7F). Human CD4⁺ T cells polarized into Th1 and Th17 lymphocytes (fig. S8A) (24) adhered significantly less to BBB-ECs when polarized in the presence of Shh (Fig. 3E), whereas adhesion of Th2 cells was significantly enhanced. To elucidate the effect of Shh on the adhesion capacity of Th cells, we studied the expression of molecules known to participate in leukocyte migration through the BBB in MS (7, 26-28). Shh decreased the expression of CD6 on Th17 cells, increased expression of CD6 and VLA-4 on Th2 and reduced IFN- γ production by Th1 cells (fig. S8, B to E). In contrast, the levels of LFA-1, VLA-4 and IL-17 remained similar to the control in Th17 cells, while expression of CD6, LFA-1 and VLA-4 were unaffected in Th1 cells (fig. S8F). Downregulation of CD6 and IFN- γ was confirmed in Th1 and Th17 cells expanded in the presence of ACM and treated with an anti-Shh functional blocking antibody (fig. S8, G and H).

Given the immune-modulating effect seen on BBB components in Hh activating conditions, we assessed the influence of Hh pathway blockade in experimental autoimmune encephalomyelitis (EAE), the animal model of MS, by repeated injections of the Hh pathway antagonist GDC-0449. Hh antagonism significantly increased disease severity (Fig. 3F and fig. S9A), the extent of demyelination (Fig. 3G), the amount of leukocytes accumulating in the CNS (Fig. 3H), and the production of IFN- γ and IL-17 in T cells infiltrating the CNS (Fig. 3I). The magnitude of T cell activation in the GDC-0449 treated animals was unique to the CNS compartment as the degree of T cell activation in the periphery did not differ between both groups (fig. S9, B and C). Collectively, these data support an endogenous immune quiescent role of the Hh pathway at the level of the BBB by protecting the CNS against entry of pro-inflammatory lymphocytes.

We next evaluated the effect of an inflammatory environment on the expression of Hh components in the cellular elements of the BBB. HFAs inflamed with tumor necrosis factor (TNF) and IFN- γ increased Shh expression as compared to control (Fig. 4A), while BBB-ECs grown in ACM and treated with TNF and IFN- γ increased their expression of Ptch-1 and Smo (Fig. 4B). To further support these findings, the expression of Shh, Ptch-1 and Smo was also determined in the human CNS. In control brain and in normal-appearing white matter (NAWM) of MS patients, astrocyte endfeet surrounding parenchymal vessels displayed Shh immunoreactivity and BBB-ECs expressed Smo and Ptch-1 (Fig. 4, C and D, and fig. S9D). Shh

immunoreactivity, however, was strikingly enhanced in hypertrophic astrocytes throughout active demyelinating MS lesions (Fig. 4, C and D). In NAWM, Ptch-1 expression was mainly restricted to ECs surrounded by astrocytic endfeet (Fig. 4D), whereas in active MS lesions, Ptch-1 expression increased on ECs and was also detected on infiltrating leukocytes (Fig. 4D). Smo expression in NAWM was predominant in the vasculature and although Smo was detected in macrophages and brain ECs in active lesions (fig. S9D), no significant upregulation was observed. Finally, Gli-1 was found in the cytoplasm of ECs in NAWM, while in active MS lesions Gli-1 was detected in the nucleus of both ECs and infiltrating leukocytes (Fig. 4D). These data suggest that upon inflammatory stimulation, astrocyte-secreted Shh induced expression of Hh receptors in BBB-ECs, which led to the upregulation and translocation of the Hh transcription factor Gli-1 into the nucleus of BBB-ECs.

Astrocyte- and pericyte derived soluble factors, such as those inducing Wnt signaling (29, 30), are essential for the development and maintenance of an impermeable BBB *in vitro* (5, 31, 32). Astrocytes express Shh (18, 19) but until now its function, in the context of BBB integrity and CNS immunity, remained unexplored. The Hh pathway is involved in epithelial barrier formation in the submandibular gland, where lumen formation and junctional protein assembly is a Shh-dependent process (33). Our findings also correlate with this essential role of Shh, as Hh neutralization reduced the expression of junctional proteins in primary cultures of human BBB-ECs and in the CNS of Shh^{-/-} embryos and Tie2-Cre; Smo^{ec} mice. In addition, the Shh-driven reduction in chemokine and CAM expression by BBB-ECs, in the binding capacity of proinflammatory T cells and in the expression of pathogenic immune mediators demonstrate that Shh acts as an endogenous anti-inflammatory effector of the neurovascular unit. Since Shh is upregulated in acute brain and nerve injury (34, 35) and as shown here in neuroinflammation, we postulate that inflammation activates Shh production in astrocytes in order to promote BBB repair and counter-balance inflammatory events induced during lesion formation and thus restores physiological and immunological BBB competence.

Overall, our data provide compelling evidence suggesting a dual protective role for the Hh pathway at the level of the BBB, by promoting barrier formation and by acting as an endogenous anti-inflammatory system. In addition, these novel findings open new avenues in the design of therapeutics to control leukocyte migration into the CNS or conversely, to improve delivery of therapeutic agents in the CNS compartment.

