Netrins enhance blood-brain barrier function and regulate immune responses at the blood-brain barrier

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ABBREVIATIONS

$A_{2B}AR$ = adenosine 2B adenosine receptor

$A_{2B}AR^{-/-}$ = A2BAR knockout mice

Ang-1,-2 = angiopoietin-1, -2

AJ = adherens junction

BBB = blood-brain barrier

BBB-ECs = blood-brain barrier derived endothelial cells

DCC = deleted in colorectal cancer

DSCAM = Down Syndrome Cell Adhesion Molecule

EAE = experimental auto-immune encephalomyelitis

ECM = extracellular matrix

FGF = fibroblast growth factor

EC = endothelial cell

fMLP = f-formyl-methionine-leucine- phenylalanine

HIF-1α = hypoxia inducible factor 1 alpha

IFNγ = interferon gamma

iNOS = inducible nitric oxide synthase

IL-6 = interleukin-6

IL-8 = interleukin-8 / CXCL-8

LPS = lipopolysaccharide

Mo = monocytes

MΦ = macrophages

MCP-1 = Monocyte chemotactic protein-1 / CCL-2

MIP-1α = Macrophage inflammatory molecule-1 alpha / CCL-3

MPO = myeloperoxidase

MMP = matrix metalloproteinase (-2, -9)

MS = multiple sclerosis

N1 = netrin-1

$N1^{+/-}$ = netrin-1 heterozygous mice

N4 = netrin-4

NADPH oxidase = nicotinamide adenine dinucleotide phosphate-oxidase

NG1 = netrin-G1

NG2 = netrin-G2

NGL = netrin-G ligand

NO = nitric oxide

NVU = neurovascular unit

O_2^- = superoxide

$ONOO^-$ = peroxinitrate

PDGF = platelet-derived growth factor

PDGF-R = platelet-derived growth factor receptor

PMN = polymorphonuclear cells / granulocytes

RGM = repulsive guidance molecule

ROS = reactive oxygen species

RNS = reactive nitrogen species

TGFβ = transforming growth factor beta

Th1 = IFNg-secreting T helper cells

Th2 = IL-5-secreting T helper cells

Th17 = Th17-secreting T helper cells

TJ = tight junction

TNFα = tumor necrosis factor alpha

Treg = CD4+CD25+FoxP3+ regulatory T cells

Unc5h = uncoordinated 5 homolog

VEGF = vascular endothelial growth factor

WT = wild type mice

ZyA = Zymosan A

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The following findings are novel contributions to scientific knowledge that have not been published or described before. For all work presented in this thesis the author is the primary researcher and experimenter.

- I discovered netrins as endothelial barrier strengthening factors during development, adulthood and neuro-immune disease.
- Netrin-1 and -4 increase junctional protein expression in mouse and human brain-endothelial cells, thereby reducing barrier permeability in the developing, adult, and inflamed blood-brain barrier (BBB).
- Inflammation regulates netrin expression by cerebral microvascular endothelial cells. While netrin-1 levels increase in brain-derived microvessels and in blood serum, endothelial netrin-4 expression is reduced during inflammatory conditions *in vitro* and during the chronic autoimmune diseases multiple sclerosis and the animal model of CNS inflammation experimental autoimmune encephalomyelitis (EAE).
- Besides barrier-enhancing functions, netrins have anti-inflammatory effects on brain-derived endothelial cells and decrease their cytokine and chemokine secretion.
- However, netrin-1 does not affect cell adhesion molecule expression by brain endothelial cells and does not alter T cell migration through the *in vitro* BBB.
- Endogenous netrin-1 expression increases in dendritic antigen presenting cells upon differentiation and maturation.
- Netrin-1 increases immune cell adhesion; T cells adhere to an immobilized netrin-1 substrate, but not to netrin-4.
- For the first time, we show netrins' function during autoimmune disease. We found that intravenous netrin-1 therapy decreases disease incidence, delays EAE disease onset and severity by increasing endothelial barrier stability and reducing recruitment of myeloid cells into the central nervous system at the early stages of the disease.

- In the chronic phases of EAE, netrin-1 promotes an accumulation of T lymphocytes in the CNS, and increases their activation, which results in additional atypical disease symptoms such as ataxia and hind limb spasticity.

CONTRIBUTION OF OTHER AUTHORS BY ALPHABET:

- **Bin, Jenea** setting up and providing netrin-1 knockout mice.
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- **Terouz, Simone** provided human brain sections performed the Luxol Fast Blue stain on EAE brains, and helped stain and quantify plasma proteins in netrin-1 knockout brains.

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ABSTRACT

During development, netrin guidance cues control cell motility and cell adhesion. Cell-adhesion between endothelial cells at the blood-brain barrier makes the endothelium impermeable to blood-derivatives and immune cells. To establish and maintain this barrier during development, and adulthood, and as well as during disease, brain endothelial cells must develop and sustain these strong adhesive contacts, through expression of tight junction molecules. However, we do not know whether netrins support inter-endothelial cell adhesion at the blood-brain barrier. Given this, we hypothesize that netrin tightens the blood-brain barrier during development, adulthood, and protects it during disease.

Methods: To test this, we used both human adult primary brain-derived endothelial cells and newborn netrin-1 knockout mice and evaluated netrin's effect on inter-endothelial cell adhesion and barrier permeability. We also assessed netrins' therapeutic potential to maintain the barrier and limit immune cell infiltration into the central nervous system (CNS) during experimental autoimmune encephalomyelitis (EAE).

Results: Our results demonstrate that brain endothelial cells express netrins where they function in three ways. They help to form a tighter blood-brain barrier during development. They also maintain and protect the adult barrier by increasing the expression of endothelial junction molecules, thus promoting inter-endothelial adhesion and reducing protein leakage across the barrier. Netrins also reduce blood-brain barrier breakdown and diminish initial myeloid cell infiltration into the brain and spinal cord during EAE, which delays disease onset and ameliorates disease severity. However, during the chronic phase of EAE, netrin-1 treated mice have higher numbers and more activated T cells in their CNS and exhibit an ataxic gait and limb spasticity.

Discussion: We conclude that netrins enhance BBB stability, but have dual functions on immune responses during neuroinflammatory disease. These findings favour the hypothesis that if netrin function was to be manipulated as a therapeutic, early short-term approaches would likely be the most effective.

RÉSUMÉ

Au cours du développement, les molécules de la famille des nétrines contribuent à la morphologénèse des organes en contrôlant la motilité et l'adhérence cellulaire. L'adhérence cellulaire entre les cellules endothéliales est une caractéristique importante de la barrière hémato-encéphalique (BHE), ce qui rend l'endothélium imperméable aux molécules sanguines et aux cellules immunitaires. Pour établir et maintenir cette barrière au cours du développement, à l'âge adulte et au cours de la maladie, les cellules endothéliales du cerveau doivent développer et maintenir ces contacts adhésifs en exprimant des molécules de jonction serrées. Cependant, nous ne savons pas si les molécules de la famille des nétrines influencent l'adhérence cellulaire inter-endothéliale de la BHE. Nous avons donc émis l'hypothèse que les nétrines resserrent la BHE au cours du développement, à l'âge adulte, et la protège au cours de la maladie.

Méthodes: Pour valider notre hypothèse, nous avons utilisé des cellules endothéliales primaires dérivées des cerveaux humains adultes ou des cerveaux de souris nouveau-nés déficientes en nétrine-1 et évalué l'effet de la nétrine sur l'adhésion cellulaire endothéliale et inter-perméabilité de la barrière. Nous avons également évalué le potentiel thérapeutique des nétrines a restaurer la barrière et l'infiltration de cellules immunitaires limite dans le système nerveux central (SNC) pendant encéphalomyélite allergique expérimentale, un modèle animal de sclérose en plaques.

Résultats: Nos résultats démontrent que les nétrines sont exprimées par les cellules endothéliales du cerveau, exprimes nétrines. Au cours du développement les nétrines aident à assurer l'étanchéité de la BHE. Chez les adultes, ils maintiennent et protègent la barrière adulte en augmentant l'expression des molécules de jonctions serrées, favorisant ainsi l'adhérence inter-endothéliale et diminuant les fuites de protéines à travers la BHE. Dans la pathologie de l'EAE, le rôle des nétrins diffère en fonction de la phase de la maladie. Au cours de la phase aigue, les nétrines atténuent la perte de l'intégrité de la BHE et diminuent l'infiltration des cellules myéloïdes dans le SNC. Ceci retarde l'apparition de la

maladie et réduit sa sévérité. Au cours de la phase chronique de l'EAE, les souris traitées avec netrin-1 ont un plus grand nombre des cellules T activées dans leurs SNC et présentent une démarche ataxique ainsi qu'une spasticité des membres.

Discussion: Nous concluons que les nétrins améliorent la stabilité de la BHE. Ces résultats suggèrent que les nétrines peuvent être envisagée comme agent thérapeutique dans les maladies neuroinflammatoire. Dans ce cas une approche précoce et à court terme serait probablement plus efficace.

INTRODUCTION LITERATURE REVIEW

1. THE BLOOD-BRAIN BARRIER – AN OVERVIEW

Vascular networks are transport highways for nutrients, waste products, and immune cells. They replenish tissues with anabolic imports, export cells' catabolic waste products, and carry immune mediators to sites of injury. To allow this traffic across blood vessels to occur, vascular endothelial cells permit molecules to passively and actively diffuse or be transported through this semi-permeable barrier. Blood vessels in the central nervous system (CNS), however, create an impermeable barrier. They strictly and selectively control molecule exchange to protect the integrity and optimal function of the CNS. To achieve this high barrier capacity, endothelial cells within the CNS have to be equipped with structures and molecules that link them tightly to one another. This creates an anatomical impermeable wall between the blood and the brain, the *blood-brain barrier* (BBB) [1].

1.1. Why is the vasculature of the CNS so special? - Impermeability

CNS-derived endothelial barriers possess greater structural and functional complexity compared to endo- and epithelial barriers in the periphery [1]. As a result, brain-capillaries are ~50 to 100 fold tighter than capillaries in the periphery [2]. The formation of a highly complex and impermeable seal between the blood and the brain parenchyma is essential to maintain an optimal neuronal microenvironment and to protect the brain from damage.

The BBB functions in two main ways: Firstly, it creates a structural fence by lacking trans-cellular pores (fenestrations), and by tightly connecting neighboring endothelial cells with complex adhesive junctions leading to low trans- and para-cellular diffusion, respectively. This limits blood-borne molecules, foreign chemical compounds (xenobiotics), and circulating immune cells from entering the CNS, and restricts electrolytes from freely diffusing into and out of the CNS. Secondly, it actively provides the CNS with macro- and micronutrients, peptides, proteins and ions through a variety of selective transport systems

(including glucose transporters [GLUT-1], Na^+, K^+ ATPases, or endocytic uptake by insulin and transferrin receptors) [3,1,4,5]. The BBB also prevents accumulation of toxic substances and metabolites by carrying them from the brain parenchyma back into the blood via drug efflux pumps (ATP-binding cassette [ABC]-transporters, i.e. multiple drug resistance protein-1 [MRP-1/ABCB1], P-glycoprotein)[3,6]. Because building a fence and actively shuttling molecules across this fence requires energy, the brain-endothelium is equipped with a high number of mitochondria, another hallmark of the BBB [5].

1.2. What makes the blood-brain barrier tight? - Inter-endothelial adhesion

Unique BBB properties derive not only from strong and complex inter-endothelial adhesion, which are mediated by tight junction (TJ) and adherens junction (AJ) proteins, but also from the physical support within the neurovascular unit. The neurovascular unit mainly consists of endothelial cells, pericytes, and astrocytes. Within this complex, these vascular and neural cell types directly interact with each other and secrete factors that stimulate and reinforce inter-endothelial adhesion [5,7,8] (Figure i). In fact, capillaries and post-capillary venules, small vessels, where endothelial cells stay in direct contact with pericytes, astrocytic endfeet, and basement membrane proteins to synergistically enhance the barrier, the BBB exhibits maximum barrier capacity [9,10]. There, tight and adherens junction complexes mediate strong inter-endothelial adhesion [11], which microscopically appear to form connective strands that resemble zipper-like structures.

Tight junction complexes encompass transmembrane proteins as well as intracellular proteins that provide a link to the actin cytoskeleton. BBB endothelial cells express two types of four-span transmembrane proteins: claudin-1, -3, -5, -11, -12 and occludin [3,4,12], and a single-span transmembrane protein: junctional adhesion molecules (JAM)-A, all of which bind homotypically to one another. Claudin-5 appears to be of particular importance for establishing the BBB [13]. Its absence results in plasma protein extravasation into the brain, while its increased expression enhances trans-endothelial electrical resistance [14]. Transgene

expression of claudin-1, which naturally only occurs in meningeal vessels, limits protein diffusion through the BBB and ameliorates pathologies related to BBB-breakdown [13]. While claudins establish barrier properties, occludins exhibit regulatory effects on the BBB. Compared to blood vessels outside the CNS, occludin is highly expressed and widely distributed at the BBB [13,15,16]. However, mice lacking occludin exhibit normal BBB permeability [17]. JAM-A also supports inter-endothelial adhesion, however, its role in BBB permeability is less clear [18,19]. Other proteins also locate to BBB junctional complexes as well, but despite known roles in epithelial tight junction complexes, their role remains unclear at the BBB [3,4,20-22].

By connecting to a variety of intracellular adaptor and signaling proteins, claudins, occludins and JAMs assemble, maintain and regulate TJ complexes [12]. Intracellular TJ proteins are essential to connect transmembrane TJ proteins to the actin-cytoskeleton. They help to recruit integral TJ proteins and assemble them into complexes. Intracellular zonula occludens (ZO-1, -2, -3), MAGI, and MUPP1 function as adapter proteins and scaffolds to effectively support protein interaction and signaling. In epithelial cells, ZO-1 helps to recruit claudin-5 to the junction therefore allowing its association with other claudin-proteins [12,23-25]. In fact, ZO-1 and -2 knockouts fail to assemble TJ strands [23].

While tight junctions play an integral role in promoting and maintaining barrier properties, adherens junctions (AJ) help to establish initial TJ complexes during barrier-genesis [26] and stabilize the barrier. Cadherins are transmembrane AJ proteins that bind to other cadherins or to growth factor receptors on neighboring cells. They transduce signals into the cell and activate the cytoplasmic associated adaptor proteins: β-catenin and p120 catenin. β-catenin in turn binds to α-catenin, which provides linkage to the actin cytoskeleton to promote vascular stability [5,26]. Brain-endothelial cells express vascular endothelial (VE)-cadherin [4] and neuronal (N)-cadherin [26]. VE-cadherin is crucial for AJ assembly and maintains vascular integrity and barrier properties [27,28]. Mice with mutated VE-cadherin die before birth [29,30]. Additionally, blocking VE-cadherin homotypic adhesions with antibodies results in a leaky barrier [31] increases

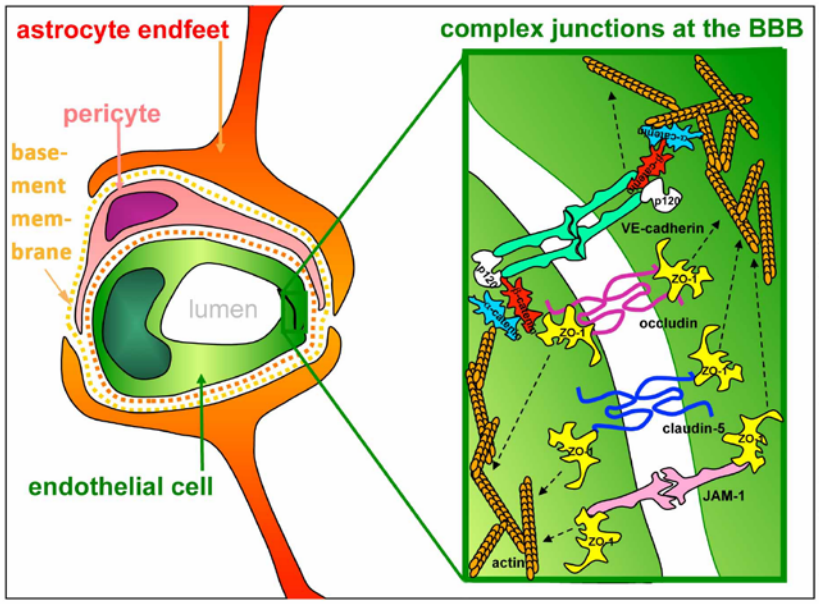
extravasation of macromolecules [32] and causes hemorrhages in adult mice [27,33]. N-cadherin, on the contrary does not specifically locate to AJ complexes but shows rather diffuse expression patterns in endothelial cells. Instead of supporting inter-endothelial adhesion, N-cadherin appears to maintain endothelial-pericyte contact [34-37]. P120-mutant mice lack endothelial N-cadherin and pericytes are no longer efficiently recruited to the vessel. Pericytes also promote endothelial N-cadherin expression through the S1P secretion. P120 also plays an important role by stabilizing and integrating cadherins. Loss of p120 leads to lower or absent expression of VE and N-cadherin [35]. And mice with a deletion of endothelial p120 exhibit cerebral hemorrhages and die during embryonic development [35].

Both, tight and adherens junction complexes communicate with each other to enhance barrier properties [38]. Blood flow through the vessel enhances barrier stability by exerting shear stress on the endothelial wall. VE-cadherin is crucial for sensing and translating shear stress (through blood flow) by promoting occludin phosphorylation, recruitment, and increasing ZO-1 and claudin-5 expression to create more effective tight junctions.

Intracellular binding of VE-cadherin to β-catenin increases claudin-5 expression by counteracting β-catenin's claudin-5 gene repressive activity [39]. In epithelial cells, interaction occurs also between TJ and AJ proteins. In this manner, ZO-1 and α-catenins interact intracellularly [12].

In summary, endothelial cells in the CNS connect tightly to one another through transmembrane proteins. These tight and adhesion junction proteins, in turn, collaborate with other intracellular junction proteins and anchor to the cytoskeleton to establish strong adhesion between endothelial cells.

Figure i



At the NVU, pericytes, astrocytic endfeet, and basement membranes closely associate with endothelial cells and support the establishment of strong inter-endothelial adhesion. This adhesion is mediated by tight and adhesion junction proteins, which encompass both transmembrane (JAM-1, claudin-5, VE-cadherin, occludin) and intracellular proteins (ZO-1, p120, α-catenin) that stabilize the actin cytoskeleton.

1.3. Adhesion between immune cells and the blood-brain barrier endothelium

Although the BBB is tightly sealed, it can interact with immune cells and allow their passage into the CNS.

Inflammatory stimuli, such as TNFα, activate endothelial cells, including those at the BBB, so that they increase their expression of cell adhesion molecules (CAMs)[18,40-44]. Unlike TJ or AJ molecules, which increase inter-endothelial cell adhesiveness to protect the endothelial barrier, CAMs support the adhesion between immune and endothelial cells and thus promote the initial steps to immune cell extravasation. Brain-derived endothelial cells express platelet endothelial CAM (CD31 = PECAM-1, binds to itself or αvβ3 integrin), vascular CAM (VCAM-1, binds to very late antigen-4 [VLA-4 = CD49d/CD29 = α4β1 integrin]), intercellular CAM (ICAM-1, binds to lymphocyte function associated-1 [LFA-1 = CD11aCD29 = αLβ2 integrin]), neural CAM (NCAM L1, binds homotypically to NCAM, melanoma CAM (MCAM = CD146), binds to MCAM and to an yet unidentified heterotypic ligand), and activated leukocyte CAM (ALCAM = CD166, binds to ALCAM or CD6 and another type of CAM), the E-

and P-selectins (E-selectin, binds to sialylated Lewis X [s-Le^x] sugar groups; P-lectin binds to P-selectin glycoprotein ligand-1 PSGL-1) [40-44].

Immune cells express various binding partners that can bind to CAMs. Through these binding partners leukocytes can interact with the endothelium both in a homotypic fashion, via CAMs, or heterotypic via integrins, and glycosyl-rich ligands [45,46]. Consequently, when endothelial CAM expression is high, circulating leukocytes become tethered to selectins, which slows down their rate of flow in the blood stream. This allows leukocytes to further engage with endothelial ICAM-1 and VCAM-1 via integrins $\alpha L\beta 2$ and $\alpha 4\beta 1$, respectively. This in turn enables them to slowly roll along the endothelial surface and screen it for chemokines [47,48]. When leukocytes bind to chemokines, a rapid inside-out signaling occurs, which causes integrins to change conformation and become high affinity receptors for endothelial CAM ligands [49]. This tight integrin-mediated adhesion permits immune cells to stop, protrude, and cross the endothelium [50-53]. Leukocytes can invade the CNS through the specialized choroid plexus epithelium, or leptomeningeal vessels to enter the cerebrospinal fluid (CSF), or through parenchymal capillaries and post-capillary venules to migrate into the perivascular space. However, lesions usually build around small veins and venules. Immune cell migration can also occur transcellularly, whereby BBB permeability and integrity may stay intact [54]. However, most frequently, leukocytes pass the endothelial barrier paracellularly, which requires temporary disruption of inter-endothelial connections.

2. WHAT FACTORS REGULATE THE BLOOD-BRAIN BARRIER?

2.1. How does the blood-brain barrier develop?

During early embryogenesis, vascular development begins and gradually forms a complex system of blood vessels that branches throughout the entire body to provide it with oxygen, nutrients, and hormones and to remove carbon dioxide and metabolites. Vascular development starts with a process called vasculogenesis [55]. During this time, endothelial precursor cells, angioblasts, proliferate and migrate away from the mesoderm in response to vascular endothelial growth factor (VEGF) [56] and hedgehog morphogens [57]. They aggregate and form into endothelial tubes that anastomose into a primitive vascular plexus. Following this *de novo* tube generation, the angiogenic factors VEGF and angiopoietin (Ang), via Flk-1 (receptor tyrosine kinase, binds VEGF) [55,58,59] and Tie-2 (receptor tyrosine kinase, binds Ang-1 and Ang-2), further encourage endothelial cells to grow and sprout capillaries from this plexus to generate a highly branched vascular network. This process is distinct from vasculogenesis and is termed angiogenesis [60,61]. Following precise stereotypical patterns, vascular sprouts continue to grow and branch and reach into the developing brain at around embryonic day (E) 11 [20,62-64].