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- Supporting Online Material**
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- Fig 1.** Shh decreases BBB-EC permeability via Smo engagement and induction of Gli-1 and SOX-18. Changes in TEER (A), clearance (B) and permeability to ¹⁴C Sucrose (C) and BSA-FITC (D) of human BBB-EC monolayers in conditions stimulating or abrogating the Hh pathway. BBB-ECs were treated for 24h with astrocyte-conditioned media (ACM), hrShh, ACM plus Cyclopamine or Purmorphamine, and permeability was recorded over 3h (n=3-8, in triplicate). (E) Flow cytometric analysis of Gli-1 expression in human

BBB-ECs. Percentages and mean fluorescence intensity (MFI) in red refer to values of control (untreated cells), and blue refer to values of treated cells. **(F)** MFI for Gli-1 in BBB-ECs shown in **E** ($n=4$). **(G)** qPCR analysis of Gli-1 (upper panel) and SOX-18 (lower panel) mRNA expression in BBB-ECs treated with hrShh ($n=2$ experiments in triplicate). **(H)** Epifluorescence photomicrographs of CNS sections from C57Bl/6 mice treated with Cyclopamine for 6h (upper panels). Dextran-FITC and fibrinogen (arrowheads) accumulate around vessels (asterisk) as compared to vehicle-treated mice (HP β CD, lower panels). Nuclei (DAPI, blue). Scale bars, 30 μ m. **(I)** Quantitative analysis of perivascular extravasation of 70 kDa Dextran-FITC and fibrinogen in Cyclopamine and vehicle-treated animals. For all, mean \pm SEM. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Fig 2. Shh promotes junctional protein expression. **(A)** qPCR analysis of *PTCH1*, *CLAUDIN5* and *OCCLUDIN* mRNA extracted from human BBB-ECs grown with astrocyte-conditioned media (ACM) or hrShh for 24h. **(B)** WB (left) and semi-quantitative analysis (right) of claudin-5, occludin, JAM-A, claudin-3 and VE-cadherin in human BBB-ECs treated with ACM or ACM + Cyclopamine for 24h. **(C)** Characterization of CNS vessels in P19 wild type (WT) and Tie2-Cre; *Smo*^{c/c} mice. Immunofluorescence for GFAP (astrocytes), PECAM-1, claudin-5, claudin-3, occludin, ZO1, p120 (all BBB endothelium markers), fibrinogen and immunoglobulins G (IgGs, indicators of plasma protein leakage) (left, images) and quantification (right, graphs) ($n=3-5$ animals). White rectangles indicate areas shown on the right of each panel. Arrowheads on occludin panels indicate vessels. Error bars, mean \pm SEM. *, $P<0.05$; ***, $P<0.001$. Nuclei (TOPRO-3, blue). Scale bars: 30 μ m.

Fig 3. Hh activity promotes BBB immune quiescence. **(A)** Chemokine secretion (IL-8/CXCL8, left; MCP-1/CCL2, right) by human BBB-ECs treated with hrShh, Purmorphamine or both ACM and Cyclopamine. **(B)** ICAM-1 expression by human BBB-ECs treated under similar conditions. **(C and D)** Effect of Hh pathway engagement on human CD4⁺ T lymphocyte adhesiveness (1 h) to **(C)** and migration across **(D)** human BBB-ECs. A similar effect was recorded with the Hh antagonist SANT-1. **(E)** Adhesion of human Th17, Th1 and Th2 cells polarized in the presence or absence of hrShh to human BBB-ECs. **(F to I)** Effect of pharmacological Hh neutralization with the *Smo* antagonist GDC-0449 (GDC; 25 mg/Kg, injected at days 0, 4, 8 and 12, arrowheads) on clinical severity of EAE **(F)**, on spinal cord demyelination (arrowheads indicate border of demyelinating lesions) **(G)**, on the number of leukocytes infiltrating the CNS **(H)**, and on the production of IFN- γ and IL-17 by CNS infiltrating CD4⁺ T cells **(I)**, as compared to vehicle (DMSO). Panels A to F and H represent the mean \pm SEM ($n=3-8$

experiments, in triplicate). Panels F to I, 25 animals per condition. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. Scale bars: 50 μ m.

Fig 4. Hh components are upregulated under neuroinflammatory conditions *in vitro* and *in situ*. **(A)** Expression of Shh was determined in resting (red histogram) and inflamed (green histogram; TNF + IFN- γ treated for 24h) human astrocytes (HFAs) by flow cytometry. Isotype is shown in black. **(B)** Ptch-1 (left) and *Smo* (right) expression in human BBB-ECs grown with astrocyte-conditioned media (ACM, light grey) or ACM + TNF + IFN- γ (dark grey) (**A** and **B**, $n=4$). **(C)** Expression of Shh, Ptch-1 and *Smo* in CNS sections from human control (left panels) and MS patients (right panels). Arrowheads indicate astrocyte bodies. Arrows indicate astrocyte endfeet and asterisks blood vessels. The black dotted line delineates the border between an active MS lesion and the adjacent normal-appearing white matter (NAWM). **(D)** Shh and GFAP (arrowheads) colocalization in NAWM (upper left panels) and active MS lesions (upper right panels). Ptch-1 expression on ECs of NAWM (middle left panel) and active lesions (middle right panel). In active MS lesions, Ptch-1 is also detected in infiltrating cells (arrowheads). The lower panels demonstrate Ptch-1 and Gli-1 expression in ECs of NAWM and active lesions, where arrows indicate ECs and arrowheads infiltrating cells. Nuclei (TOPRO-3, blue). Scale bars, 30 μ m.