When endothelial cells reach into the CNS, they adopt a BBB-phenotype and begin barrier genesis. The mere exposure to a neural environment, containing astrocytes and other neural constituents, determines endothelial cells to develop BBB characteristics [1,65]. As soon as vascular sprouts enter the developing neural tube, endothelial cells carry BBB-specific markers (GLUT-1 and P-glycoprotein) [20,66]. Between E11 and 13, brain-endothelial cells lose trans-cellular pores and express tight junction proteins [63,67]. Hedgehog (secreted by astrocytes, signals through Smo/Ptch receptors on endothelial cells) and Wnt7a (signals through the Wnt/β-catenin signalling pathway) are among the early factors that induce expression of tight junction molecules [18,62,68,69]. Over time, vascular and neural cells assemble into the neurovascular unit, where astrocytes, pericytes and endothelial cells interact to maximally support barrier function. Barrier function increases as microvessels become impermeable to ions as transendothelial

electrical resistance, a measure for barrier capacity, dramatically increases just before birth [20]. Before birth the barrier also becomes impermeable to plasma proteins and small (under 1kDa) tracer molecules [70-72].

2.2. Astrocytes and pericytes support inter-endothelial adhesion

Astrocytes are closely associated with vascular endothelial cells, contacting almost the entire parenchymal surface of the endothelium with their astrocytic endfeet [1,2]. They adjust the vascular network to meet the neural cells' demand for oxygen and nutrients. To control and balance vascular expansion and maintain barrier properties, astrocytes secrete both angiogenic factors (vascular endothelial growth factor [VEGF] and Ang-2) [73-76], which allow vessel growth and therefore loosen and deconstruct the barrier, and factors that enhance adhesion among endothelial cells to stabilize the barrier through various growth factors such as transforming growth factor beta (TGFβ), glial-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), or other factor such as sonic hedgehog, angiopoietin-1, and angiotensinogen [77-84]. In turn, angiotensinogen, Ang-1, meteorin, AKAP12, thrombospondin (Tsp-1, and -2), SSeCKS, bFGF, GDNF, and hedgehog are barrier-promoting factors that not only stimulate TJ protein expression but also suppress angiogenic signalling[77-84]. Astrocytes are essential to establish the BBB, as brain-derived endothelial cells deprived of astrocyte-support quickly down regulate BBB-specific markers p-glycoprotein and TfR and lose their BBB-identification [85,86]. Co-culture with astrocytes or supplementation with astrocyte-conditioned media reverses this and induces TJ molecule expression in endothelial cells [77,87,88], even in endothelial cells of non-neural origin [1,2,82,89].

Pericytes surround endothelial surfaces of small arterioles, venules and capillaries of all body tissues, but they are most abundant on blood vessels in the CNS [90]. They enhance vessel stability and regulate blood flow through their contractility. However, pericytes regulate not only cerebral perfusion but also BBB permeability [90,91]. Pericytes provide growth factors, such as TGFβ, platelet-derived growth factor (PDGF), Ang-1 and -2, sphingosine-1-phosphate (S1P), and

secreted molecules that support BBB assembly and maintenance [34,90,92-94]. Loss of pericytes from vessel walls leads to partial astrocyte detachment from the vessels, decline and rearrangement of vascular tight junction proteins, and increased BBB leakiness to plasma proteins [15,90].

In a tricelluar system, astrocytes and pericytes support the cerebral microvasculature and increase barrier function more effectively, compared to when endothelial cells are co-cultured with astrocytes or pericytes alone [10,95]. However, to successfully assemble into a NVU, pericytes, astrocytes and endothelial cells require an anchoring and growth-accommodating environment. The basement membrane provides this by serving as a scaffold and reservoir for growth factors. It can be distinguished into an endothelial and an astrocyte-derived parenchymal basement membrane [50,96]. Together they build a thin but dense layer, composed of extracellular matrix (ECM) proteins, including laminins, fibronectin, collagen (type IV), tenascin, and proteoglycans (agrin, brevican, dystroglycan, perlecan, versican and heparin sulfate proteoglycan [HSPGs]) [3]. NVU cells anchor to these matrix proteins via integrins [97-100], which strengthens the barrier. In fact, basement membranes devoid of agrin result in BBB-leakage [101]. Thus, all components of the neurovascular unit contribute to maintain and develop a tight BBB. They increase junctional strength by providing an anchoring matrix, by up-regulating TJ and AJ molecule expression or promoting their recruitment and organization into cholesterol-enriched, raft-like microdomains (lipid rafts) [77,102,103], where TJ proteins assemble and efficiently interact with other TJ or AJ proteins [20,104].

2.3. Pathological conditions destabilize the blood-brain barrier

Under pathological conditions such as hypoxia or inflammation cells of the NVU become activated and can promote barrier loosening by destabilizing endothelial junctions [10,11,105]. Although this seems paradoxical and may harm some neural cells, these mechanisms are a compromise to protect the CNS from nutrient deprivation or from microbial destruction. Hypoxic activation of the endothelium

decreases vascular integrity to allow vessel growth. And a weakened barrier permits immune cells to enter the CNS and clear threatening infections [106-108].

Blood-brain barrier breakdown is in fact a hallmark of many CNS pathologies and can have dramatic consequences. BBB collapse takes place during traumatic brain injury causing hematoma, swelling, and immune activation [109]. Multifocal BBB damage occurs during multiple sclerosis (MS) [5,110] a chronic autoimmune disease of the CNS. In MS, misprogrammed T cells become auto-aggressive against myelin components and cross the BBB into the CNS, where they accumulate in the perivascular space and recruit other immune cells to mount the attack [46,111-113].

2.4. Inflammatory responses damage the blood-brain barrier

During neuro-inflammatory responses immune cells cross the BBB and produce potent inflammatory mediators, both of which destabilize the BBB. They decrease barrier function by endocytotically removing TJ or AJ proteins from the cell surface [12] or modulating their ability to assemble within junctional complexes through specific phosphorylation [77,114-116]. Depending on which specific residues get phosphorylated, junctional proteins such as occludin, and JAM-1 can lose or gain the capacity to remain within lipid rafts or to mediate barrier properties. Tyrosine residue phosphorylation of VE-cadherin, for example, generally destabilizes the barrier [26].

To overcome the endothelial barrier, leukocytes deconstruct junctional protein complexes and break down ECM proteins [54,117,118]. Only freshly activated T cells can interact with the BBB and cross it through para- or transendothelial migration [119], while resting non-activated leukocytes have a very limited capacity to enter into the CNS [117]. This enhanced migratory ability of activated leukocytes can be explained by their increased expression of adhesion molecules, compared to non-activated leukocytes. This in turn allows for stronger adhesion and interaction with the endothelial wall, which is a crucial step to transmigrate across the endothelium.

By producing matrix metalloproteinases (MMPs), leukocytes have the ability to cleave ECM molecules enzymatically [119-121]. They also secrete cytokines that alter junctional protein expression [119,122,123]. TNFα, and IL-1β additionally activate matrix metalloproteinase (MMP2 and MMP9) secretion by pericytes [124] or endothelial cells [125,126]. These MMPs enzymatically degrade tight junction proteins such as occludin, ZO-1, claudin-5 and basal lamina components [18,127-129]. This destabilizes the barrier and increases its permeability to other blood-derived factors, and further facilitates subsequent immune cell infiltration [130,131]. Another mechanism of barrier weakening during inflammation occurs through internalization and removal of tight junction proteins from the endothelial cell surface [132-134].

Once at the site of injury, immune cells secrete a range of cytokines, anti-microbial proteins and reactive oxygen and nitrogen species (ROS, hydrogen peroxide H_2O_2, Hydroxyl radical $HO_2^{\cdot}$, superoxide anion $O_2^{\cdot-}$; and RNS, peroxinitrate ONOO), which are generated by enzymes like NADPH oxidase and MPO, in a reaction called the oxidative burst [135]. Whilst this effectively clears pathogen, it also causes damage to the surrounding healthy tissue because these reactive species [136,137] oxidize lipids, proteins and DNA leading to cell activation [138], cell damage and death [139,140]. ROS cause BBB endothelial cells to lose their expression of claudin-5, occludin and ZO-1, thus damaging the BBB [141,142]. Oxidative stress also affects other cells of the neurovascular unit, such that pericytes disappear from the BBB and consequently weaken barrier function [143]. At the BBB, both the process of transendothelial migration and the release of harmful immune mediators disrupt the barrier.

Cytokines can also directly damage the barrier. One of the most potent pro-inflammatory vascular regulators is TNFα. During an inflammatory response, activated macrophages rapidly up-regulate and secrete large amounts of TNFα [144]. TNFα loosens inter-endothelial adhesions and increases barrier permeability through several mechanisms. It disrupts and disorganizes TJ complexes and promotes endothelial cell contraction [125,145]. Additionally, within a few hours TNFα induces MMP-2 and -9 release from endothelial cells and pericytes [124]. It

decreases and represses the expression of tight junction proteins occludin, claudin-5, and ZO-1 [122,123,125,145]. Other cytokines such as IFNγ or IL-1 can synergistically contribute to TNFα-mediated barrier disruption [145,146]. Furthermore, activated Th1 and Th17 cells, which are pro-inflammatory types of T helper cells, disrupt the barrier through IL22 and IL17 by decreasing occludin and ZO-1 expression to then infiltrate into the CNS [119]. Inflammatory mediators additionally destabilize the barrier by promoting endothelial cells to contract, potentially through the activation and up-regulation of MLCK (myosin light chain kinase phosphorylates myosin to promote actin cytoskeleton contraction and stress fibre formation) [133,146,147]. This results in actin cytoskeleton remodeling [148,149] and altered sub-cellular distribution of AJ and TJ molecules [150]. The loosened barrier in turn encourages the passage of immune cells into the CNS. For example, disruption of homophilic VE-cadherin binding between endothelial cells destabilizes adherens junctions and promotes neutrophil extravasation into the peritoneum [31,32].

The reciprocal interaction between the endothelium and immune cells is intricate and affects endothelial cells but also astrocytes, pericytes and the CNS-residing immune population, microglia. Immune players can activate astrocytes, pericytes, and microglia, causing them to further diminish barrier stability. Upon activation, astrocytes, microglia and pericytes can further enhance BBB breakdown. Astrocytes release ATP, prostaglandins, and IL-6; microglia produce TNFα and ROS; pericytes up-regulate MMPs [1,89,105,124,151]. At this time, we know many factors that can destabilize the barrier, but we know only few factors to maintain and protect the BBB.

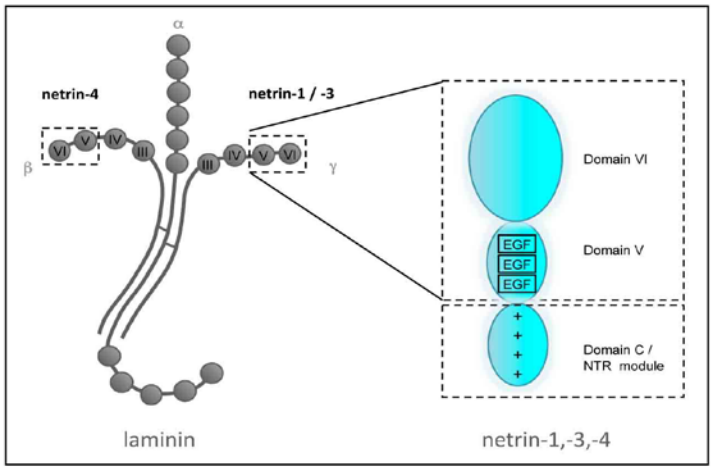
3. DO NETRINS REGULATE THE BLOOD-BRAIN BARRIER?

This thesis tests the idea that netrin, known as a developmental neural guidance cue, has the ability to support and protect blood-brain barrier integrity. During development, they regulate cell migration, survival, and adhesion in the central nervous system and other organs including the vasculature [152]. And in the adult, they have anti-inflammatory effects and regulate vascular growth in various pathological conditions.

3.1.What are netrins?

Netrins comprise a small family of laminin-related proteins that are best known for directing cell and axon migration in the embryonic nervous system. In mammals, three secreted forms have been identified: netrin-1, netrin-3, and netrin-4 [153,154,13,14]. Two additional membrane-bound netrins (netrin-G's carboxyl terminal domain encodes a sequence for a glycosylphosphatidylinositol (GPI) for linkage to the plasma membrane) exist: netrin-G1 and netrin-G2, but they appear to have rather neural specific functions (they function as receptors for netrin-G ligands [NGL] and support synapse stabilization) [153,155,156].

Figure ii



The netrin domains V-VI share structural homology with the V-VI domains of laminins. Netrin's C terminus is rich of basic amino acids.

All netrins are composed of approximately 600 amino acids and all are members of the laminin-superfamily of proteins [157]. Netrins have a three-part

domain structure: amino terminal domains VI and V, which are homologous to domains VI and V of laminins, and a netrin carboxyl terminal C domain, which is not related to laminins (Figure ii). Netrin's C domain, also called the netrin-like (NTR) module, is rich in basic amino acids and binds heparin with high affinity [158].

3.2. How can netrins be so versatile? - Netrin receptors

By binding to a diverse repertoire of receptors, netrins mediate a variety of functions (Figure iii) [153,154]. Classical netrin receptors in mammals include deleted in colorectal cancer (DCC) [159,160], the DCC paralogue neogenin [161,162], four Unc5 homologues, Unc5A-D, and Down syndrome cell adhesion molecule (DSCAM) [163]. All are single pass transmembrane proteins and all are members of the immunoglobulin (Ig) superfamily. DCC mediates cell and axon chemoattraction in response to secreted netrins. Neogenin mediates netrin dependent cell-cell adhesion, and some evidence also supports a chemoattractive axon guidance receptor function [138]. In addition to directing motility, DCC and neogenin promote cell-cell and cell-substrate adhesion upon sensing netrin, thereby supporting proper tissue organization [164-172].

On the contrary, the vertebrate Unc-5 homologues, Unc5A-D, signal chemorepulsion in response to secreted netrins. Interestingly, chemorepulsion often requires co-expression of DCC and an Unc5 homologue, which can form a heteromeric complex to functionally collaborate [173]. However, examples of DCC independent chemorepulsion to netrin have also been documented and the difference between DCC dependent and DCC independent chemorepulsion remains unclear [174,175].

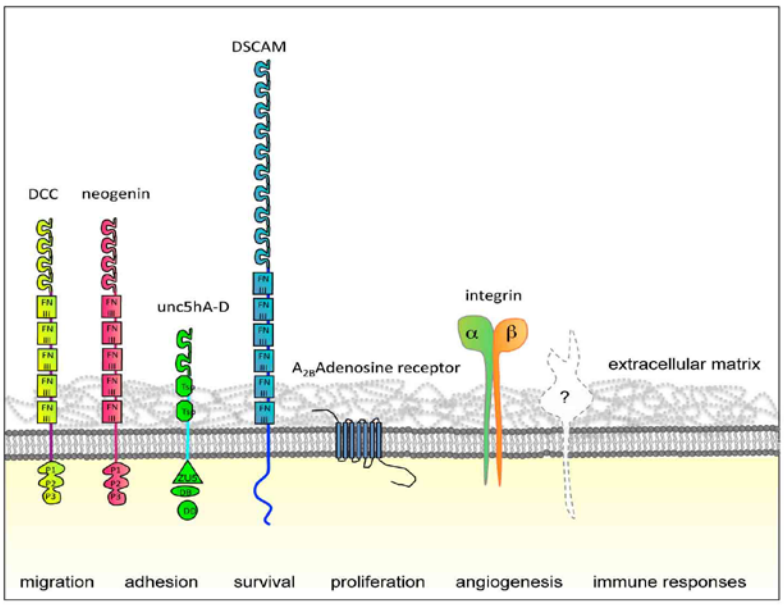
A number of other putative netrin receptors and netrin binding proteins have been described. Integrins, which are alpha-beta heteromeric transmembrane receptors and mediate cellular adhesion to various extracellular matrix proteins, function as netrin receptors and contribute to tissue organization [176-180]. In particular, the laminin-binding integrins α6β4, α2β1, α3β1 and α6β1 bind netrins [181]. Secreted netrins also interact with extracellular matrix proteins such as

heparin [158,182], raising the possibility that they may be bound to cell surfaces by heparin sulphate proteoglycans (HSPGs). Domain VI of netrin-4, but not other netrins, binds to domain VI of laminins, potentially integrating netrin-4 into basement membranes [183].

More controversial remains the involvement of A_{2B} adenosine receptor (A_{2B}AR) as a netrin-receptor [184,185]. While reports suggests that netrin-1 binding to A_{2B}AR promotes neurite outgrowth [185] and mediates netrin's anti-inflammatory function [186-188], several other groups failed to reproduce these findings [138,184,189].

Taken together, cellular responses depend on the balance and type of netrin-receptors, available to bind netrin at the cell surface.

Figure iii



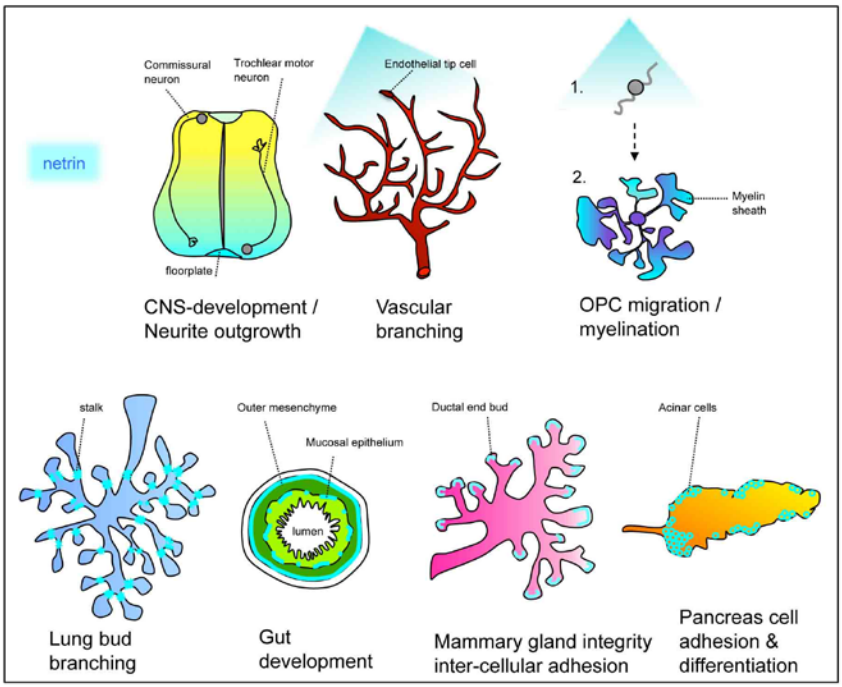
Netrins can bind to a variety of different receptors. Classical netrin receptors include DCC, neogenin, DSCAM and unc5 homologues A-D. Integrins, the adenosine receptor A_{2B} and extracellular matrix proteins are alternative netrin binding partners. Netrin may also associate to yet unidentified netrin-receptor.

3.3. How do netrins function during development? (A)

During development, many cell types express netrin to regulate cell migration, cell-cell and cell-matrix adhesion, and cell survival [154]. Researchers first discovered netrin-1 in the embryonic central nervous system (CNS), where it directs neurons along proper trajectories [174,190-192] (Figure iv). Outside of the nervous system netrins support proper vascular patterning [193-196], and contribute to

the maintenance of organ morphology in the developing lung [197], mammary gland [170], pancreas [198], salivary gland and kidney [183] (Figure iv).

Figure iv



Netrin has versatile functions during development. It guides axonal growth cones,regulates vascular sprouting, repels oligodendrocyte precurser cells (OPCs), and oligodendrocyte myelination. It controls early lung branching, cell adhesion and migration in bowl and pancreas. And netrin stabilizes cellular adhesion at the ductal end buds in the mammary gland.

At the ventral midline of the embryonic neural tube, netrin-1 is highly expressed by floorplate cells that attract DCC-expressing growth cones of commissural neurons but repel unc5-expressing trochlear motor neurons [199,200,154]. The absence of netrin-1 in knockout mice has dramatic consequences for axon guidance in the developing embryo [201,202]. These mice lack major commissures (corpus callosum, hippocampal commissure, pontine nucleus, defective anterior commissure, malformed fimbria) and die within a few hours after their birth [202].

3.3.1. Netrins adhesive functions during development

Netrins also stabilizes other cellular structures by promoting cell-cell and cell-substrate adhesion. Although netrin is a secreted molecule it can function as an immobilized substrate by remaining closely associated to cellular membranes or components of extracellular matrix [178,183,203,204]. When cells bind to netrin-1

immobilized on a substrate, they adhere with sufficient strength to generate a pulling force toward the netrin-1 surface [166,169,205]. *In vivo*, netrins' adhesive properties ensure proper morphogenesis in the lung, pancreas, and at the terminal end bud in the developing mammary gland [170,176,179,197,198]. During formation of the mammary gland, netrin-1 promotes the adherence of prelumenal epithelial cells to neogenin-expressing cap cells in terminal endbuds [170]. In developing muscle, netrin-3 and neogenin contribute to myotube formation and stimulate adhesive signaling to create larger myofibrils [164,172]. Vascular smooth muscle cells, important for arterial vessel contractility, also adhere to netrin-1-coated surfaces via neogenin [168]. Netrin-4, on the other hand, serves as an adhesive substrate for lymphatic endothelial cells, although the receptor mediating this function remains unknown [206,207]. Via integrins ($\alpha2\beta1$, $\alpha3\beta1$) pancreatic epithelial cells adhere to netrin-4, which in turn may facilitate the differentiation of pancreatic epithelial progenitor cells into islet cells [179]. Therefore, netrins not only contain sequences homologous to the extracellular matrix laminin superfamily [178,208], they also interact with laminins [158,183,209] and function as ligands for integrins [176-180,192], which mediate cell-matrix and cell-cell adhesion. This supports the argument that netrins play a role in cellular adhesion; however knowledge of netrins' adhesive properties is relatively limited and deserves further investigation.

3.3.2. Netrins' effect on the developing vasculature

To date, we know little about netrins' functions in the embryonic vasculature [193,195,196,210-212]. Netrins exert important effects on the vascular system, controlling sprouting angiogenesis. They guide endothelial tip cells, cells at the leading tip of outgrowing vascular branches, to arborize and elaborate the vascular network [168,193,195,213]. Endothelial tip cells also sense their environment and protrude or retract their processes [194,196] in response to chemotactic cues [152,214] similar to neuronal growth cones.

However, opinions diverge on whether netrin supports or inhibits sprouting of endothelial branches. One group has reported that netrin-1 inhibits extension and arterial arborization of endothelial tip cells through unc5B [193,194,196]. In these studies, netrin-1 applied to avian embryos inhibited angiogenesis [193,194,196], and loss of netrin-1a [194] in zebrafish embryos increased sprouting angiogenesis. In contrast, a different group has consistently reported evidence that netrin is pro-angiogenic. Knockdown of netrin-1a [211,215] or netrin-4 [212] resulted in missing or incorrectly branched parachordal and intersegmental vessels in zebrafish embryos. Thus, how netrins contribute to the elaboration of the vascular network is still a matter of debate.

3.4. How do netrins function during adulthood?

Beyond embryonic development, adult neural and non-neural tissues also express and respond to netrins. In the adult spinal cord, netrin-1 promotes adhesion between neurons and oligodendrocytes, where oligodendrocytes wrap around axons with a protective myelin-sheath, to ensure proper signal transmission. Both mature oligodendrocytes [203,216] and many types of neurons express netrin-1 [194,216-218]. In fact, netrin is enriched at the interface between the axon and the myelinating oligodendrocyte, where it maintains adhesive oligo-axonal interactions and structural integrity at the paranode [171] (Figure iv). In the absence of netrin-1 or DCC, mature paranodes become disorganized and paranodal loops detach from the axon [171].

Because this thesis focuses on studying netrins' effect on the BBB endothelium during health and inflammatory disease, it is appropriate to focus on netrins' two main functions in the adult: regulating vascular growth, and modulating inflammatory responses by reducing immune cell motility. To evaluate netrins' effect on vascular angiogenesis, most researchers used models of ischemia, such as cerebral ischemia [163,219,220], hind limb ischemia, lung hypoxia, and heart ischemia [221-223,224] (for details refer to Table 2). When investigating netrin's role as an anti-inflammatory factor, most studies focused on acute inflammatory responses that largely involve the recruitment of monocytes and

granulocytes during inflammatory bowel disease (IBD) [225], acute lung and kidney injury [187,226-230], and arthrosclerosis [231]. In summary, in most of those pathologies, netrin ameliorated disease symptoms by acting either as a pro-angiogenic factor or as an anti-inflammatory mediator.

3.4.1. Netrins' effects on the adult vasculature (B)

Netrin-1 and netrin-4 are expressed by the adult epithelium and vascular endothelium of several organs, including the eyes [204,213,232], ovaries [208], submandibular glands [183], lungs [186,233], kidneys [228], intestines [234], placenta [195,235-237], and the brain [219]. Similar to their role during development, netrin-1 and -4 modulate the adult vasculature (Figure iv), where they mostly promote vascular growth during hypoxia or ischemia. Netrin-therapy results in vascular branching, and increases vessel density, thus ameliorating pathological symptoms in diabetes, hind limb ischemia [211], ischemic hearts [221-223] and cerebral ischemia (stroke) [218-220]. Although some report netrin to have anti-angiogenic effects, most researchers ascribe pro-angiogenic functions to netrins.

Normally angiogenesis is quiescent in the adult endothelium [61]. However, under pathological conditions, such as hypoxia, ischemia, or wound healing the endothelium becomes activated to restart angiogenic growth. Additionally, angiogenesis occurs to regulate endometrium growth, placental development, and organ growth. All of these are conditions of an increased demand for oxygen, nutrients and other factors: originating either from limited oxygen supply or underperfusion as seen in hypoxia and ischemia, respectively, or due to higher demands of oxygen in situations of growth (cancer) or repair (inflammation, wound healing).

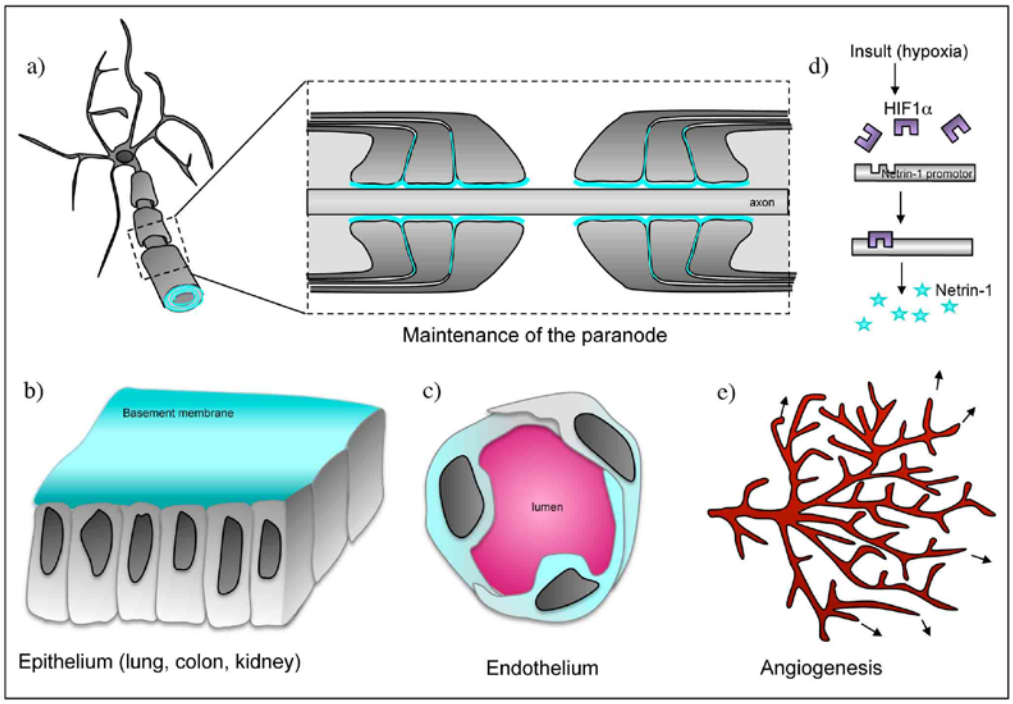
During angiogenesis, a proangiogenic stimulus activates endothelial cells to become motile and extend filopodia, which allows them to branch out. The leading tip cell continues to move away from the capillary as cells behind it migrate in and divide, forming a stalk. Then, the sprout begins to hollow out to form a tube [238]. In this process, pinocytic vesicles merge into larger vacuoles that then fuse with vacuoles of neighboring cells to build a continuous lumen that runs

through the capillary sprout [239]. Capillary sprouts then spontaneously develop internal pinocytic vesicles that then join with one another and with neighbouring cells to build a tubular network. Throughout this process, many cells share a single lumen; however, they do not share cytoplasm and remain separate cells.

To move away and branch out from the vessel, endothelial cells a) rearrange their cytoskeleton and contract [133,240], b) enzymatically deconstruct the supporting basement membrane that normally provides vessel stability [99,241,242], and c) loosen inter-endothelial connections [75,243,244]. Thus it is generally accepted that angiogenesis involves a relative compromise of endothelial barrier permeability. Nevertheless, angiogenesis is a highly regulated process, in which a vast number of factors [245-247] stay in balance to remodel the vascular network and its surrounding extracellular matrix, yet maintain relative vessel integrity and limit permeability [241,243,248,249]. Angiogenesis is regulated by growth factors (VEGF, PDGF, FGF), adhesion molecules (integrins, cadherins, JAM), ECM proteins (collagen, fibrinogen, laminin, thrombospondin, fibrin), proteinases (plasmin, tissue plasminogen activator, MMP-2, MMP-9), and proteinase inhibitors (tissue inhibitors of metalloproteinases = TIMP), maturation, morphogenic and guidance cues (angiopoetins, ephrins, Wnt, Notch, netrin, semaphorin, slit, NogoB), and numerous intracellular signalling and transcription factors. Many of the vascular modulating factors are stored within the ECM and can be released or activated by enzymatic cleavage.

However, when levels of proangiogenic factors rise acutely and are released suddenly in large amounts [250], such as during acute disease or ischemia, the endothelium responds drastically and becomes hyperpermeable so that plasma proteins leak into the surrounding tissue [243].

Figure v



During adulthood, netrin-1 (aqua-coloured) maintains adhesive oligo-axonal contacts at the paranode, a). Its expression localizes to epithelial basement membranes, b) and vascular endothelial cells, c) and netrin-1 gene expression is induced by hypoxia, d). Both, netrin-1 and -4 function as angiogenic factors for the adult vasculature, e).

3.4.1.1. Netrins' pro-angiogenic functions

Hypoxic [186] or ischemic stress [219] triggers the activation of the angiogenic transcription factor hypoxia inducible factor-1 alpha (HIF-1α) (Figure v). HIF-1α then induces transcription of pro-angiogenic factors, including netrin-1 [186,251]. Similar to other angiogenic factors, netrin-1 also promotes angiogenesis. *In vitro*, both arterial and venous derived endothelial cells migrate, proliferate, and form tubes in response to netrin-1 [162,168,213,217,221,252] or netrin-4 [207,211,212,219] (Table 3). Netrin-1 increased sprouting angiogenesis when applied to aortic [210,215] or placental [195] explants. Implantation of netrin-1-containing hydropellets into the corneal pocket of the eye [168,213], or injection into the aorta [223], or muscle [252] all increased vessel density and vascular outgrowth. Transplantation of mesenchymal stem cells, transfected with either Shh [221] or netrin-1[222,252] into the heart muscle or hind limbs protected the heart from infarction and increased vessel density, blood

flow, elevated levels of nitric oxide or VEGF and reduced scar tissue formation. Others injected netrin-1-encoding viral vectors into the brain [217,218,220] or skeletal muscle [211] prior to an ischemic insult, and observed enhanced vascularization and increased blood flow. In consequence to netrin's angiogenic properties, netrins improved nerve conduction in hind limb ischemia and in polyneuropathic co-morbidities in db/db diabetic mice [211], ameliorated heart function in infarcted hearts [221], and enhanced placental vascularization, thus positively affecting fetal growth, survival and development [195,236,237,253]. In a similar fashion, netrin-4 promotes angiogenesis under ischemic conditions in brain [219], hind limbs [211] and lymphatic vessels [207]. In the ischemic brain, netrin-4 increases vessel density and improves behavioral recovery when applied through transplanted osmotic pumps [219]. Transgenic mice, overexpressing netrin-4 in keratinocytes exhibit redder skin and have a higher lymphatic vessel density [207]. These mice also appear to be smaller and lack a fur coat.

At this moment, we know little about how netrin mediates its effects on endothelial cells [210]. One possible mechanism by which netrin-1 promotes angiogenesis in ischemic hearts may be through DCC signaling. DCC augmented synthesis of the cardio-protective nitric oxide, by upregulation of eNOS, and promoted mitogenic signaling via activation of ERK1/2 [210,223]. This pathway was further enhanced as cardiomyocytes up-regulated DCC expression in response to nitric oxide [223]. Netrin-4's angiogenic functions also involve activation and phosphorylation of intracellular signaling molecules: Src family kinases, FAK, Akt, Jnk, and ERK1, 2 [165,207,212,254]. Although many researchers reported that endothelial cells express unc5B and/or neogenin, most studies could not identify the netrin receptor responsible for netrins' angiogenic functions. In these cases it may be important to consider other non-classical receptors, receptor complexes or unknown receptors that may mediate netrin's effects.

3.4.1.2. Netrins' anti-angiogenic functions

Netrin has also been documented to inhibit angiogenesis. Han et al. reported that repeated daily applications of netrin-1 to the alkali burned eye reversed corneal neovascularization [232]. While it may appear paradoxical that netrin has both pro- or anti-angiogenic functions, these diverse functions may be dependent on netrin's concentration [213], with low (nanomolar) concentrations of netrin promoting and high (micromolar) concentrations inhibiting angiogenesis [194,213,196,255].

3.4.2. Netrin-1 has anti-inflammatory properties during acute injury (C)

Besides regulating angiogenesis during adulthood, researchers also recognized that netrins possess anti-inflammatory properties and explored netrin's immune-regulatory potential in several inflammatory pathologies. During acute inflammatory responses, netrin-1 therapy dampens immune responses and reduces tissue damage in lungs, kidney, gut, and atherosclerotic plaques [138,186-188,211,219,220,223,225-229,231,256-259] (Figure vi). Netrin-1 inhibits immune cell migration into injured tissues and decreases levels of chemokines, cytokines and other potent inflammatory mediators. Interestingly, the expression of netrin-1 itself is regulated by inflammation [138,186,187,225,233].

3.4.2.1. Netrin-1 expression is regulated during immune responses

Netrin-1 expression increases in the inflamed colonic, renal, and pulmonary epithelium and endothelium [138,186,225,227,256,260], and in activated macrophages [138]. Several groups also observed elevated levels of secreted netrin-1 in supernatants from activated macrophages [138] and in urine from mice with acute kidney injury [227,229,230,261]. This increase in netrin-1 expression is regulated by a transcription factor downstream of many inflammatory stimuli, nuclear factor kappa B (NF-κB) [187,260,138,262,263]. However, others showed contradictory results, demonstrating that inflammation reduced netrin-1 levels in lungs and colon [187,188,233]. Although we know little about how and under which conditions netrin-1 expression is regulated, it appears to depend on the integration of NF-κB and HIF-1α during inflammation and hypoxia, processes that are closely associated [264-266].

Interestingly, netrin-1 levels also rapidly increase in urine during acute kidney injury [227,230,257,267]. This has encouraged researchers to assess netrin's potential as an early biomarker of acute kidney injury in humans [229,230,257]. Results showed that, urinary netrin-1 levels rapidly increased in patients with heart-related kidney failure and in patients who ultimately will develop acute kidney injury as a complication after cardio-pulmonary bypass surgery.

3.4.2.2. Netrin-1 inhibits immune cell migration

Netrin-1 can promote chemo-attractive or –repellent responses in neural and non-neural cells. In the immune system, chemokines control leukocyte guidance. They are small proteins with chemotropic functions, that attract immune cells to a site of injury or a secondary lymphatic tissue [118]. Chemokines can be distinguished into two groups, CCL or CXCL, according to the configuration of a cysteine residue. Examples are interleukin IL-8/CXCL8, IP10/CXCL10, monocyte chemotactic protein-1 MCP-1/CCL2 and macrophage inflammatory protein-1 alpha MIP-1α/CCL3. Both are ligands that bind to G-protein coupled chemokine receptors (CCR or CXCR). Other factors such as growth factor containing serum and bacterial peptides (fMLP, N-formyl-methionine-leucine-phenylalanine) are also known to be chemoattractive.

Netrin-1 reduced immune cell migration in various inflammatory models *in vivo* [186-188,226,228,233,258,268]. Intravenous or inhalation therapy with netrin-1 decreased the number of immune cells within injured lungs of mice [187] and swine [226]. It rapidly reduced the inflammatory response and ameliorated lung tissue damage [186,188,226]. Neutrophils and monocytes no longer invaded kidneys, damaged by ischemia-reperfusion injury with intravenous netrin-1 application [227,228,258]. In turn, mice lacking one netrin-1 allele exhibited more severe signs of ischemic kidney damage (indicators of renal tissue injury are increased levels of plasma creatinine, increased excretion of urinary K^+ and cast formation) [227]. However, intravenous injection of netrin-1 reversed these signs of inflammation and decreased tissue necrosis, apoptosis, cytokine and chemokine secretion, leukocyte infiltration, and renal tissue damage [227,228,258]. Netrin-1 promoted a

similar drop in total number of immune cell infiltrates in the inflamed peritoneum [188,233]. Reduced levels of endogenous netrin-1 in heterozygous mice increased immune cell infiltration across the mucosal epithelium or endothelium in lung [186,187], kidney [227], peritoneum [188], and intestines [256]. In its presence, netrin-1 stopped predominantly innate immune cell types such as monocytes and granulocytes from migrating towards strong chemoattractants (fMLP) in *in vitro* migration assays [186,233]. Netrin-1 also cancelled chemoattractive responses of monocytes to IL-8 [233] and macrophages to MCP-1, CCL-19, CCL-21 [138], suggesting that netrin-1 inhibits a broader spectrum of chemokines.

In absence of a chemokine, netrin-1 alone did not repel immune cells [138,233], which argues against the idea that netrin-1 is a chemorepellent. Additionally, regardless of whether netrin-1 was added to the bottom, top or both chambers in a Boyden chamber migration assay, netrin-1 inhibited motility towards chemokines, [138,233]. This indicates that netrin-1 provides no directional immune cell guidance but rather paralyzes the cell. Van Gils and colleagues supported this theory by demonstrating that cells treated with netrin-1 remained round and did not protrude lamellipodia, as netrin inhibited actin-polymerization, required for cell migration [138]. Thus, netrin-1 restrains chemokine mediated actin cytoskeleton assembly required for immune cell motility.

Both Unc5B [138,233,258] and $A_{2B}AR$ [186-188] have been proposed to mediate netrins inhibitory effects on immune cell migration. However, it is still debated which of these receptors signals the netrin response. While $A_{2B}AR$ knockout mice remained unresponsive to netrin-1's anti-inflammatory effects [186-188], in other models, blockade of Unc5B inhibited *in vitro* migration of leukocytes [138,233,258].

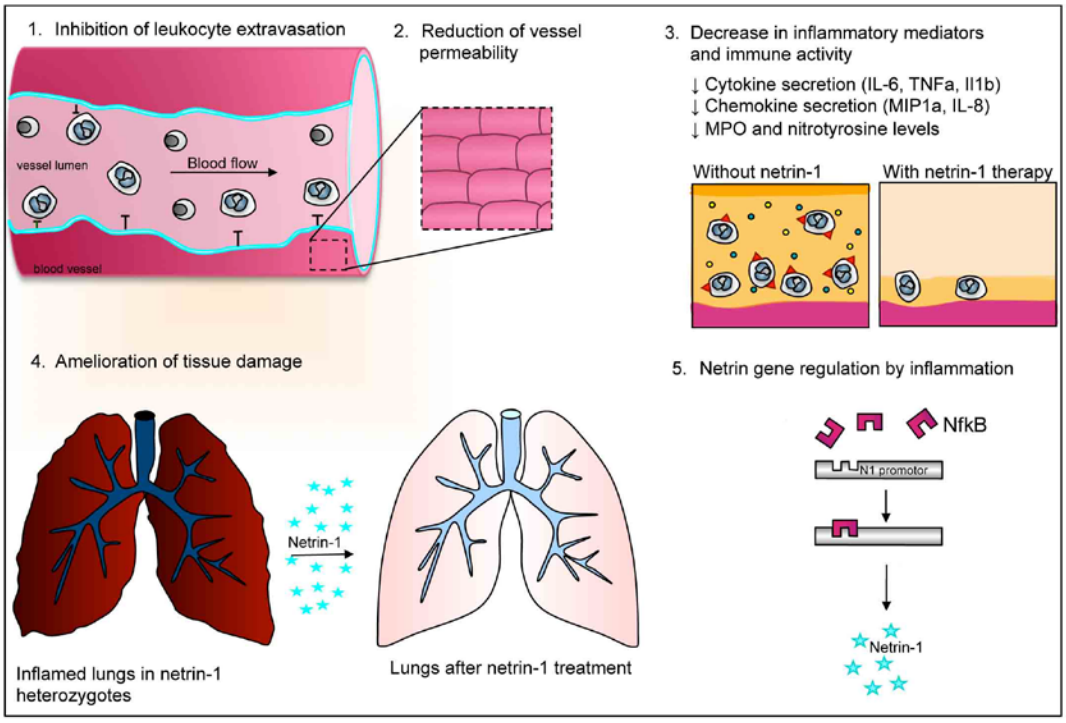
3.4.2.3. Netrin-1 reduces immune cell activation and inflammatory mediators

To identify and quantify leukocyte migration and infiltration, most groups used myeloperoxidase (MPO) as a marker for neutrohilic granulocytes [186-188,226]. Because MPO is an enzyme needed to generate the oxidative burst, in which granulocytes release potent cytotoxic mediators and generate reactive oxygen and nitrogen species (ROS/RNS), decreased MPO levels might not only be a sign of

fewer immune cell infiltrates but also an indicator of lesser granulocyte activation. In fact, mice with only one allele of netrin-1 displayed increased MPO enzymatic activity [187] and overexpression of netrin-1 in atherosclerotic vessels decreased nitrotyrosine, an indicator for the presence of ROS and RNS, within the aorta [268]. Activated neutrophils also appeared to slightly decrease superoxide levels in presence of netrin-1, however this effect was not significant [233]. Others observed that netrin-1 dampened immune cell activation by down regulating the co-stimulatory marker CD86 on myeloid antigen presenting cells [259]. Besides netrin's ability to reduce immune cell infiltration and activation, it also reduces levels of acute response cytokines tumour necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and primarily myeloid-targeted chemokines MIP-1α, MCP-1 and IL-8 in bronchio-alveolar lavages, peritoneal lavages, blood serum, and kidney tissue [186-188,226,258,259].

Although netrin-1 therapy ameliorates acute disease pathology [188,226,233,256] it remains to be elucidated on which cell types netrin-1 exerts its multiple anti-inflammatory effects. In contrast to netrin-1, only one research group has examined whether netrin-4 has anti-inflammatory functions as well. No effect was detected in a model of acute kidney injury [228].

Figure vi



Netrin-1 has anti-inflammatory effects. 1.) It inhibits leukocyte migration, 2.) reduces endothelial permeability, 3.) reduces levels of inflammatory mediators, and 4.) reduces tissue damage. Netrin-1 is also regulated by inflammation. 5.) NfkB binds to the netrin-1 promotor and induces netrin-1 transcription

4. DO NETRINS STRENGTHEN THE BLOOD-BRAIN BARRIER AND PROTECT IT DURING IMMUNE ATTACK?

4.1. Do netrins regulate inter-endothelial adhesion during BBB development? (A)

We know that netrins are expressed in the developing CNS. They regulate vessel growth and arborization [193-195,211], and they function as adhesive proteins. However, we do not know whether netrin facilitates endothelial cell barrier function, particularly at the specialized CNS vasculature, promoting adherence to one another and the development of a tight BBB. Netrin is a potential candidate to strengthen the BBB, because the intracellular domain of unc5 netrin-receptors contains a domain homologous to proteins that promote adhesive barrier strength [269]. Unc5, the tight junction protein ZO-1, and the actin-cytoskeleton–linking protein ankyrin, all harbor a ZU-5 domain [270], however, the function of this domain remains unknown. Furthermore, hedgehog signaling, which increases inter-endothelial adhesion at the developing BBB and tightens the barrier, induces netrin-1 expression [221,271].

4.2. Does netrin maintain endothelial barrier integrity during adulthood? (B)

While we know that netrin has adhesive properties and promotes angiogenesis during adulthood, and angiogenesis changes endothelial permeability, a limited number of studies have addressed whether netrin also affects endothelial barrier function. Netrin-1 increases connectivity of human microvascular endothelial cells *in vitro*. Notably, it reduced protein diffusion through the endothelial barrier and enhanced barrier functions during hypoxia [186]. Furthermore, despite of its angiogenic properties during cerebral ischemia, netrin-4 did not appear to increase BBB permeability [219]. In contrast, in ischemic brains treated with netrin-4 the level of serum albumin extravasation was perhaps even reduced, compared to untreated mice. Hoang and colleagues quantified the area of protein leakage within the infarct area, which remained the size of the initial infarct area, and concluded that netrin-4 does not affect BBB permeability. To measure BBB

permeability, one could have quantified albumin levels by measuring its staining intensity within the infracted brain area, which would have provided information about the amount of protein that has diffused into the brain. However, they did not measure actual albumin levels within that area. Therefore, despite netrins' angiogenic functions it appears to maintain or potentially reduce serum protein-leakage in the CNS. Additionally, although netrin-4 overexpressing mice showed increased dermal vessel density and local application of VEGF to the skin increased vascular permeability, compared to control mice treated with VEGF [207], the researchers who carried out this study did not normalize endothelial permeability to vessel density. Another group demonstrated that simultaneous application of netrin-4 to skin treated with VEGF did not alter VEGF-induced permeability [255]. However, it has to be noted that Eveno and his group used a very high dose of netrin-4 (up to 10 μg). Furthermore, overexpression of netrin-4 reportedly decreased ZO-1 expression *in vivo* [207]. However, regardless of netrin-4's presence or absence, staining for ZO-1 did not show any strand-like patterns, typical for tight junction molecules, instead it resembled and narrowly co-localized with the lymphatic marker LYVE-1.

Evidence for netrins' ability to regulate vascular barriers remains sparse, lacks detail, and is controversial. Here, we propose to test the idea that netrin regulates vascular permeability, by promoting cell adhesion, and maintaining endothelial barrier integrity, even during angiogenesis. In support of this, vascular smooth muscle cells [168] adhere to an immobilized netrin-1 substrate via neogenin. But whether netrin influences the adhesion of endothelial cells remains controversial [168,206].

4.3. Does netrin-1 have anti-inflammatory and barrier-protective properties at the BBB? (C)

It has been shown that netrin has anti-inflammatory properties during acute pathologies [186], and that under non-inflammatory conditions it potentially maintains endothelial barrier stability [219]. However, it had not been determined whether netrin protects the endothelial barrier under inflammatory conditions in

the CNS that are associated with BBB breakdown. Furthermore, it is not known whether netrin has anti-inflammatory effects at the BBB during inflammatory diseases of the CNS. Anti-inflammatory effects of netrin at the BBB may reduce immune cell migration across the BBB and diminish immune cell accumulation in the CNS.

A limited number of studies have described netrin in inflammatory conditions of the CNS. In the spinal cord of rats with experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, exhibiting BBB breakdown, immune cell infiltration and demyelination, increased netrin-1 expression was detected and was highest at the peak of the disease, compared to control animals [272]. Similarly, netrin-1 expression increased in experimental autoimmune neuritis (EAN), a T cell mediated peripheral nerve injury model [273]. However, these studies are purely descriptive and we do not know how netrin functions in these chronic neuro-inflammatory conditions.

5. BENEFITS OF STUDYING NETRIN'S FUNCTION AT THE BBB

A) Studying the effect of netrin-1 on BBB-permeability and integrity in the developing CNS will provide insight into whether netrin-1 promotes the adhesion of vascular endothelial cells. If netrin promotes blood-brain barrier integrity, then our findings will have identified a novel factor to promote barrier genesis, among the few known to regulate it.

B) Furthermore, if netrin continues to have adhesive properties at the mature BBB, then it may be possible to apply netrin to maintain and protect this important endothelial barrier.

C) Lastly, investigating netrin's influence during neuroimmune disease, of which BBB breakdown and immune cell infiltration are hallmarks, we will not only answer whether netrin reduces immune cell infiltration and prevents immune-mediated damage to the CNS, we will also know whether netrin protects and supports BBB stability. Such a study may also provide insight into the effect of netrins on adaptive immune cells, because EAE is an autoimmune disease that is

mediated by auto-reactive T and B cells. This will help us understand how or whether netrin influences T cell behavior.

6. EXPERIMENTAL APPROACH

A) We compared newborn wild type and netrin-1 knockout mice to evaluate whether the absence of netrin-1 loosens inter-endothelial adhesiveness at the blood-brain barrier. To do so, we quantified levels of endothelial junctional protein expression by western blot and measured plasma protein extravasation into the central nervous system by *in situ* immunostaining.

B) To then delineate netrin's effect on barrier stability of the adult BBB, we applied netrin-1 and netrin-4 to primary cultures of human brain-derived endothelial cells and quantified the effect on tight junction protein expression and tracer molecule diffusion through an *in vitro* permeability model of the BBB.

C) Using experimental autoimmune encephalomyelitis as a disease model, characteristic for barrier breakdown and immune cell extravasation, we tested whether netrins have anti-inflammatory potential at the BBB and whether netrins ameliorate EAE disease symptoms. We quantified whether netrin-1 would promote barrier integrity during inflammatory disease by staining and quantifying brain and spinal cord sections for levels of extravasated plasma proteins. Using flow cytometry analysis and immunostaining, we also counted immune cell numbers in the CNS and characterized their phenotype. Finally, we determined EAE severity by scoring control and netrin-1 treated mice according to the degree of paralysis during the course of the disease.

7. HYPOTHESES

A) We speculate that netrin-1 increases endothelial barrier stability.

B) We also propose that netrins continue to maintain and promote barrier properties during adulthood.

C) Finally, we hypothesize that netrins have anti-inflammatory effects at the blood-brain barrier and protect it from damage and during immune-mediated CNS disease.

8. SUMMARY OF MAIN FINDINGS

A) We demonstrate that newborn mice lacking netrin-1 develop a normal brain microvasculature; however, these mice have a more permeable blood-brain barrier, compared to wild type mice. Netrin-1 knockout mice showed increased plasma protein leakage across the barrier, which coincided with altered expression levels of junction proteins by endothelial cells.

B) Adult human brain-endothelial cells express both netrin-1 and netrin-4. They reduce barrier permeability and up regulate tight and adhesion junction protein expression and enrich them in membrane microdomains, to form functional junctional complexes.

C) Furthermore, netrins protect the BBB and have anti-inflammatory effects during EAE. Netrin decreased the secretion of inflammatory mediators by brain-endothelial cells, reduced immune cell infiltration into the CNS, diminished BBB leakage, and reduced the severity of the neuro-autoimmune disease EAE.

MATERIALS AND METHODS

MATERIALS and METHODS

Netrin-1 knockout mice phenotyping and genotyping, and tissue harvesting.
To assess the effect netrin-1 loss during BBB development, we characterized mice at postnatal day 0 (P0) from parents that were both heterozygous for netrin-1 in a CD-1 background (obtained from Marc Tessier-Lavigne [Genentech]) [190]. Heterozygotes carry an intracellular beta galactosidase reporter gene and a gene insertion encoding a transmembrane CD3 protein in one of their netrin-1 alleles. We performed all animal procedures in accordance with the "Canadian Council on Animal Care" guidelines for the use of animals in research. After giving birth, the mother was killed by cerebral dislocation. Newly born offspring were quickly isolated and kept warm on a heating pad. We identified netrin-1 knockout mice quickly by their symmetrical limb movements, and inability to move back onto their belly, we began experimental manipulations in these mice first because netrin-1 knockout mice die quickly after birth. We then sampled both tails (for genotyping) and brains from P0 newborns for quantitative immunohisto-fluorescence analysis of the brain vasculature.

We confirmed the offspring's' genotype by a) quantitative analysis of beta galactosidase and netrin-1 protein normalized to the amount of actin by western blot (from brain), b) semi-quantitative PCR for beta galactosidase DNA (extracted from tails), normalized to levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), c) semi-quantitative enzymatic conversion of X-galactose (X-Gal) into a blue reaction product catalyzed only in presence of beta-galactosidase positive tail tissue. Briefly, for the X-Gal staining, tails were first equilibrated in a phosphate buffer at pH7.4, supplemented with 2mM $MgSO_4$ and 5mM EGTA. Then tails were immersed in a phosphate buffer, containing 2mM $MgSO_4$ and Na Deoxycholate and NP-40 to provide an optimal environment for the final enzyme reaction. In the last step, we added pre-warmed (37° C) 5mM $K_3Fe(CN)_6$ and 5 mM $K_4Fe(CN)_6$ and 1 mg/ml X-gal to the tails. After 30 min and 1 hour, two people independently recorded the color intensity observed in the tail (high intensity = knockout, low intensity = heterozygote, no staining = wild type).

Newborn mice were anesthetized with 60 µl of 3.8 % chloral hydrate i.p. per mouse and perfused transcardially with 1 ml of ice-cold PBS. We then removed the brain, cut it sagittally into two halves. One half was embedded in optimal cutting temperature (OCT) Tissue Tec and then fresh frozen at -80° C for immunohistostaining analysis. The other half was homogenized, lysed in 2% SDS buffer, sonicated and quantified (BCA protein quantification kit, Pierce) for western blot analysis. Tissue blocks were then evaluated by immunohisto-fluorescence to quantify extravascular diffusion of plasma proteins.

Protein lysates were also analyzed by western blot for the expression of TJ and AJ proteins. All procedures with animals were performed in accordance with the "Canadian Council on Animal Care" guidelines for the use of animals in research.

Isolation and culture of human brain-derived microvascular endothelial cells and collection of human foetal astrocyte conditioned media.

With informed consent and ethical approval (ethical approval number HD04.046), human temporal lobe material was obtained from patients who underwent surgical treatment for intractable temporal lobe epilepsy. Primary human brain-derived endothelial cells (BBB-ECs) were isolated as described elsewhere [131,274,275]. In short, the tissue was removed from the meninges, minced, resuspended and washed several times in phosphate-buffered saline (PBS) to remove residual blood and was then homogenized. Sequentially, the homogenate was filtered three times through meshes of different pore sizes (first through a 350µm and then twice through 112µm pore-sized meshes). Cells obtained after the third filtration were then treated with collagenase type 4 (1mg/ml) and plated on 0.5% gelatin-coated culture dishes in endothelial cell culture media (M199 cell culture media, with 10% foetal bovine serum [FBS], 10% human normal serum, 1% Insulin-transferrin-selenium, 5µg/ml EC-growth supplement). Plated cells were expanded after cell colonies had visibly formed. Cultures were then tested for cell specificity with markers specific for endothelial cells, astrocytes and glia cells.

Experiments were performed with BBB-ECs from at least four different tissue samples.

To harvest astrocyte conditioned media (ACM), human foetal astrocytes (obtained with the approval of the Canadian Institutes of Health Research [CIHR] the human foetal repository in the Albert Einstein College of Medicine, Bronx, NY) were isolated and grown in complete DMEM (Invitrogen), supplemented with 10 % FBS [77,276]. ACM was harvested every 7 days from confluent astrocyte monolayers, filtered and then added to the BBB-EC when indicated.

Preparation of human and mouse tissue-sections for qualitative immuno-histostaining for endothelial netrin expression.

Adult mice (C57BL/6), with or without EAE, were anaesthetized and PBS-perfused. Brains and spinal cords were removed, embedded in OCT compound and frozen [44]. Human brain tissue was obtained post-mortem from individuals diagnosed with multiple sclerosis (MS). We identified lesion sites and normal appearing white matter (NAWM) by the presence or absence of immune cell infiltrates and demyelination, respectively.

OCT embedded frozen CNS tissues were cut into 7 μm sections, dried and then fixed with acetone and 70% ethanol (both at -20°C). Tissue sections were blocked with 10 % FBS in PBS, permeabilized with 0.05% Tween in PBS and incubated with primary antibody (diluted in 3% FBS), followed by washes, secondary antibody incubation and final washes. The tissue was mounted in Mowiol.

Primary antibodies: Mouse anti GFAP-Cy3 (1:2000, SIGMA), FITC-Esculentum-lectin (1:2000, Vector Laboratories), rabbit anti Caveolin (1:100, Santa Cruz), Goat anti netrin-4 (1:50, RnD Systems), rat anti netrin-1 (1:100, RnD Systems) goat anti neogenin (1:50, Santa Cruz C-20), goat anti unc5B (1:1000 RnD Systems), rabbit anti unc5C (1:10000 provided by Tompson Lab), TOPRO (1:300). Alexa-Fluorochrome-conjugated secondary antibodies (Invitrogen) were diluted 1:500 in PBS. As isotype controls for netrin-1 and -4 staining, we applied the same concentration of rat IgG2a and goat IgGs in place of rat anti netrin-1 and

goat anti netrin-4 antibodies, and added their respective secondary antibodies. Qualitative microscopic evaluation was performed using a LEICA confocal microscope.

Table 1 Specific markers for cells at the BBB

Endothelial cells	Lectin / von Willebrandt factor 8 (vWF8) / caveolin-1
	g-glutamyl transpeptidase (gGT) / glucose transporter-1 [Glut-1] / P-glycoprotein (Pgp/Abcb1)
Neurons	Synaptophysin (Syn
Pericytes	PDGFR
Astrocytic endfeet	Aquaporin 4 (AQP4)
Basement membrane	Endothelial laminin 8 / Astrocytic laminins 1 and 2

Quantitative analysis of vessel density and morphology in brain-sections of wild type and netrin-knockout mice.

OCT embedded frozen brain tissues were cut into 7 μm sections, dried and then fixed with acetone and 70% ethanol (both at -20°C) (see also section: 'Netrin-1 knockout mice phenotyping and genotyping, and tissue harvesting').

Tissue sections were blocked with 10 % FBS in PBS, permeabilized with 0.05% Tween in PBS and incubated with FITC-Esculentum-lectin (1:2000, diluted in 3% FBS, Vector Laboratories), followed by washes, and final tissue mounting in Mowiol.

We counted the number of lectin positive vessels per microscopic fields (140 μm x 140 μm, recorded with a LEICA confocal microscope) for 4 animals per group with each a total of 10 fields. To quantify the width of the meningeal network we measured the width of the lectin and ZO-1-positive vascularized area (measuring point were always perpendicular to the brain surface) using the Image J software.

***In vivo* BBB permeability to serum proteins in netrin-1 knockout mice and EAE mice.**

To measure serum protein leakage in blood vessels from netrin-1 knockout versus wild type mice (CD1 background), we sacrificed P0 mice from two netrin-1 heterozygous parent mice (see section: "Netrin-1 knockout mice phenotyping and genotyping, and tissue harvesting"). We stained brains from both mouse groups with antibodies against mouse plasma proteins: immunoglobulins, fibrinogen, and apolipoprotein B, together with markers for blood vessels (lectin, PECAM, laminin).

Similarly, to assess BBB integrity in netrin-1 or PBS treated mice during EAE, we quantified the extravasation of type G immunoglobulins (IgG) and fibrinogen by staining fresh frozen spinal cord sections and measuring their fluorescence intensity and area of leakage with the Image J software.

Primary antibodies: rabbit anti laminin (1:1500, DAKO), FITC-Esculentum-lectin (1:2000, Vector Laboratories), rat anti CD31 (1:1500, BD Bioscience), Goat anti apoB (1:300, Abcam), rabbit anti fibrinogen (1:1500, Innovative Research), goat anti mouse Alexa 546 conjugated; Molecular Probes), TOPRO (1:300, Invitrogen). Alexa-Fluorochrome-conjugated secondary antibodies (donkey anti mouse Alexa 546, donkey anti goat Alexa 546, donkey and anti rabbit Alexa 555 antibodies conjugated for plasma proteins; anti rabbit, anti rat Alexa 488 conjugated, Molecular Probes for vessels) were diluted 1:500 in PBS. As isotype controls for the blood plasma protein stain, we applied the same concentration of rabbit IgGs and goat IgGs in place of rabbit anti fibrinogen and goat anti apoB antibodies, and added their respective secondary antibodies. To control for the staining with donkey anti mouse IgGs conjugated with Alexa 546, we applied a donkey anti rabbit Alexa 546 antibody.

Microscopic evaluation was performed using a LEICA confocal microscope. Plasma protein leakage was determined by multiplying fluorescent (mean pixel intensity) and the area of leakage (number of pixels) of the respective fluorescent serum protein marker, using the Image J software.

RNA isolation, reverse transcription (RT), quantitative PCR (qPCR) for netrin and netrin-receptor screening on BBB-ECs and immune cell subsets.

We profiled netrin and netrin receptor expression by BBB-ECs and different subsets of immune cells. For this purpose BBB-ECs were cultured to confluency, then treated for 24 hrs in fresh ECM-media, supplemented without any treatment or with either recombinant Shh (100 ng/ml R&D systems), 40% ACM or TNFα and IFNγ (both at 100 U/ml). Similarly, purified immune cell subsets, were either harvested *ex vivo* (monocytes or CD4 or CD8 T cells) or activated *in vitro in* RPMI media. CD4 or CD8 T cells were activated for 3 days *in vitro* with or without 2.5 μg/ml okt3 (anti CD3 antibody, eBioscience). Monocytes were activated with 25 ng/ml IL-4 and 100 ng/ml GMCSF on days 1, 3, and 5 to harvest immature dendritic cells on day 6. For generation of mature dendritic cells, cells received an additional treatment of IL-4, GMCSF and LPS on day 7, and were harvested on day 9. Subsequently, BBB-ECs were trypsinized, immune cells pelleted, washed and then lysed with TRIzol (Invitrogen). RNA was isolated with the Mini-elute columns ® (Qiagen) according to manufacturer's instructions and treated with DNAse (Qiagen). To obtain cDNA, a total of 2 μg per RNA samples, random hexaprimers (Roche) and Moloney murine leukemia virus-RT enzyme (Invitrogen) were used for the RT reaction at 42° C. To measure the expression levels of netrins and netrin-receptors, cDNA samples were prepared in duplicate with the respective TaqMan FAM-labeled MGB probes (netrin-1: Hs00924151_m1; netrin-3 Hs00352403_g1; netrin-4: Hs00221915_m1; DCC: Hs00180437_m1; neogenin: Hs00933949_m1; unc5h-A: Hs01113563_m1; unc5hB: Hs00402127_m1; unc5h-C: Hs00186620_m1; unc5h-D: Hs00369888_m1; all by Applied Biosystems), together with human Beta actin-VIC as an endogenous reference control (to control for variability in amounts of RNA and for the reaction itself), and amplified and measured with the ABI PRISM 770 Sequence Detection System (Applied Biosystems). The cycling program was set to 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C in a final volume of 15 μl, including 1 μl of cDNA,

TaqMan Probe, VIC actin, 1x TaqMan Universal Master Mix (Applied Biosystems). Each probe was tested for its efficiency by including a serial dilution of cDNA from human foetal brain homogenates (positive control) to obtain a standard curve.

The ΔC_T method (C_T = cycle threshold = cycle at which gene of interest is detected in a linear range; ΔC_T = difference between C_T of the gene of interest and the C_T of the internal gene control actin) was used to compare absolute levels of mRNA.

Protein isolation and western blot analysis of netrin / netrin receptor / TJ protein expression by BBB-ECs.

BBB-ECs were cultured to confluency, treated for 24 hours, (kept the conditioned supernatant post 24 hours for ELISA analysis), and then scraped off and lysed with SDS lysis buffer (final 2% SDS) supplemented with protease inhibitors. The lysate was sonicated and quantified with the BCA detection kit (Pierce) for polyacrylamide gel-electrophoresis (Biorad).

To test the expression of netrin and netrin receptors under barrier promoting or destabilizing conditions, confluent BBB-ECs were treated for 24 hours with either 40 % astrocyte conditioned media (ACM) or a mix of tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) (each at 100 U/ml GIBCO), respectively, compared to untreated BBB-ECs.

To test the expression of tight junction molecules, confluent BBB-ECs were treated for 24 hours with either recombinant netrin-1, or recombinant netrin-4 (each at 100 ng/ml from RnD Systems), or 40 % ACM, or a mix of TNFα and IFNγ (at 100 U/ml each; GIBCO).

To test the expression of TJ proteins in presence of netrin and pro-inflammatory cytokines, we treated confluent BBB-ECs with a mix of TNFα and IFNγ (at 0.1 U/ml each; GIBCO) alone or together with either netrin-1 or netrin-4 (recombinant proteins each at 100 ng/ml from RnD Systems) for 24 hours.

Twenty micrograms of denatured protein were loaded per lane, separated by 10% SDS-PAGE, and blotted onto a PVDF-membrane (Biorad). After

blocking with 5% skim milk in TBST (Tris, NaCl, 0.1 % Tween), the primary antibody was applied, followed by a three-step wash with TBST, incubation with secondary horseradish peroxidase (HRP) coupled antibody and film detection of the reaction with ECL substrate (Amersham, ECL-Plus detection kit). Protein bands were then measured by densitometry using a BioRad Gel Doc System and Quantity One software.

For netrin and netrin-receptor analysis: Goat anti netrin-4 (1:500 RnD Systems), rabbit anti netrin-1 (1:5000 serum BLAZE), goat anti neogenin (1:50 Santa Cruz C-20), goat anti unc5B (RnD Systems), rabbit anti unc5C (Tompson Lab). Tight junction molecule expression was assessed with rabbit anti occludin (1:500 BD Bioscience), mouse anti JAM-1 (1:300 BD Bioscience), rabbit anti claudin-5 (1:250). A mouse anti actin (1:20000 SIGMA) antibody was used to detect beta-actin as a reference control. Secondary horseradish peroxidase (HRP) conjugated antibodies: rabbit anti goat-HRP (1:2000 DAKO), rabbit anti mouse-HRP (1:2000 DAKO), goat anti rabbit (1:5000 Jackson Laboratories), rabbit anti rat-HRP (1:2000 DAKO) were used.

Immuno-fluorescent staining analysis of netrin and TJ protein by BBB-ECs

To test the expression of netrin-1 and netrin-4, or TJ proteins by human primary BBB-ECs, we cultured BBB-ECs to confluency in 8 well plastic chamber slides and then treated them for 24 hours with either 40 % ACM, or a mix of TNFα and IFNγ (at 100 U/ml each; GIBCO), compared to untreated BBB-ECs. To test the effect of netrins on TJ protein expression we also added a treatment group with recombinant netrin-1, or recombinant netrin-4 (each at 100 ng/ml; RnD Systems). Then BBB-ECs. After 24 hour of treatment, cells were carefully washed twice with PBS and then fixed at room temperature with 70 % Ethanol. We added the following primary antibodies: goat anti netrin-4 (1:50, RnD Systems), rat anti netrin-1 (1:100, RnD Systems), mouse anti p120 (1:100, Invitrogen), rabbit anti ZO-1 (1:100, Invitrogen), rabbit anti alpha catenin (1:50, Invitrogen), and Alexa-Fluorochrome-conjugated secondary antibodies (Invitrogen), diluted 1:500 in PBS. As isotype controls for netrin-1 and -4 staining, we applied the same

concentration of rat IgG2a and goat IgGs in place of rat anti netrin-1 and goat anti netrin-4 antibodies, and added their respective secondary antibodies. Alexa-Fluorochrome-conjugated secondary antibodies (donkey anti mouse Alexa 546, donkey anti goat Alexa 546, donkey and anti rabbit Alexa 555 antibodies conjugated for plasma proteins; anti rabbit, anti rat Alexa 488 conjugated, Molecular Probes for vessels) were diluted 1:500 in PBS.

Qualitative microscopic evaluation was performed using a LEICA confocal microscope. TJ protein expression was quantified by measuring the mean fluorescent pixel intensity of endothelial TJ protein strands with the Image J software.

ELISA for detection of netrin in conditioned supernatants from BBB-ECs / human fetal astrocytes / human blood serum / CSF.

To quantify levels of soluble netrin-1 and netrin-4 in supernatants from astrocytes, and BBB-ECs, in blood serum from healthy young adults and multiple sclerosis patients or cerebral spinal fluid (CSF), we analyzed samples by ELISA. MaxiSorb 96 well ELISA plates (NUNC) were first coated with 50 µl of recombinant netrin-1, or -4 protein (serial dilution of 1000 ng/ml to 1 ng/ml R&D systems) to establish a standard curve, and undiluted blood serum, CSF, or supernatants of astrocytes or BBB-ECs and their respective media negative controls. We then incubated the plates over night at 4° C. Wells were then washed 5 times with PBS, containing 0.5% Tween20, and then blocked with 100 µl 5% fetal bovine serum in PBS for 2 hours. We then applied 100 µl of the primary detection antibody rat anti netrin-1 (1:500, R&D systems), diluted in 5% FBS (in PBS) and incubated the plates overnight at 4° C. After washing the plates 5 times with 250 µl PBS supplemented with 0.5% TWEEN, 100 µl of a secondary horse radish peroxidase (HRP)-conjugated antibody (1:3500 rabbit anti rat-HRP for netrin-1 in 5% FBS in PBS; DAKO) was added for 45 min, then washed 5 times again and incubated with the colorless TMB (3,3`,5,5` tetramethyl-benzidine; BD Biosciences) substrate. After stopping the enzymatic reaction with 50 µl 2N H_2SO_4 we quantified the absorbance of each sample at λ = 450 nm using a Bio-Tek EL800

96-well plate reader and then analyzed and calculated the netrin-1 concentration with KC Junior software (Bio-Tek, Mississauga, ON, Canada).

***In vitro* BBB permeability of BBB-ECs to tracer molecules.**

Experimental conditions were prepared in triplicates. BBB-ECs were plated on gelatin-coated 3 μm pore size Boyden Chambers at a density of $1.3x10^4$ cells per well. When cells reached confluency, they were treated with ECM media, supplemented with either 40% (vole/vole) astrocyte conditioned media (ACM), netrin-1 (100ng/ml RnD Systems) or netrin-4 (100ng/ml RnD Systems) (added to both top and bottom wells). After 24 hours, 50 μg/ml fluorescin-isothiocyanate-labeled bovine serum albumin (FITC-BSA, Invitrogen) or 50 μg/ml Alexa 647-labelled Dextran 10 kDa (Invitrogen) were added to a final volume of 700 μl in the upper chambers, while the bottom chambers contained a volume of 1.4 ml. Aliquots of 50 μl were harvested from each upper and lower chamber separately at 0, 2, and 4 hrs after tracer-molecule-addition and quantified with a fluorescence multimode plate reader (Biotek, Synergy 4). Baseline diffusion at 0 hrs was subtracted from the diffusion rates at 2 and 4 hrs.

For competitive netrin receptor blockade, we added neogenin-Fc, Unc5B-Fc or a control human Fc contract (at 5 μg/ml, from RnD Systems) together with netrin, 24 hours before the addition of tracer molecules. Plotting the fluorescence intensity ratio of bottom to top chamber over time generated graphs for BBB permeability.

Lipid raft isolation.

For each treatment condition, two T150 flasks of confluent BBB-ECs were washed, scraped off and pooled with ice-cold PBS. All procedures were performed on ice. Lipid rafts were isolated according to a previous protocol [77]. Briefly, the obtained pellet was resuspended and solubilized in 1% Brij58 in separation buffer, supplemented with protease inhibitors (BaculoGold BD Bioscience). The lysate was then homogenized and mixed one to one with 85% sucrose (final 42.5% sucrose) to be carefully overlaid (using a 19 G needle and

syringe) with a second layer of 35% sucrose and a third layer of 5% sucrose to be ultra-centrifugation carried out for 24 hours at 39,000 rpm at 4° C in a Beckman SW41 rotor. From top to bottom, 12 fractions of 1 ml each were harvested and evaluated for protein concentration with a BCA protein assay kit (Pierce), as was cholesterol content with the Amplex red cholesterol assay kit (Molecular Probes). Western blot analysis confirmed the presence of lipid raft markers CD59 or cholera-toxin-GM1.

Flow cytometry analysis proliferation of and netrin and CAM expression by BBB-ECs. [this subtitle isn't quite right]

For the evaluation of intra- and extracellular netrin by primary cultures of BBB-ECs, cells were scraped off with cold 2mM EDTA in PBS. For the extracellular stain, antibodies diluted in FBS/PBS were incubated for 30 min at 4° C (1:100 rat anti netrin-1, 1:50 goat anti netrin-4, both RnD Systems or rat IgG2a or goat IgG isotype controls); whereas for the intracellular stain, cells were first fixed and permeabilized with 4 % paraformaldehyde and 0.1 % saponin. After washing three times with PBS, supplemented with 0.1% FBS, cells were incubated with secondary antibodies (goat anti rat A488 or rabbit anti goat A488, Invitrogen) and washed again three times before analysis.

In order to test netrins effect on BBB-EC proliferation, trypsinized cells were labeled for Carboxy-fluorescein Succinimidyl Ester (CFSE) (1:1000) in DMSO for 10 min at 37 C, and the cells were washed three times with media, supplemented with 10% FBS. After CFSE staining a sample was analyzed for staining intensity at time zero. Cells were then divided into different treatment groups and incubated for 3 days and analyzed again by flow cytometry.

To test CAM-expression, endothelial cells were grown to full confluency and then washed, and treated with either 40 % astrocyte conditioned media, 100 ng/ml netrin-1 or netrin-4 for 24 hrs, or with 100 U/ml of TNFα and IFNγ. Cells were recovered by scraping off. Harvested cells were incubated with FACS antibodies: ICAM-1 (CD54)-FITC, MCAM (CD146), or isotype controls for 30 min at 4° C, washed three times with FBS/PBS and then visualized on a BD

LSRII flow cytometer. Analysis was done with the BD LSRII flow cytometer. Data acquisition analysis was performed using FlowJo Software. For quantitative analysis, the geometric mean of fluorescence intensity was compared to the isotype control and plotted on a graph.

ELISA for cytokine/chemokine secretion by BBB-ECs.

We quantified levels of secreted cytokines IL-6, GM-CSF and chemokines IL-8, MCP-1, IP10 in BBB-EC supernatants to assess whether netrins have anti-inflammatory effects on BBB-ECs. To obtain supernatants we treated confluent BBB-ECs for 24 hrs with fresh media, supplemented with either recombinant netrin-1, netrin-4 (each at 100ng/ml R&D Systems), or with TNFα and IFNγ (each at 100 U/ml, GIBCO).

We followed the manufacturer's ELISA kit protocols for IL-6, GM-CSF, IL-8, MCP-1, and IP10 (BD Biosciences BD OptEIA) detection. In short, a capture antibody was adsorbed to the well surface of the ELISA plate (NUNC maxisorb). This capture antibody then captures the specific cytokine in the test sample that is added. A biotinylated detection antibody, specific for the targeted cytokine, is then added together with a streptavidin-coupled HRP enzyme. Only in the presence of the targeted cytokine the detection antibody can bind, which in turn links to HRP via biotin-streptavin. Only in the presence of the HRP will the TMB substrate (BD Biosciences) together with peroxidase be converted into a colored product. By adding 2N H_2SO_4 the enzymatic reaction was then stopped before test samples are saturated. We quantified the absorbance of each sample at $\lambda = 450$ nm using a Bio-Tek EL800 96-well plate reader and then analyzed and calculated cytokine and chemokine concentrations with KC Junior software (Bio-Tek, Mississauga, ON, Canada).

Immune cell isolation and T cell skewing

Peripheral blood mononuclear cells (PBMCs) were isolated from young healthy adults and purified by Ficoll Hypaque density gradient centrifugation and MACS-bead separation (positive selection for CD14, CD4, CD8, and negative selection

for CD4CD45; all from Miltenyi Biotec; to evaluate netrin expression, skewing and cytokine secretion, and cell proliferation).

To skew effector T cells to either Th0, Th1, Th2, Th17 or Treg subsets, we added specific cytokines and cytokine blocking antibodies (Table 2; reagents from R&D Systems) to CD4CD45 T cells, bathed in Xvivo media (without serum) and plated in 24 well plates ($1x10^6$ CD4CD45 T cells per well). Per well, $1x10^6$ CD4CD45 T cells were activated with either 2 µg/ml CD28 and plate bound 2.5 µg/ml okt3 (eBioscience) or with $0.6x10^6$ monocytes and soluble 5 µg/ml okt3 (eBioscience). Cells were then incubated for 7 days in *vitro*.

Table 2 Cytokine and antibody concentrations for T cell skewing

Polarizing condition	recombinant cytokines added	Antibodies added (final 5 µg/ml)
Th0	IL2 (final 20 U/ml)	-
Th1	IL12 (final 10 ng/ml)	Anti IL4
Th2	IL4 (final 10 ng/ml)	Anti IFNγ, anti IL12
Th17	IL23 (final 25 ng/ml)	Anti IFNγ, anti IL4
Treg	TGFβ (final 5 ng/ml)	-

***In vitro* transendothelial migration assay.**

We assessed whether netrins regulate T cell transendothelial migration using *in vitro* Boyden chambers. Firstly, we plated $2.5x10^4$ BBB-ECs on 0.5% gelatin-coated 3µm pore sized Boyden chamber membranes (BD Bioscience). We grew BBB-ECs to confluency for 4 days in ECM, supplemented with 15% FBS and 40% ACM. One hour before adding $1x10^6$ CD4 or CD8 T cells to the upper well, we added the various treatment conditions to the bottom well: recombinant netrin1, netrin-4 (both from R&D systems at 100 ng/ml), SDF-1α (Invitrogen at 10 ng/ml) as a positive control, or media alone as a negative control.

Netrin-1 function blocking antibodies (25 µg/ml of rabbit netri-1 antiserum, provided and generated by the Kennedy lab) were added to the top compartment together with netrin 1 hour prior to adding the T cells (no wash). Following incubation, the top compartment was carefully removed and cells that migrated and accumulated in the lower chamber were counted with a

hemacytometer, taking the collected volume into account. Conditions were performed in triplicates.

Immobilized substrate adhesion assay for T cells.

To delineate whether netrins exert adhesive effects on T lymphocytes, we quantified how many T cells remain stuck on surfaces coated with different proteins. In a four well plate, a drop of 1% nitrocellulose (Biorad nitrocellulose dissolved in methanol and filtered) was allowed to dry to immobilize the different protein substrates for T cell adhesion. We coated the nitrocellulose surface with 500 µl of 2 µg/ml (1µg total protein, diluted in PBS) of bovine serum albumin (BSA) (Sigma), recombinant netrin-1, recombinant netrin-4 (both from R&D Systems), or fibronectin (Invitrogen) for 2 hours at room temperature. Subsequent to protein-coating, each well was saturated and blocked first with 1% bovine serum albumin (Sigma) and then with 1% heparin (Sigma) for each 1 hour each. After this, a netrin-specific function blocking antibody (25 µg/ml of rabbit antiserum, provided and generated by the Kennedy lab), soluble netrin receptor bodies (neogenin-Fc, Unc5-Fc, DCC-Fc, or human Fc; each at 5 µg/ml R&D systems), or a β1-integrin-specific antibody were optionally added to the well for 1 hour. Wells were then washed twice with PBS. Then $5x10^5$ T cells were added per well and allowed to adhere to the different surface protein substrates for 2 hours. Wells were then washed 3 times with ice cold PBS to wash off non-adherent cells. Cells that remained stuck on the protein substrate were fixed with 4% PFA and stained with the nuclear dye Hoechst. Cells were visualized by epifluorescent microscopy and quantified by counting the number of cells per image field using Image J software. Each condition was performed in quadruplicates to account for variability.

Videomicroscopy of T cells on an ECM – netrin-1 coated surface.

To visualize T cells motile behavior; we coated 35 mm glass bottom culture dishes first with PLL and then with extracellular matrix proteins (ECM proteins = Matrigel, thawed overnight at 4°C, then diluted in 1:200 in ice cold DMEM, coat

dish with 2 ml of diluted Matrigel for 1.5 hours at 37°C, then wash twice with PBS). We then plated $2.5x10^5$ T cells in 3 ml of 10% FBS supplemented RPMI media.

At a 10x objective magnification, we began time-lapse recording every 30 seconds for a total of 180 frames. With a microspritzer apparatus we added netrin-1 within the first frames (10-20) of recording (pipette loaded with 10 μl of netrin-1 [100 μg/ml of recombinant netrin-1 protein, RnD Systems] or PBS; each pulse released 1/10 of 1 μl at 0.3 Pa). The image frames were assembled into an avi-videoformat with the Image J software.

Experimental autoimmune encephalomyelitis and flow cytometry analysis

We performed two EAE experiments with two groups of each 12 female C57BL/6 mice. We immunized all mice at two dorsal subcutaneous injection sites with a total of 200 μg MOG(35-55) (Sheldon Biotechnology Center) in a total of 100 μl emulsion of complete Freund's adjuvant (supplemented with 2 mg/ml of *Mycobacterium tuberculosis*; Fisher Scientific) per mouse on day 0. Pertussis toxin (300 ng/mouse, diluted in sterile PBS; Sigma Aldrich) was injected i.p. on day 0 and again on day 2. For therapeutic treatment, we injected mice intravenously with either 1 μg/ 200 μl of recombinant mouse netrin-1 (R&D Systems, diluted in sterile PBS), or 200 μl of PBS alone for two consecutive days prior to disease induction and on days 0,2,5,7,9,12,14, 16, and 19.

To assess disease severity, we weighed the mice every day and used a 5 point scale scoring system: 0, normal; 1, paralyzed floppy tail; 2, loss in coordinated movement, ataxia; 3, one hind limb paralyzed; 4, both hind limbs paralyzed; 5, all limbs paralyzed and/or moribund. The person who scored and weighed the mice was 'blinded' to the identity of the treatment group. On day 14 and 20, we sacrificed and PBS-perfused mice from each group and harvested their brain, spinal cord, spleen and lymph nodes for analysis by both immunohistostaining and flow cytometry. All procedures with animals were performed in accordance with the "Canadian Council on Animal Care" guidelines for the use of animals in research.

For immunohistochemical analysis of the CNS (right brain hemisphere and halves of cervical, thoracic and lumbar spinal cords), we embedded these tissues in OCT compound and stored them at -80° C. We then cryosectioned the fresh-frozen brain and spinal cord samples into 7 μm thin sections for further staining procedures.

To evaluate the degree to which lesions are demyelinated and infiltrated, we stained brain and spinal cords with Luxol Fast Blue and with Hematoxylin and Eosin as described [77]. We then assessed the area of demyelination and infiltrated lesions by quantifying brain and spinal cord sections, stained with Luxol Fast Blue and Hematoxylin/Eosin, respectively, and analyzing sections with the Open Lab software.

To assess BBB integrity, we quantified the extravasation of type G immunoglobulins (IgG) and fibrinogen (see section: '*In vivo* BBB permeability to serum proteins in netrin-1 knockout mice and EAE mice').

We qualitatively evaluated EAE lesions for infiltration with Th lymphocytes, Tc lymphocytes or macrophages, and stained fresh frozen brain and spinal cord sections with CD4+, CD8+ antibodies (1:50 rat anti mouse CD4, BD Bioscience; 1:20 rabbit anti mouse CD8, Vector labs) or F4/80+ antibodies (1:80 rat anti mouse, BD Bioscience), respectively (see also section: 'Preparation of human and mouse tissue sections for qualitative immuno histostaining for endothelial netrin expression').

Sections stained for immune cells were also co-labeled with a marker for endothelial cells: FITC-conjugated Esculentum-lectin (1:2000, Vector Laboratories). Secondary antibodies, goat anti rat Alexa 546 conjugated, and donkey anti rabbit Alexa 555 conjugated (both diluted 1:500 in PBS, from Molecular Probes), were used to visualize immune cells, and TOPRO (1:300, Invitrogen) for staining cell nuclei. Rat IgGs were used as isotype controls. Microscopic evaluation was performed using a LEICA confocal microscope. Plasma protein leakage was determined by measuring pixel intensity per area of leakage using Image J software.

For flow cytometry analysis of mouse immune cells, we isolated cells from the CNS compartment (left brain hemisphere and halves of cervical, thoracic and lumbar spinal cords), leukocytes from draining lymph nodes, and splenocytes from the spleen. We then recorded total cell counts and stained leukocytes with Fluorochrome-conjugated antibodies against extracellular mouse epitopes for CD45, CD44, CD3, CD4, CD8, and CD11b (BD Bioscience). For intracellular staining against intracellular epitopes IL-17, and IFNγ (BD Bioscience), we treated cells with 20 ng/ml PMA (in ethanol), 1 μg/ml ionomycin (in DMSO) and 2 μg/ml brefeldin A (in methanol) in RPMI, supplemented with 10 % FBS, 2 mM glutamax and 100 U/ml penicillin / streptomycin over night (~15 hours).

We then stained cells extracellularly with Fluorochrome-conjugated antibodies against extracellular mouse epitopes for CD45, CD44, CD3, CD4, CD8, and CD11b (BD Bioscience), washed the cells with PBS, containing 0.1% FBS, and then fixed and permeabilized with 1 % formaldehyde, supplemented with 0.1 % saponin. After fixation, cells were washed twice with 0.1% FBS, 0.1% saponin in PBS and then stained with antibodies against mouse IL-17, and IFNγ (BD Bioscience). Cells were washed again three times before acquisition with the BD LSRII flow cytometer. Data analysis was performed using the FACS DIVA and FlowJo Software. For quantitative analysis, the geometric mean of fluorescence intensity was compared to the isotype control and plotted on a graph.

Gating: In the CNS, infiltrated leukocytes were distinguished from brain resident microglia by a gate on cell that was CD45-high fluorescent. Microglia, on the other hand, were identified within the CD11b positive and CD45-low positive gate. For both spleen and lymph nodes, we used the same CD45-high gating to identify leukocytes. IL-17, and IFNγ populations were gated on CD45-high, CD3 positive, and then selected for either CD4 positive or CD8 positive cells.

Statistical analysis

We repeated each experimental condition with at least three different sample preparations and analyzed data with the GraphPad Prism software. Values are represented as mean and standard error of the mean (SEM). Values were

considered statistically significant when probability (p) values were equal or below 0.05 (*), 0.01 (**), or 0.001 (***).

To compare endothelial cell responses with and without netrin treatment by western blot, qPCR, and ELISA analysis we used a paired Student t-test. For determination of netrins *in vitro* effect on BBB permeability, we used a one-way analysis of variance. To compare netrin-1 knockout mice with wild types, or netrin-1 with PBS treated EAE mice for protein extravasation or immune cell accumulation, we used an unpaired t-test.

RESULTS

RESULTS

CHAPTER (A) - NETRIN-1'S ROLE IN THE DEVELOPING BBB

Netrin-1 absence during development increases protein diffusion through the BBB.

Endothelial netrin-4 expression remains unaffected in absence of netrin-1

To study the effect of netrin on BBB development, we examined the brain-microvasculature in offsprings from netrin-1 heterozygote parents. These animals carry a non-functional netrin-1 mutant allele that contains a CD4 transmembrane and a cytoplasmic β-galactosidase reporter insertion creating a gene product that is no longer secreted. According to the Mendelian law of inheritance, offspring from these parents had either two normal alleles of netrin-1 (25 % wild types), only one full-length netrin-1 allele (50 % heterozygotes), or no functionally intact netrin-1 alleles (25 % knockouts). We screened mice for both phenotype and genotype by behavioral assessment, quantitative PCR, western blot, and β-galactosidase reporter activity assays (Figure 1a-d).

To delineate whether the lack of netrin-1 could be compensated by an increased expression of netrin-4, we quantified the intensity of netrin-4 expressed by brain-endothelial cells in netrin-1 knockout mice compared to wild types'. We found that newborn mice do not express netrin-1 in parenchymal or meningeal vessels (Figure 1e, f). In comparison, we detected netrin-4 only in larger brain-derived blood vessels within the meninges of both wild type and knockout mice, indicating that brain endothelial cells of larger vessels express netrin-4 and that netrin-4 expression is not altered when netrin-1 expression is nearly absent.

Loss of netrin-1 increases the meningeal layer width

When we looked at the number and morphology of blood vessels by lectin staining, we did not observe obvious changes in the brain microvasculature of netrin-1 knockout mice, compared to wild type mice. Netrin-1 knockout and wild type mice exhibited similar vessel density in the brain parenchyma (Figure 2a), as the number of lectin positive blood vessels counted per image field remained the

same in both genotypes (Figure 2b). Furthermore, in absence of netrin-1, the vessel thickness, morphology, and arborization of brain microvessels remained unchanged. This indicates that netrin-1 does not function as a main angiogenic factor in the developing mouse CNS.

On the contrary, when we stained endothelial vessels in the meningeal layer with the vessel marker lectin and the TJ molecule ZO-1 (Figure 2d), we observed that the width of meningeal vascular structures was significantly increased in netrin-1 knockout mice (Figure 2c).

Absence of netrin-1 leads to a more permeable BBB

At birth, the BBB is already impermeable to serum proteins [70-72]. To evaluate whether absence of netrin-1 in newly born knockout mice would affect barrier permeability, we quantified the area and intensity of serum proteins that diffused into the brain parenchyma. We quantified serum proteins of different size, with immunoglobulins type G (IgG) being the smallest (~160 kDa), fibrinogen of medium size (~340 kDa), and apolipoprotein B (apoB) being the largest protein (~510 kDa). While serum proteins were barely detectable in wild type mice, fibrinogen, IgG and apoB (Figure 3) accumulated in brains of netrin-1 knockout mice. This leakage occurred only in larger sized vessels. Thus in absence of netrin-1, more plasma proteins extravasated into the brain, which indicates that netrin-1 deficiency leads to an incompletely sealed blood-brain barrier.

Netrin-1 deficiency alters tight and adherens junction protein expression

We wondered whether the increased barrier permeability in netrin-1 knockouts resulted from a change in junction protein expression because tight and adherens junction proteins increase inter-endothelial adhesion, which promotes barrier impermeability. Indeed, the tight junction proteins occludin and JAM-1 and the adherens junction protein p120 were less abundant in parenchymal and meningeal vessels in netrin-1 knockouts compared to wild type mice (Figure 4a-d immunofluorescent staining; Figure 4g, h western blot analysis). In contrast, we observed no change in claudin-5 expression (Figure 4e, f, i). Surprisingly, levels

of the intracellular tight junction adaptor protein ZO-1 increased in knockout mice (Figure 4j, k), which may be a mechanism to compensate for the lowered levels of JAM-1, occludin, and p120 protein. Hence, netrin-1 influences the expression patterns of junctional proteins during vascular development. Despite potential compensatory mechanisms through increased ZO-1 expression, this altered tight junction protein expression is insufficient to completely seal the BBB and renders it leaky to blood-derived serum proteins.

CHAPTER (B) - NETRIN-1'S ROLE IN THE ADULT BBB

Netrins enhance and maintain blood-brain barrier function during adulthood

Human brain-derived microvascular endothelial cells express netrins

With the support of our findings in Chapter A, stating that netrin-1 can modify BBB function during development, and our rationale that netrin increases barrier stability; we first tested whether primary adult human brain-derived microvascular endothelial cells (BBB-ECs) express netrin under physiological or pathological conditions. As an initial step, we tested antibodies directed against netrin-1 and netrin-4 for their cross-reactivity. Analyses by western blot, flow cytometry, and immunocyto-fluorescent staining of recombinant netrin-1 and netrin-4 proteins demonstrated the specificity of the antibodies used (Figure 5). We then examined primary human BBB-ECs for netrin expression using quantitative PCR, western blot analysis, flow cytometry, and fluorescent immunocytostaining, all of which revealed expression of both netrin-1 and netrin-4 by these cells (Figure 6) but not netrin-3 (Figure 10g).

We screened BBB-ECs for netrin in physiological and pathological conditions. Astrocyte conditioned media (ACM) is well known to decrease BBB permeability through factors such as sonic hedgehog and angiotensin [77,102,277], whereas pro-inflammatory cytokines such as tumor necrosis factor (TNF) and interferon gamma (IFNγ) promote a breach in the BBB and therefore increase BBB permeability [52,278]. Following stimulation of BBB-EC cultures with either ACM or TNF/IFNγ, we measured netrin expression by quantitative real time PCR. Our results demonstrate that netrin-1 remains unaltered following ACM treatment but is increased upon pro-inflammatory cytokine treatment (Figure 6a). On the contrary, BBB-ECs up regulate netrin-4 with ACM treatment and down-regulate it with TNF/IFNγ, compared to untreated cells (Figure 6c). We did not detect netrin-3 expression by BBB-ECs (Figure 10g). These distinct patterns of netrin expression were also seen at the protein level, by western blot (for netrin1 Figure 6b and for netrin-4; Figure 6d) and by confocal immunocyto-fluorescence (for netrin-1 Figure 6i-k; and for netrin-4 Figure 6l-n). Thus, netrin-4 is strongly

expressed by brain endothelial cells in barrier-tightening, physiological conditions, whereas netrin-1 becomes up regulated in barrier-destabilizing conditions (Figure 6l-n). Netrin staining patterns were rather diffuse and did not appear as zipperlike structure, which is typical for tight and adherence junction proteins.

Next, we sought to determine the localization of netrin proteins made by BBB-ECs. Although netrin-1 and -4 are secreted proteins, fractionation of adult brain tissue has previously shown that netrins remain associated with cell surfaces and with the extracellular matrix in the adult CNS [203]. To evaluate the distribution of netrin proteins, we labeled BBB-ECs with netrin antibodies using methods to stain whole cell or cell surface compartments, and analyzed them by flow cytometry. We found that endothelial cells express both netrin-1 and netrin-4 (Figure 6f, h), which are present on the cell surface membrane (Figure 6e, g).

Netrins do not affect BBB-EC proliferation but increase barrier integrity *in vitro.*

We know little about netrins' effect on the vascular barrier. To determine whether netrins have a functional effect on the BBB, we assessed netrins' ability to change BBB permeability. We measured BBB-EC permeability in response to netrin-1 and -4, using an *in vitro* Boyden chamber system amenable to modulation of intercellular adhesions (Figure 7a). Using distinct fluorescently labeled tracer molecules of different size, FITC-labeled bovine serum albumin (BSA) (Figure 7b) and 10 kDa dextran conjugated to Alexa 646 (Figure 7c), we observed a significant reduction in their diffusion through a monolayer of primary human BBB-ECs, following stimulation with netrin-1 or netrin-4.

We then asked whether this barrier tightening could have been a result of netrins' previously reported angiogenic effects, which may have increased cell proliferation and consequently resulted in a denser cell layer. By using Carboxyfluorescein Succinimidyl Ester (CFSE)-labeling and flow cytometry analysis we quantified the division rate of BBB-ECs following netrin treatment. We detected no difference in fluorescent intensity in cells treated with ACM,

netrin-1, or -4 versus untreated BBB-ECs (Figure 7d), indicating that netrins have no proliferative effect on BBB endothelial cells. Therefore, netrins' barrier tightening effect was not caused by an increased proliferation, despite of previous reports about netrins ability to promote cell proliferation [207,211,254]. However, in most of these reports netrin promoted endothelial proliferation under conditions of starvation [168,206,211,212,217,221], while we performed our experiments in serum-rich culture media to most closely mimic BBB physiology. In contrast, pro-inflammatory conditions decreased the rate of BBB-EC division.

BBB-EC morphology, size, and shape also remained unchanged in the presence of netrin-1 or netrin-4, compared to untreated cells (Figure 7e). Therefore, netrins' effect on reducing tracer molecule diffusion though BBB-ECs does not involve changes in cellular morphology and results from mechanisms independent of cell division.

Netrin increases inter-endothelial adhesion by up regulating tight junction proteins in BBB-ECs.

To find out whether netrin-1 reduces barrier permeability by increasing adhesion among endothelial cells, we asked whether netrins directly or indirectly promote inter-endothelial adhesion. From our previous immunofluorescence analysis we observed that netrins are diffusely distributed on the endothelial cell surface and did not appear to localize strongly to sites of cell-cell contacts, which would otherwise resemble a pattern typical for TJ molecules. This indicates that netrins may not directly serve as adhesive factors, but perhaps indirectly, by regulating other adhesive factors, such as junctional proteins, at sites of cellular junctions.

We examined whether netrins strengthened the barrier by regulating junction protein expression at the BBB. We compared the effect of netrin-1 and netrin-4 on BBB-ECs to the effect of ACM or pro-inflammatory cytokines. Western blot analysis showed that treatment of BBB-ECs with either recombinant netrin-1 or -4 resulted in a significant up-regulation of tight junction molecules JAM-1, occludin, and claudin-5 compared to untreated cells (Figure 8a-c). This effect was similar to ACM-treated BBB-ECs. Immunocytofluorescence stainings

were consistent with our finding of increased tight junction molecule expression following netrin application by western blot. Compared to untreated cells, the fluorescence intensity (quantified as mean pixel intensity in Figure 8h) of ZO-1, p120, and α-catenin was significantly greater at inter-endothelial cell connections when we added netrin-1 (N1) or netrin-4 (N4) (Figure 8g, h). In contrast, activated endothelial cells (T/I, treated with TNF and IFN-γ) exhibited a decrease in TJ protein expression.

Concomitant application of netrin-1 or netrin-4 to TNF and IFN-γ activated BBB-ECs significantly prevented the down regulation of the TJ molecules JAM-1, occludin and claudin-5, induced by inflammatory stimuli (Figure 8d-f). However, JAM-1 expression remained unaffected by netrin-4. We conclude that both netrins promote proper expression of TJ and AJ molecules, and protect BBB-ECs against a barrier breach induced by inflammatory mediators.

Netrin increases tight junction molecule expression in lipid rafts.

TJ molecules cluster in detergent-insoluble lipid raft fractions and become enriched in and recruited to lipid rafts when endothelial cells are treated with barrier-promoting factors [102]. To determine whether netrins help to enrich TJ molecules in lipid rafts, we performed sucrose density gradient fractionation of stimulated BBB-ECs followed by Western blot analysis. Twelve fractions were harvested and analyzed for protein and cholesterol content. The peak level of cholesterol and presence of CD59, GM1-ganglioside and absence of the transferrin receptor identified fractions 4 and 5 as those enriched with lipid rafts (Figure 9a-c). The protein content in lipid raft fractions was then compared to the pellet fractions (10, 11, and 12) and compared to levels of TJ proteins among the different treatment groups. Following netrin-1 treatment of primary cultures of BBB-ECs, we found higher amounts of occludin in lipid raft signaling platforms (Figure 9f, g). However, we observed no effect on TJ molecule recruitment with netrin-4 treatment (data not shown).

BBB-ECs express netrin-receptors.

Although researchers documented the expression of netrin-receptors by vascular endothelial cells, there is substantial controversy regarding whether and which receptors are responsible for netrins' functions [165,168,170,177-179,207,210,211,219,221,223].

We detected high levels of the netrin receptor neogenin in BBB-ECs, following a dynamic pattern of expression similar to netrin-4. Neogenin is up regulated under barrier-promoting conditions but down-regulated in the inflamed brain-derived endothelium, relative to untreated cells (Figure 10a). Unc5B and unc5C were detected at moderate to low levels of expression (Figure 10c, e). We did not detect DCC, unc5A, and unc5D in BBB-ECs, but in human foetal brain homogenates that we used as a positive control to verify the functionality of probes (figure 10g). The expression of neogenin, unc5hB and unc5hC transcripts was confirmed by protein expression analysis (Figure 10b, d, f).

Blood vessels in EAE-lesions and MS plaques express netrin-1 and -4 in contrasting patterns.

We next assessed the expression and regulation of netrin-1 and netrin-4 in post-mortem brain sections of individuals with multiple sclerosis, a demyelinating disease characterized by focal inflammation and disruption of the BBB [18,279] (Figure 11a). Blood vessels associated with perivascular lesions and immune cell accumulation displayed increased netrin-1 immunoreactivity (Figure 11a). In contrast, the relative level of netrin-4 immunoreactivity was reduced or absent from vessels within MS lesions (Figure 11b) when compared to expression levels in adjacent normal-appearing blood vessels found in the same section (Figure 11b). We found a similar expression pattern in the animal model of MS, experimental autoimmune encephalomyelitis (EAE). The normal adult mouse brain microvasculature expressed netrin-4 (Figure 12b) but not netrin-1 (Figure 12a). And upon EAE induced inflammation, the brain-endothelium reduced netrin-4 (Figure 12b) and increased netrin-1 expression (Figure 12a).

Because the netrin-1 staining pattern in the MS-lesioned vasculature located to endothelial cells and to vessel lumen, we tested the presence of netrin-1 in serum from MS patients and compared it to amounts in serum from healthy donors, using ELISA analysis (Figure 11c). Although, neither netrin-1 nor netrin-4 were detectable as soluble forms in media conditioned by *in vitro* cultured BBB-ECs we detected significantly elevated netrin-1 levels in blood serum from MS patients (4500 pg/ml), compared to healthy controls (2500 pg/ml). Netrin-4 was not detected in serum. We also tested netrin-1 levels in cerebrospinal fluid (CSF) and observed overall higher levels (~8000 pg/ml) compared to blood serum (Figure 11d). However, we could not detect a significant difference in CSF-netrin-1 in MS patients, as compared to CSF samples from non-inflammatory controls.

We speculate that netrin-1 is produced in sufficient amounts by BBB-ECs under *in vivo* inflammatory conditions so that it rises above the levels that remain attached to cell surfaces and can be secreted into the blood. Otherwise, netrin-1 and netrin-4 remain associated with the plasma membrane of BBB-ECs.

Furthermore, because the BBB requires support from closely adhering astrocytic endfeet [1], we sought to determine whether netrins are expressed by astrocytes. However, we detected neither netrin-1 nor netrin-4 protein in primary human foetal astrocytes by western blot analysis, regardless of whether they were untreated or inflamed (Figure 13a, b). Similarly, we did not detect netrins in conditioned media of primary cultures of these astrocytes, using ELISA analysis. Consistent with this, we were unable to detect netrin-1 or -4 in astrocytes, stained with the astrocyte specific marker glial fibrillary acetic protein (GFAP) within mouse brain sections (Figure 13c). We conclude that astrocytes are not a significant source of netrin protein associated with the BBB.

CHAPTER (C)-NETRINS' ROLE IN IMMUNE RESPONSES AT THE BBB

Netrins have anti-inflammatory functions at the blood-brain barrier

Netrins reduce endothelial cytokine and chemokine secretion

Recent reports show evidence that netrin-1 has anti-inflammatory properties and dampens immune responses by down regulating cytokines and chemokines, and reducing leukocyte infiltration [138,186-188,227,233,268]. We assessed whether netrin possesses anti-inflammatory functions at the blood-brain barrier, which may decrease immune cell infiltration into the CNS. When we exposed BBB-ECs to either netrin-1 or netrin-4, we found that BBB-ECs secreted significantly lower levels of the cytokines IL-6 and GM-CSF and of the chemokines IL-8, MCP-1 and IP-10 (Figure 14).

Netrins do not affect endothelial CAM expression or T cell migration across the *in vitro* BBB

To evaluate whether netrins have the ability to inhibit T cell migration across the brain-derived endothelium, we first tested whether netrins influence endothelial expression of cell adhesion molecules (CAMs), because immune cells use CAMs to adhere to the endothelium. Treatment with netrin-1 or netrin-4 did not impact the expression of CAMs by untreated (Figure 15a, b) or activated (Figure 15c, d, e) brain-endothelial cells. Levels of ICAM-1, MCAM, and ALCAM remained unchanged, which is consistent with Tadagavadi et Al.'s finding that netrin-1 does not modify renal ICAM expression [258]. Furthermore we evaluated whether netrin affects the expression of the human major histocompatibility complex (MHC) class I protein HLA-A, B, C, but did not observe a change in expression (Figure 15f).

From other *in vivo* and *in vitro* models, we know that netrin-1 reduces myeloid cell migration [138,186-188,227]. However, we know very little about netrins' effect on T lymphocyte motility [233]. Here, we assessed netrins' potential to inhibit T cell migration across BBB endothelial cells, because of their key role in many

autoimmune diseases, such as EAE and MS. We used an *in vitro* model of the BBB by growing a monolayer of BBB-ECs in Boyden chamber in the presence of ACM and measured the number of T cells that migrated across the endothelial monolayer in absence or presence of netrin-1 or netrin-4 (Figure 15g,h).

T cells can be largely distinguished into CD4-positive helper (Th) T cells or CD8-positive cytotoxic T cells. Here we examined the migratory behavior of both T cell populations. We added netrin in both upper and lower chamber as previous studies demonstrated that netrin has no directional effect on leukocyte migration [233]. We tested transendothelial migration of both CD4-positive helper T cells and cytotoxic CD8-positive T cells and found that neither netrin-1 nor netrin-4 had a significant effect on T cell migration.

T lymphocytes express neogenin and Unc5B

In the previous chapter we showed that netrin-1 is up regulated by blood vessels within lesioned and immune infiltrated areas of MS and EAE brains. Both are autoimmune diseases mediated by autoaggressive T cells, which cross the BBB and damage the CNS. We wondered whether T cells have the ability to bind or respond to netrin, because in our previous findings netrin did not affect T cell motility. To test whether T cells are equipped with netrin receptors we quantified levels of DCC, neogenin, and unc5 homologues A-D expressed by CD4 and CD8 T cells. By quantitative real time PCR analysis, we found that CD4 and CD8 T cells express both neogenin and unc5B (Figure 16e, f). Furthermore, we also screened T cells for netrin-1, -3, and -4 to delineate whether immune cells have the potential to interact with one another in a netrin-dependent manner. We found that only netrin-4 was netrin expressed by CD4 and CD8 T cells (Figure 16d).

Mature and activated dendritic cells express netrin-1

Next, we asked whether antigen-presenting cells, such as dendritic cells, express netrin. In presence of IL-4 and GM-CSF, we differentiated *ex vivo* monocytes to immature dendritic cells (DCs). To obtain activated, mature DCs we added LPS (LPS = lipopolysaccharide, a component of gram-negative bacteria that highly

activates immune cells through toll like receptors and other pattern recognition receptors) to immature DCs cultures. Our findings show that *ex vivo* monocytes expressed barely any netrin or netrin receptors. Only when differentiated and activated to become mature LPS -activated DCs, they up regulated netrin-1 expression (Figure 16a). This finding is supported a previous finding, which described that macrophages up-regulate netrin-1 expression upon activation [138]. Additionally, neogenin and Unc5B were up regulated in mature DCs (Figure 16b, c) compared to circulating monocytes, which do not express any type of netrin or netrin-receptor.

T cells adhere to netrin-1 but not to netrin-4 substrates

Next we assessed whether netrins support T cell adhesion to a netrin-coated surface, as a model for a potential netrin-1-rich matrix or netrin-expressing cell. T cells and antigen presenting myeloid cells, for example need to engage in adhesive contact formation to efficiently communicate with one another. We know that activated macrophages [138] and mature dendritic cells express netrin-1, and T cells are equipped with netrin-receptors. To study netrins' capacity to function as an immobilized adhesive substrate for T cells, we plated T cells on various substrates: bovine serum albumin (BSA) as a negative control, netrin-1, netrin-4, or fibronectin a known adhesive substrate and positive control for our adhesion assay [280]. In the presence of netrin-1, three to ten times more T cells remained bound to the surface, compared to a BSA-coated surface (Figure 17a, b). We observed this effect on CD3-, CD4- and CD8-positively selected T cell populations. Adhesion to netrin-1 was reversed when we pretreated netrin-1-coated surfaces with a function blocking antibody recognizing netrin-1, but not with control antibodies, demonstrating the specificity with which T cells bind to netrin-1 (Figure 17c,e). However, when we blocked the adhesive netrin receptors neogenin or beta-1 integrin, with function blocking antibodies against beta-1 integrin or neogenin Fc-receptor constructs, we could not inhibit netrin-1's adhesive function on T cells (Figure 17d). Interestingly, while netrin-1 promotes T cell adhesion, T cells did not adhere to a netrin-4 substrate (Figure 17a, b).

We next tested T cells' crawling behavior on a netrin-1 versus a matrigel control substrate, using time lapse microscopy, and observed that T cells on the netrin-1 coated surface moved less and appeared to have a more round shape, indicating that netrin-1 inhibited motile behavior of T cells (Figure 17f). Thus, while netrin-1 reduced T lymphocytes' motile behavior, it increased their adhesion to a netrin-enriched surface.

Netrin-1 does not alter T cell polarization

T cells contact and communicate with antigen presenting cells (APCs). To interpret signals from the local environment and to polarize into an appropriate T cell effector phenotype, Netrin-1 may influence the communication between T cells and APCs, because APCs have the capacity to become a source of netrin (Figure 16), and T cells adhere to netrin-1 (Figure 17). Thus netrin-1 may support or prolong the contact between T cells and APCs, which in turn could influence Th cell activation and differentiation (Figure 18a).

Th lymphocytes ($CD4^+$) can differentiate into either pro-inflammatory IFN-γ producing Th1 or Il-17-producing Th17 cells, or into anti-inflammatory Th2, or immune-suppressing regulatory T cells (Tregs). While Th1 and Th17 cells strongly activate innate immune players, IL-4 secreting Th2 cells and Tregs suppress other immune cells and down-regulate immune responses. Tagadavi *et al*. have previously shown that *in vitro* activated $CD4^+$ T cells secreted less IFNγ and IL-17 in the presence of netrin-1 [258].

We added polarizing cocktails and antigen presenting cells to T cells and studied whether adding netrin-1 affects expansion of pro-inflammatory Th1, Th17, or anti-inflammatory Th2 phenotypes. When we counted the number of effector Th lymphocytes, treated with or without netrin-1, by flow cytometry, we observed that netrin-1 had no effect on the amount of IFN-γ, IL-17, or IL-4 producing T cells, generated in each skewing condition (Figure 18b, d). We also measured whether addition of netrin to a mix of activated T helper cells (Th0) and APCs would change levels of chemokines MCP-1 and IL-8, but we did not observe an effect (Figure 18c).

Netrin-1 has dual functions in EAE

In previous chapters, we reported that netrin-1 stabilizes and protects the BBB by up-regulating tight junction protein expression under inflammatory conditions (Figure 8). Additionally, netrin-1 has anti-inflammatory properties on BBB endothelial cells by reducing their secretion of cytokines and chemokines *in vitro* (Figure 14).

To test whether netrin-1 has anti-inflammatory and BBB-stabilizing effects *in vivo*, we applied intravenous netrin-1 therapy to EAE-induced mice (immunized with MOG35-55, complete Freund 's adjuvant, and pertussis toxin). Hallmarks of EAE are BBB-breakdown and immune cell accumulation in the CNS, which leads to disease symptoms such that mice become paralyzed with progressing disease, starting with paralysis in the tail, and reaching paralysis in both hind limbs and weakness in forelimbs. We injected netrin-1 prior to disease induction, and three times weekly after immunization (Figure 19a). While untreated MOG-immunized C57/B6 mice became sick at day 11, netrin-1 treated mice exhibited a delayed disease onset, a less steep incline of disease severity (Figure 19a), and had a 50% lower rate of disease incidence compared to PBS treated mice (Figure 19b). In the netrin-1 treatment group, fewer mice developed a low or intermediate score, compared to the control EAE group (Figure 19c, d).

However, when we assessed mice at the chronic phase of disease (day 20), netrin-1 treated mice had signs of atypical EAE, being noticeably more ataxic and spastic, which we did not observe in control mice. This suggests that netrin-1 has dual functions in EAE, whereby it delays disease at the onset but promotes additional symptoms to the typical paralysis as disease progresses.

To investigate the underlying differences in immune activity and extent of CNS damage in netrin-1 treated mice at disease onset and in the chronic phase of the disease, we looked at the degree of demyelination and immune cell accumulation in spinal cords with Luxol Fast Blue and Haematoxylin & Eosin staining, respectively (Figure 20a), and analyzed the number and type of infiltrated immune cells within the CNS (brain and spinal cord) by flow cytometry

(Figure 20b). At the beginning of the disease, on day 14, netrin-1 treated mice had milder disease symptoms, which coincided with reduced CNS tissue damage, fewer and smaller lesions, and overall fewer immune cell infiltrates, particularly of myeloid origin (F4/80+ macrophages, and CD11b+ antigen presenting cells) (Figure 19i; Figure 20b,c). We also detected fewer CNS resident immune cell populations, microglia, in netrin-1 treated mice (Figure 19h). In contrast, control mice exhibited multifocal areas of immune cell infiltration, consisting of large numbers of both lymphoid (CD3) and myeloid (CD11b) immune cells (Figure 20b, c). When we compared mice during the chronic disease phase, we observed that infiltrates began to resolve and lesions transformed into scarred tissue in control mice. On the contrary, netrin-1 treated mice exhibited multifocal lesioned areas and higher number of lymphoid type immune cells. High levels of CD8 T cells were present in the CNS (Figure 19k) and migrated past the lesion sites, deep into the CNS parenchyma (Figure 20c). We also noted that at this time, immune cells were more activated with netrin-1 treatment (Figure 19g). Therefore, netrin-1 has dual functions during the chronic autoimmune disease EAE. It ameliorates disease severity at the onset, but worsens EAE during its chronic phase.

Netrin-1 changes CD8 T cell phenotypes during EAE

We previously theorized that netrin-1 may promote adhesion between APCs and T cells (Figure 16 and 17) which could change T cell activation and behavior. However, we did not observe any difference in Th phenotype skewing *in vitro* (Figure 18). To understand, whether T cells become differentially activated in presence of netrin-1 *in vivo*, we analyzed Th and Tc lymphocytes isolated from the CNS (Figure 21a-d), spleen (Figure 21e-h), and lymph nodes (Figure 21i-l) for cytokines IL-17 and IFNγ.

When we compared cytokine levels in the lymphoid organs of netrin and control mice, we saw no difference between these 2 groups on day 14 and day 20. In contrast, in the CNS of netrin-1 treated mice, we detected less T cells on day 14, compared to control mice. This is consistent with the lowered numbers of CNS-infiltrated CD4 and CD8 T cells in netrin-1 treated mice (Figure 19j, k). Most

strikingly, netrin-1 treatment reduced the number of IL-17 secreting CD8 T cells in the CNS (Figure 21d) and spleen (Figure21h), but not in the lymph nodes (Figure 21l) on day 14. This suggests that netrin-1 treatment affects CD8 T cells already in the periphery, whereby systemic netrin-1 injection probably reached the spleen more effectively than the inguinal lymph nodes.

With disease progression into the chronic phases (day 20), overall amounts of IL-17 and IFNγ secreting immune cells decreased again and were similar in the PBS versus netrin-1 treatment groups. However, we noticed a trend towards increased levels of IFNγ secreting CD8 T cells in the CNS of netrin-1 treated mice on day 20 (Figure 21c).

Netrin-1 reduces BBB breakdown

To evaluate whether therapeutic treatment of EAE mice with netrin-1 protects the BBB and increases its integrity, we stained sacral, lumbar, thoracic, and cerebral spinal cord sections from netrin-1 or PBS-treated mice for extravasated plasma proteins fibrinogen, and type G immunoglobulins (IgG) (Figure 22). At the onset of disease (day 14), we observed a dramatic difference between the PBS and netrin-1 treated mice (Figure 22a). In the netrin-1 group, we barely detected plasma protein extravasation, which can be partly explained by the low numbers of lesions and immune cell infiltrates. However, even in the presence of immune cell infiltrates, vessels remained largely impermeable to serum protein extravasation. On the contrary, PBS treated mice exhibited massive leakage of fibrinogen and IgGs. Graphs, depicting the fluorescence intensities of plasma protein quantified per area of leakage illustrate the BBB-sealing effect of netrin-1 treatment (Figure 22c).

Although we observed a larger number of immune cells within the CNS during chronic phases of the disease (day 20), we still observed that vessels within lesioned areas from netrin-1 treated mice remained less permeable to IgGs and fibrinogen, compared to control mice (Figure 22b,d). Therefore, netrin-1 has a protective effect on the BBB, by decreasing its permeability to serum proteins during inflammatory disease.

FIGURES

AND FIGURE LEGENDS

Figure 1: Identification of netrin-1 knockout mice. Using PCR analysis (a), we genotyped tail DNA from P0 offsprings (from two netrin-1 heterozygote parents) and identified netrin-1 heterozygotes (het) and knockouts (ko) by the presence of a β-galactosidase gene insertion, and wild types (wt) by the absence of this gene insertion. GAPDH served as a loading control. Additionally, we quantified brain homogenates for the amount of β-galactosidase protein by western blot (b). Native netrin-1 protein was faintly detected only in heterozygote and wild type mice by western blot analysis (75 kDa) (c). The antibody against netrin-1 also detected the β-galactosidase / netrin-1 fusion protein (220 kDa) in netrin-1 knockout and heterozygote mice (c). We used β-actin as a loading control for western blot analysis (b, c). We also performed a colorimetric quantification of β-galactosidase activity in mouse-tails by adding a substrate to the tissue, which turns blue in presence of b-galactosidase (d). We distinguished knockout mice from heterozygotes by the relative higher amount of β-galactosidase compared to heterozygote mice.

Brain sections from both knockouts and wildtypes showed that brain microvessels (stained with lectin in green) do not express netrin-1 (stained in red) (e). Similarly, netrin-4 (red) is absent from these parenchymal vessels (green, upper panel) (f), but can be found in some larger meningeal vessels (lower panel). TOPRO-staining in blue indicates nuclei. (wt = wild type, het = heterozygote, ko = knockout). Scale bars: 20 μm. n=4 wild types, n=4 knockouts.

Figure 1

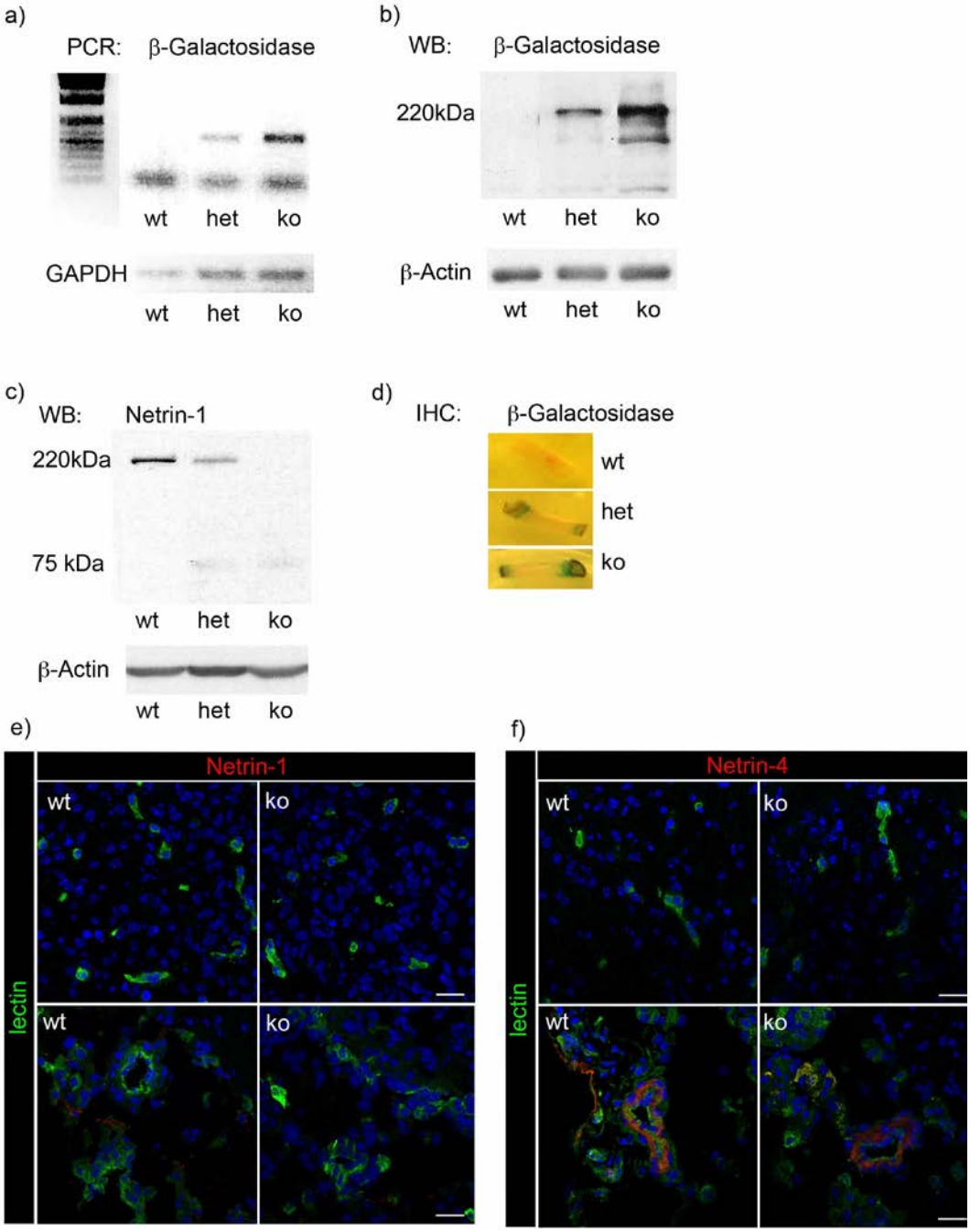

a)
PCR: β-Galactosidase
wt het ko
GAPDH
wt het ko
b)
WB: β-Galactosidase
220kDa
wt het ko
β-Actin
wt het ko
c)
WB: Netrin-1
220kDa
75 kDa
wt het ko
β-Actin
wt het ko
d)
IHC: β-Galactosidase
wt
het
ko
e)
Netrin-1
wt
ko
lectin
wt
ko
f)
Netrin-4
wt
ko
lectin
wt
ko

Figure 2: Absence of netrin-1 increases meningeal layer width in the developing brain. We observed no difference in the number of lectin-positive microvessels from wild type (wt) or knockout (ko) mice (a). The bar graph represents the number of vessels counted per 10 fields (140 μm x 140 μm) for 4 animals per group (b).

However, we observed that the meningeal layer expanded in netrin-1 knockouts, compared to wild types (c, d). We quantified the width of the meningeal area (lectin [green] and ZO-1-positive (red) vasculature (d), and confirmed that netrin-1 knockout mice have a significantly wider meningeal network (c). (wt = wild type mice, ko = knockout mice). Scale bars 40 μm (a), 20 μm (d). n=4 wild types, n=4 knockouts.

Figure 2

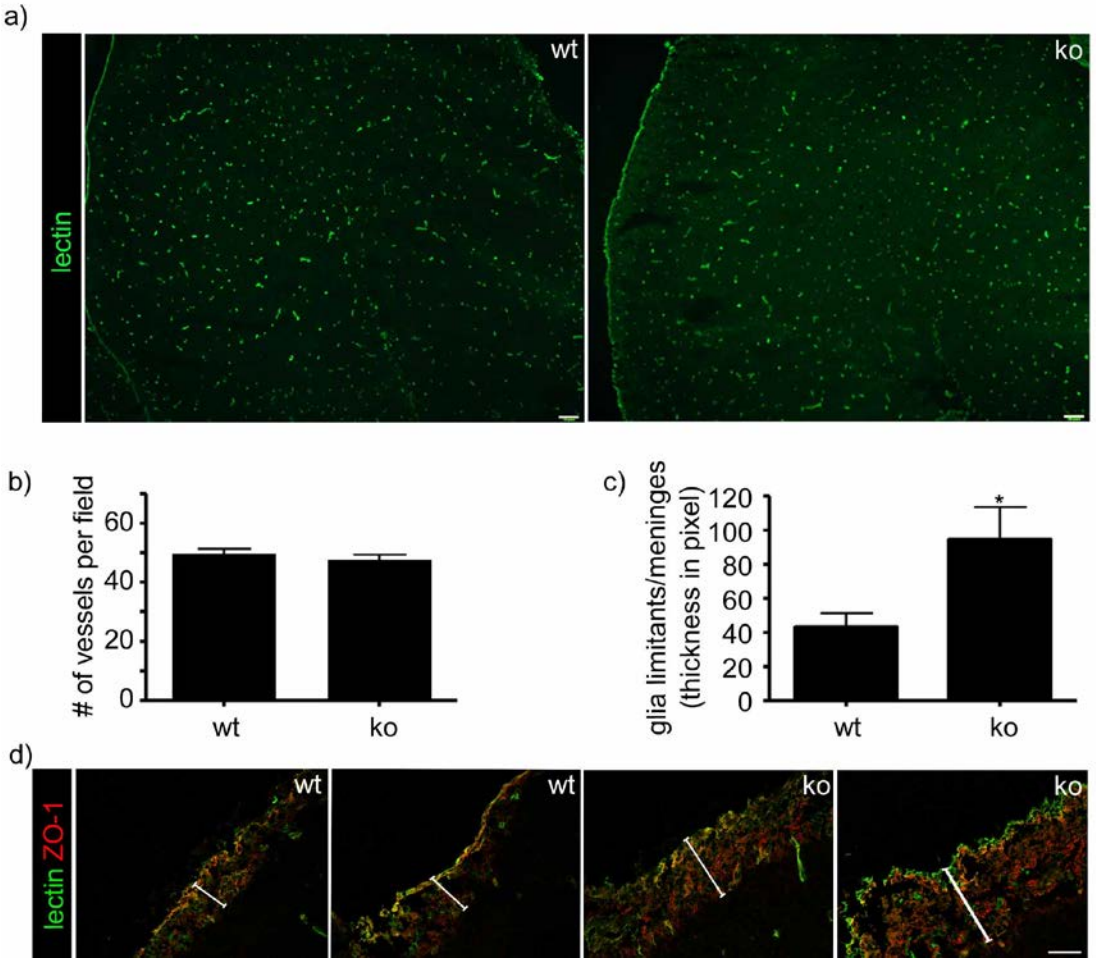

a)
lectin
wt
ko
b)
# of vessels per field
60
40
20
0
wt
ko
c)
glia limitants/meninges
(thickness in pixel)
120
100
80
60
40
20
0
*
wt
ko
d)
lectin ZO-1
wt
wt
ko
ko

Figure 3: Netrin-1 knockout mice show increased BBB permeability to plasma proteins. The effect of netrin-1 deficiency on the developing BBB was evaluated by measuring BBB permeability to blood-derived serum proteins. PECAM, laminin, and lectin were used to visualize the vascular endothelium (all red) (a). We found that extravasation of apoB, IgG, and fibrinogen (all green) (a) into the brain-parenchyma was increased in newborn netrin-1 knockout mice (ko) compared to wild types (wt). To highlight fibrinogen leakage from parenchymal vessels, we zoomed into a section showing fibrinogen extravasation from parenchymal vessels (lower right corners of bottom row panels) (a). Extravation was quantified and presented in graphs (b, c, d) by multiplying the mean pixel fluorescent intensity of serum protein and the area of leakage.

We used goat IgGs, anti rat secondary antibodies alone, and rabbit IgGs (together with the nuclei marker TOPRO in blue) as isotype controls for apoB, IgG and fibrinogen, respectively (e). Scale bars: 20 μm (a, b). (apoB = apolipoprotein B, IgG = type G immunoglobulins). n=4 wild types, n=4 knockouts.

Figure 3

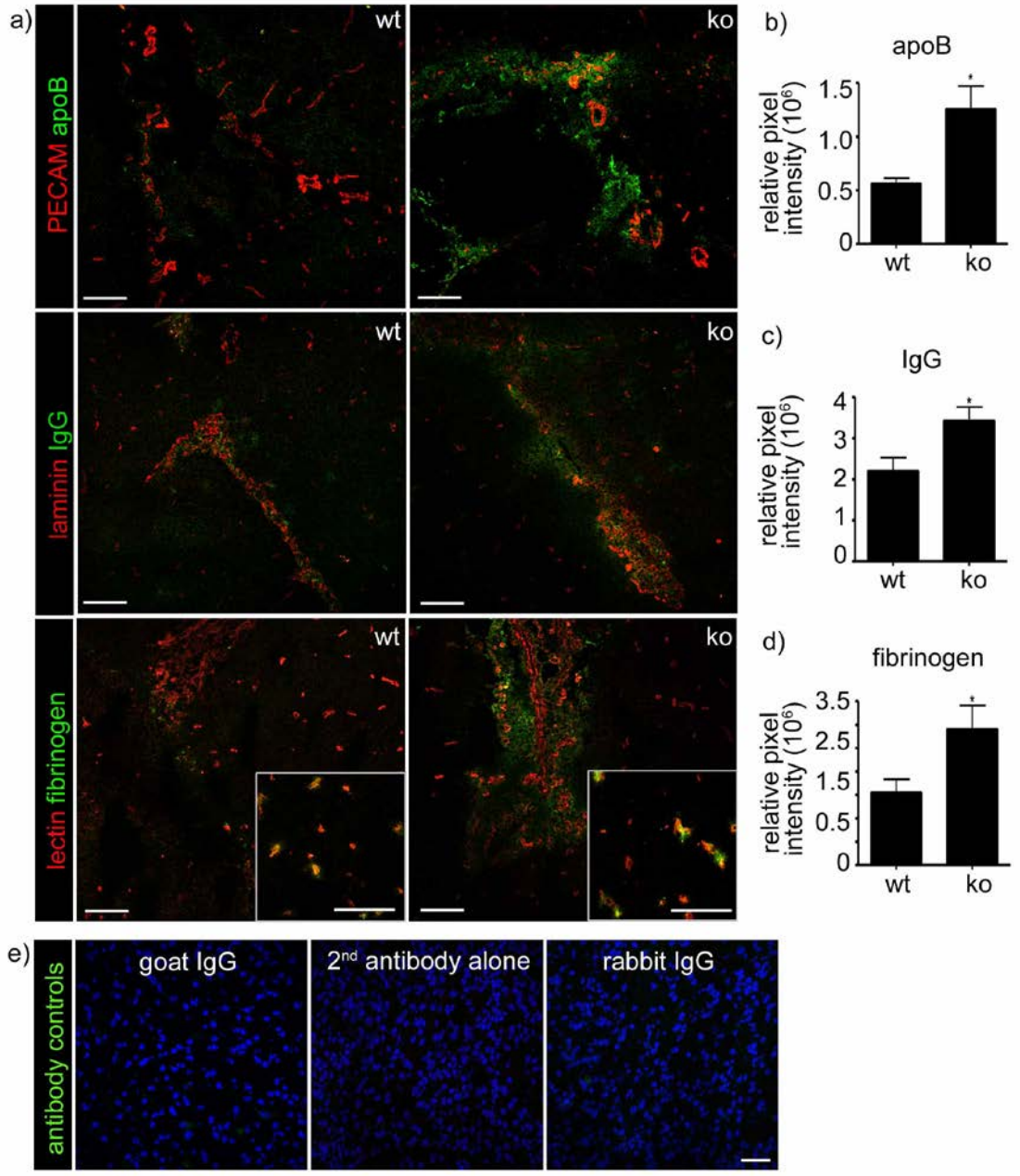

a)
PECAM apoB
wt
ko
laminin IgG
wt
ko
lectin fibrinogen
wt
ko
b)
apoB
relative pixel intensity (10^6)
1.5
1.0
0.5
0
wt
ko
c)
IgG
relative pixel intensity (10^6)
4
3
2
1
0
wt
ko
d)
fibrinogen
relative pixel intensity (10^6)
3.5
2.5
1.5
0.5
0
wt
ko
e)
antibody controls
goat IgG
2nd antibody alone
rabbit IgG

Figure 4: Netrin-1 knockout mice exhibit altered junction protein expression. Absence of netrin-1 reduced the expression of TJ proteins occludin (a, b, g), p120 (c, d), and JAM-1 (h), but increased ZO-1 expression (j, k), and had no effect on claudin-5 (e, f, i). We quantified the fluorescence intensity of the tight junction strands (a, c, e, j) from the *in situ* stained parenchymal (wt-p, ko-p) and meningeal (wt-m, ko-m) brain tissues and presented them in graphs (b, d, f, k) (n=4 mice per group). To confirm this we measured the relative band intensity for occludin, JAM-1, and claudin-5 of brain homogenates from wild type (wt), heterozygote (het) and knockout (ko) mice by western blot analysis (g-i). Scale bars: 20 μm for parenchymal and 5 μm meningeal vessels (a, c, e, and j). (wt-p = wt parenchymal vessels, ko-p = ko parenchymal vessels**,** wt-m = wt meningeal vessels, ko-m = ko meningeal vessels). n=4 wild types, n=4 knockouts.

Figure 4

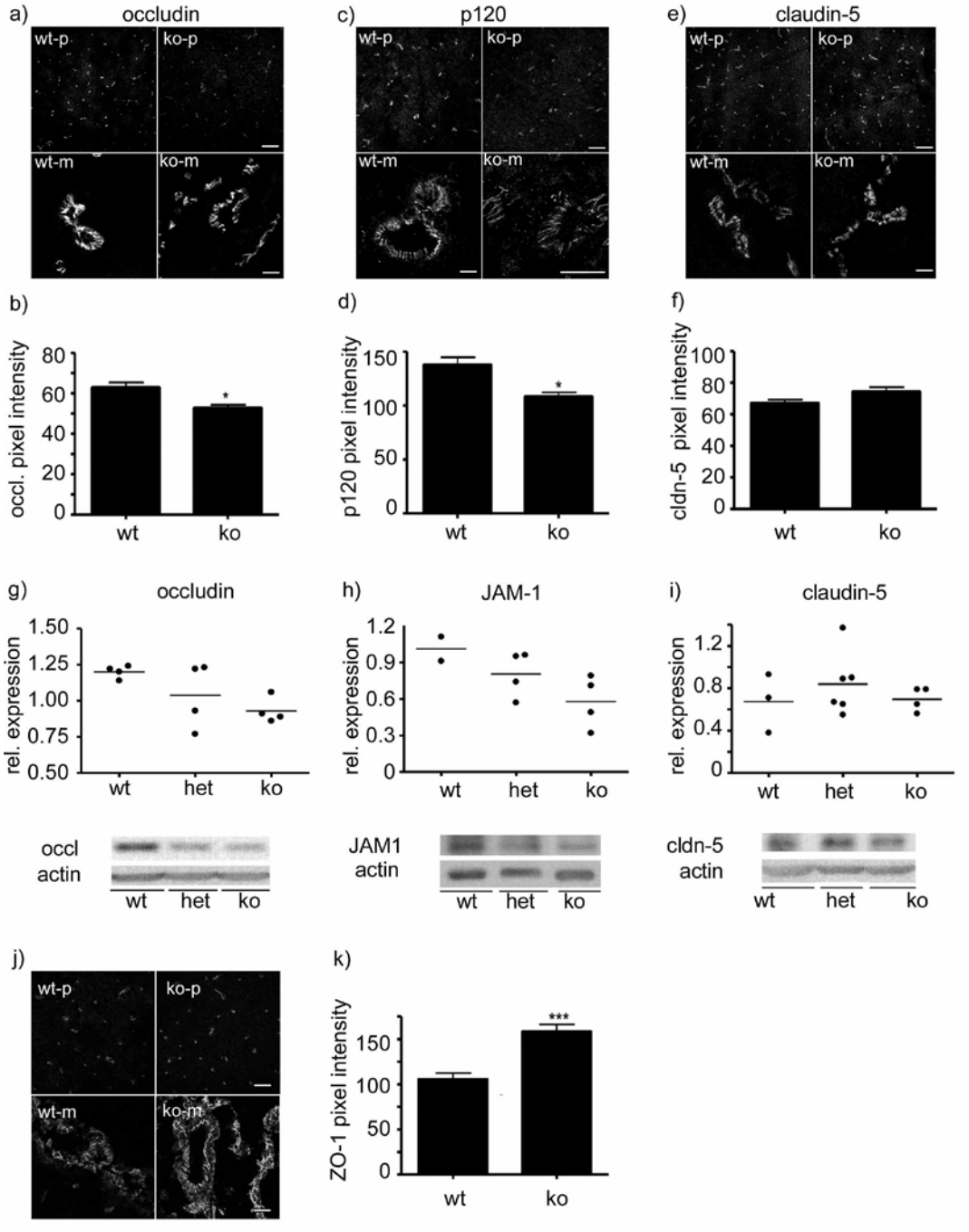

a)
occludin
wt-p
ko-p
wt-m
ko-m
c)
p120
e)
claudin-5
b)
occl. pixel intensity
d)
p120 pixel intensity
f)
cldn-5 pixel intensity
wt
ko
g)
occludin
rel. expression
h)
JAM-1
i)
claudin-5
het
occl
actin
JAM1
cldn-5
j)
k)
ZO-1 pixel intensity

Figure 5: Antibodies against netrin-1 or nerin-4 are specific. Recombinant netrin-1 protein and netrin-1 expressed by netrin-1 transfected HEK cells (HEK^{N1}) was exclusively recognized at 75 kDa by a monoclonal rat antibody (a) and a rabbit serum (c) against netrin-1. In BBB-ECs, we detected netrin-1 only under inflammatory conditions (T/I), but barely under resting conditions (R) (c). However, the size of netrin-1 protein dropped to about 60-70 kDa, which may have been a result of proteolytic cleavage as a consequence of inflammatory cell activation, which causes the release of proteolytic enzymes such as MMP2 and MMP9.

In turn, a polyclonal goat antibody against netrin-4 (b) recognized netrin-4 at about 80 kDa when added as recombinant protein (N4 at 50, 25 ng) or expressed by BBB-ECs, but it did not bind to netrin-1 recombinant protein (N1 at 50, 25 ng) or to netrin-1 when transfected in HEK cells (HEK^{N1}).

We used the polyclonal anti netrin-1 rabbit serum (c) for western blot analysis, and the monoclonal rat anti netrin-1 antibody for immunofluorescent staining analysis (d, f).

By both immunocytofluorescent staining (d) and flow cytometry analysis (e, f), we detected netrin-1 only the in netrin-1-transfected HEK^{N1} cells but not in untransfected HEK cells, compared to the isotype control (f). Netrin-1 was not detectable in HEK cells stained with antibodies against netrin-4 (e). (HEK=human embryonic kidney cells, N1=netrin-1, N4=netrin-4)

Figure 5

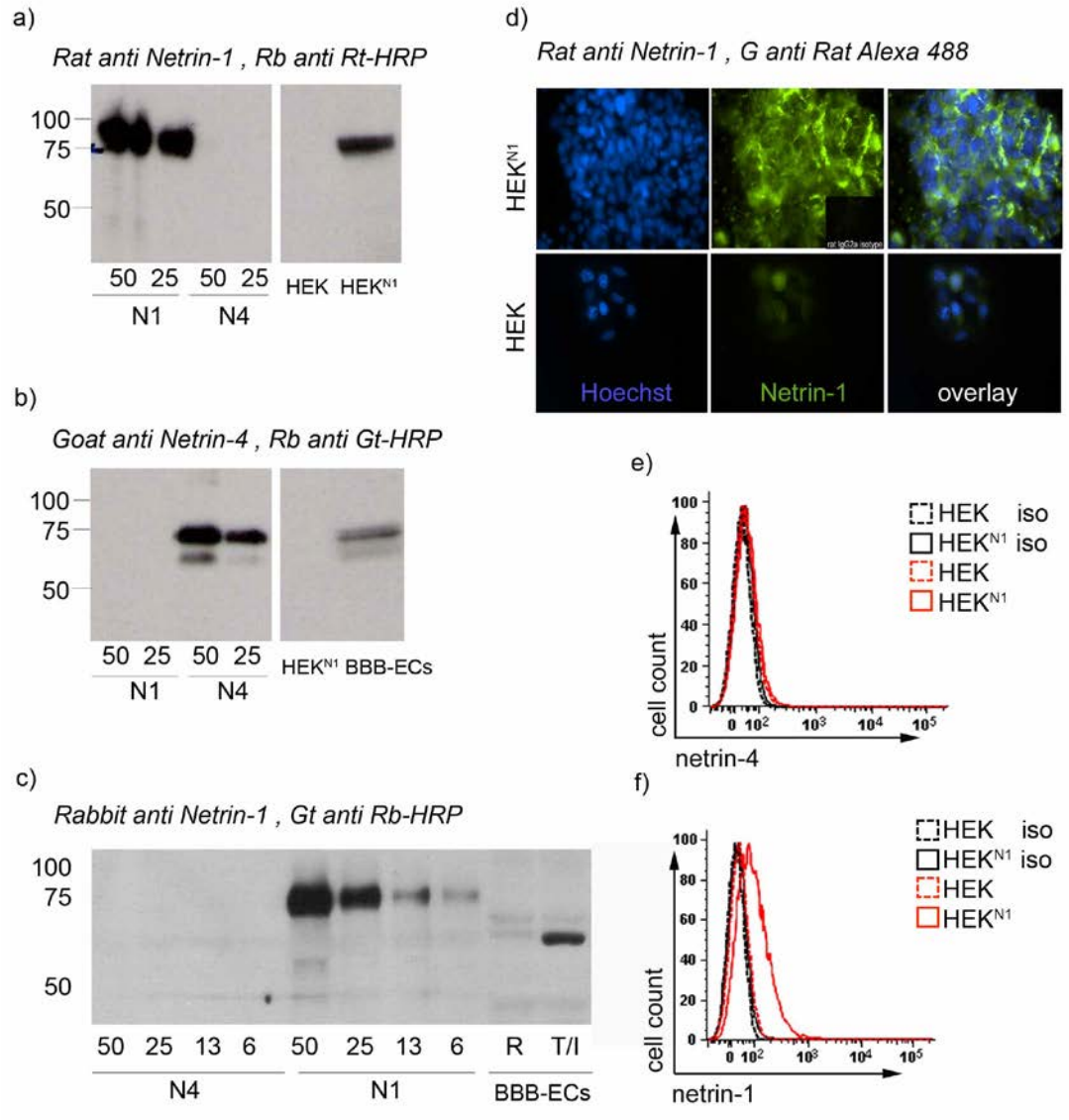

a)
Rat anti Netrin-1 , Rb anti Rt-HRP
100
75
50
50 25 50 25
HEK HEKN1
N1 N4
b)
Goat anti Netrin-4 , Rb anti Gt-HRP
100
75
50
50 25 50 25
HEKN1 BBB-ECs
N1 N4
c)
Rabbit anti Netrin-1 , Gt anti Rb-HRP
100
75
50
50 25 13 6 50 25 13 6 R T/I
N4 N1 BBB-ECs
d)
Rat anti Netrin-1 , G anti Rat Alexa 488
HEKN1
HEK
Hoechst
Netrin-1
overlay
e)
HEK iso
HEKN1 iso
HEK
HEKN1
cell count
netrin-4
f)
HEK iso
HEKN1 iso
HEK
HEKN1
cell count
netrin-1

Figure 6: Primary human BBB-ECs express netrin-1 and -4. Netrin-expression was measured by quantitative PCR (a, c) and western blot (b, d). Treatment of BBB-ECs with inflammatory cytokines (T/I) increased expression of netrin-1 (a, b). Conversely, netrin-4 transcript (c) and protein (d) were increased with barrier-promoting factors (ACM) but decreased with T/I. Netrin mRNA levels (a,c) are presented as the difference in cycle threshold (ΔCt) of netrin relative to actin. Netrin-protein levels were quantified from protein-band intensities of western blot photographs and represented as the percentile change of netrin expression compared to expression levels under untreated (R) conditions, normalized to 0% (b,d). All values are normalized to actin.

By flow cytometry (e-h), we detected both netrin-1 (e, f) and netrin-4 (g, h) in entire cells (filled circle) (f, h) and associated to the cell surface (empty circle) (e, g), compared to respective isotype controls, shown as a shaded graph. By immuno-fluorescent staining we visualized both netrin-1 (i-k) and netrin-4 (l-n) expression (green) by BBB-ECs and detected a diffuse staining pattern. Netrin-1 expression increased with T/I (k) over untreated BBB-ECs (i) and resembled an irregular honeycomb structure typical of junctional molecules. On the other hand, netrin-4 levels increased with ACM (m) but decreased with T/I (n), compared to untreated cells (l). Antibody isotope controls for both netrin-1 (rat IgG2a isotype) and netrin-4 (goat IgG isotype) showed minimal fluorescent background. Nuclei were visualized with TOPRO (blue). (R=untreated, ACM=astrocyte conditioned media, T/I=TNFα/IFNγ, N1=netrin-1, N4=netrin-4). Scale bars: 10 μm (k-p). n<5 BBB-EC preparations in min. n=3 qPCR or western blot experiments, n=3 BBB-EC preparations for flow cytometry or immunostaining analysis.

Figure 6

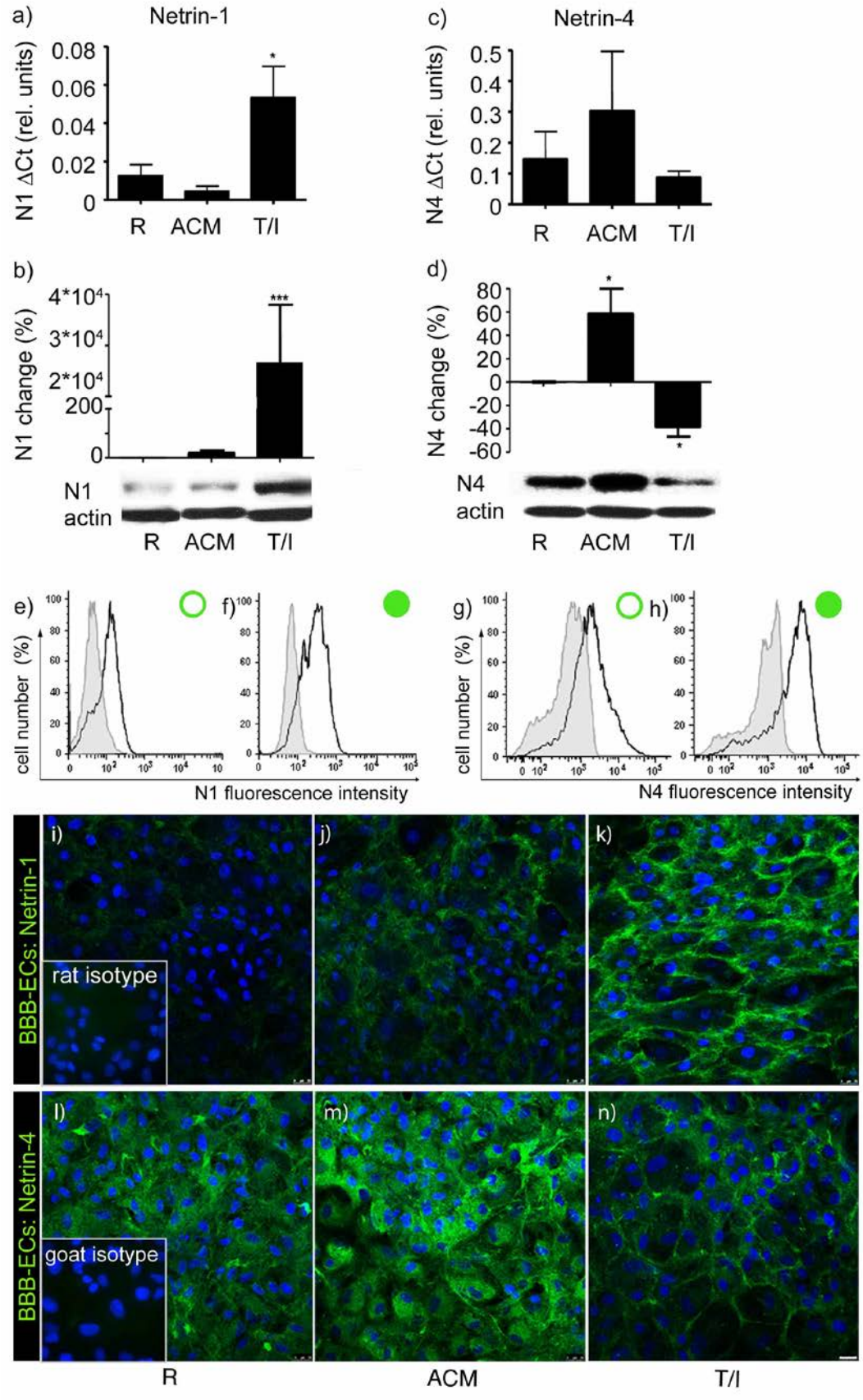

a)
Netrin-1
N1 ΔCt (rel. units)
0.08
0.06
0.04
0.02
0
R
ACM
T/I
c)
Netrin-4
N4 ΔCt (rel. units)
0.5
0.4
0.3
0.2
0.1
0
R
ACM
T/I
b)
N1 change (%)
4*10⁴
3*10⁴
2*10⁴
200
0
N1
actin
R
ACM
T/I
d)
N4 change (%)
80
60
40
20
0
-20
-40
-60
N4
actin
R
ACM
T/I
e)
f)
g)
h)
cell number (%)
N1 fluorescence intensity
N4 fluorescence intensity
BBB-ECs: Netrin-1
i)
j)
k)
rat isotype
BBB-ECs: Netrin-4
l)
m)
n)
goat isotype
R
ACM
T/I

Figure 7: Netrins reduce endothelial barrier permeability *in vitro* but do not impact BBB-EC proliferation or morphology. Netrins' effect on BBB permeability was tested by quantifying diffusion of tracer molecules through a monolayer of differentially treated BBB-ECs over several hours in a modified Boyden chamber permeability assay (a). Addition of recombinant netrin-1 (cross) and netrin-4 (triangle pointing downwards) reduced BSA (b) and D10 (c) tracer molecule diffusion. The addition of netrin-1 or netrin-4 promoted a 16 % decrease in BSA diffusion through the endothelial barrier, compared to ACM (empty square) at about 20 %.

By flow cytometry analysis, proliferation of BBB-ECs is not affected in presence of recombinant netrin-1 or netrin-4 (d). Proliferation was measured by recording the intensity of CFSE, a marker that binds to proteins and that dilutes progressively with each cell division. On day 1, cells showed an intense labeling with CFSE (red line), which diluted as cells divided by day 3. Treatment with ACM (light green line), N1 (light blue line) or N4 (dark blue line) was comparable to proliferation at baseline, but treatment with T/I (dark green line) decreased proliferation. Therefore, proliferation is not a contributing factor for netrins' effect on decreasing BBB permeability. Furthermore, we did not detect any change in BBB-EC morphology (e) when we added netrin to BBB-ECs compared to untreated or ACM treated cells. Scale bars: 30 μm (e). (R = untreated, ACM = astrocyte conditioned media, N1 = netrin-1, N4 = netrin-4, T/I = TNFα/IFNγ). n<10 BBB-EC preparations in independent permeability assays for netrin-1, n=5 for netrin-4, n=3 BBB-EC preparations for proliferation assays.

Figure 7

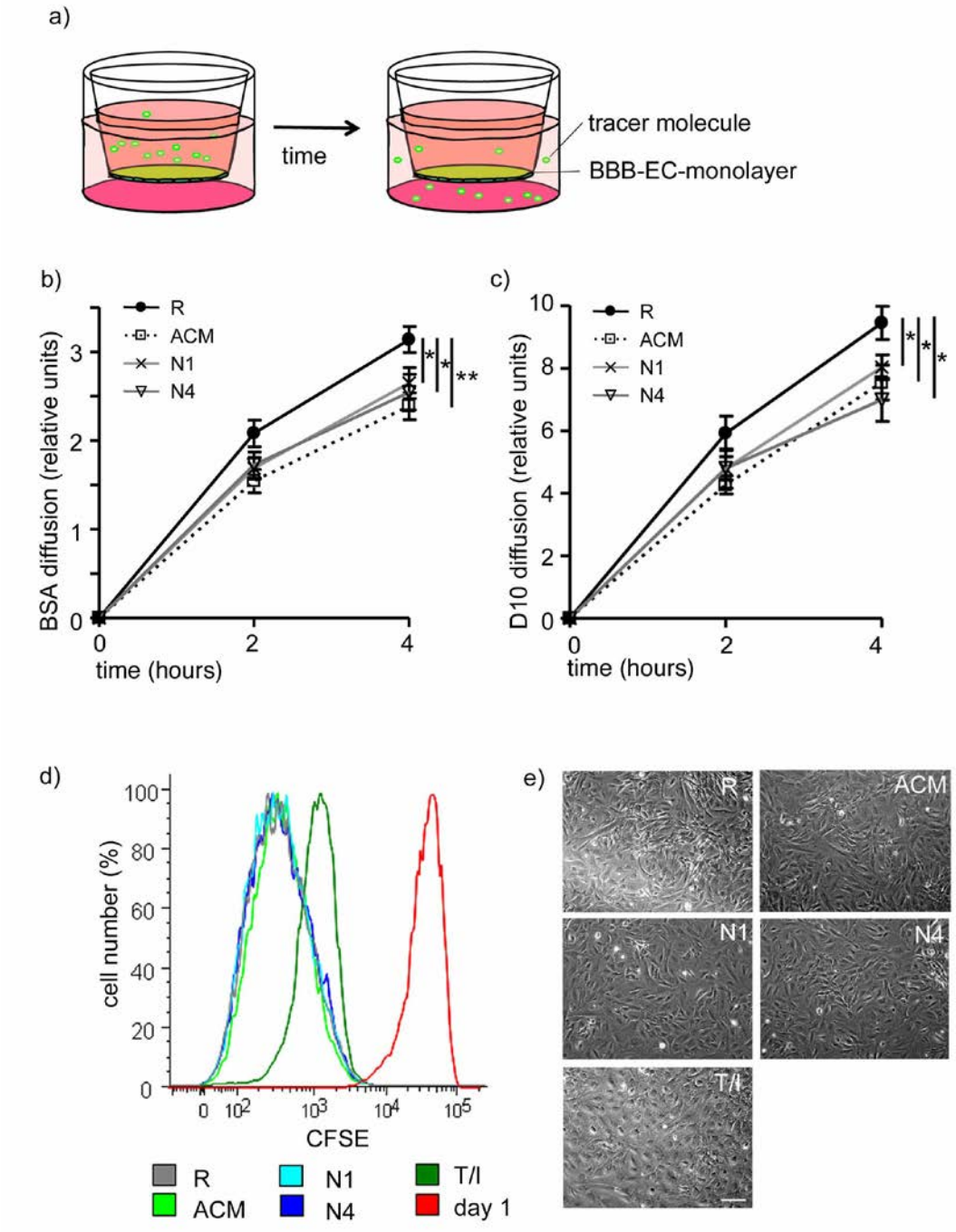

a)
time
tracer molecule
BBB-EC-monolayer
b)
R
ACM
N1
N4
BSA diffusion (relative units)
time (hours)
c)
D10 diffusion (relative units)
time (hours)
d)
cell number (%)
CFSE
R
ACM
N1
N4
T/I
day 1
e)
R
ACM
N1
N4
T/I

Figure 8: Netrins increase junctional protein expression by BBB-ECs. Endothelial cells increased expression of JAM-1 (a), occludin-5 (b), and claudin-5 (c) upon treatment with either ACM or netrin-1, and to a lesser extend with netrin-4, compared to untreated BBB-ECs. Treatment with T/I induced a reduction in TJ molecule expression. Graphs (a-c) represent the quantitative percentile change of expression of western blot photographs, normalized to actin and relative to untreated cells (R, at 0%). Immunofluorescence analysis confirmed increased expression of ZO-1, p120 and α-catenin by BBB-ECs when treated with ACM, netrin-1 or netrin-4, compared to untreated cells (g). With inflammatory activation (T/I), expression of ZO-1, p120 and α-catenin diminished and its pattern became fragmented and irregular. Fluorescent pixel intensity was quantified and plotted for ZO-1, p120, and α-catenin, which showed that netrin-1 and netrin-4 treatment increased the expression of the above TJ proteins (h).

Netrin-1 prevented the loss of JAM-1 (d), occludin (e) and claudin-5 (f) that otherwise occurred with proinflammatory activation of BBB-ECs. Graphs (d, e, and f) represent the quantitative percentile change of expression of western blot photographs normalized to actin and relative to inflamed cells (T/I at 0%). Scale bars: 10 μm (g). (R = untreated, ACM = astrocyte conditioned media, N1 = netrin-1, N4 = netrin-4, T/I = TNFα/IFNγ). n<5 BBB-EC preparations in independent western blot experiments, n=3 BBB-EC preparations for immunostaining analysis.

Figure 8

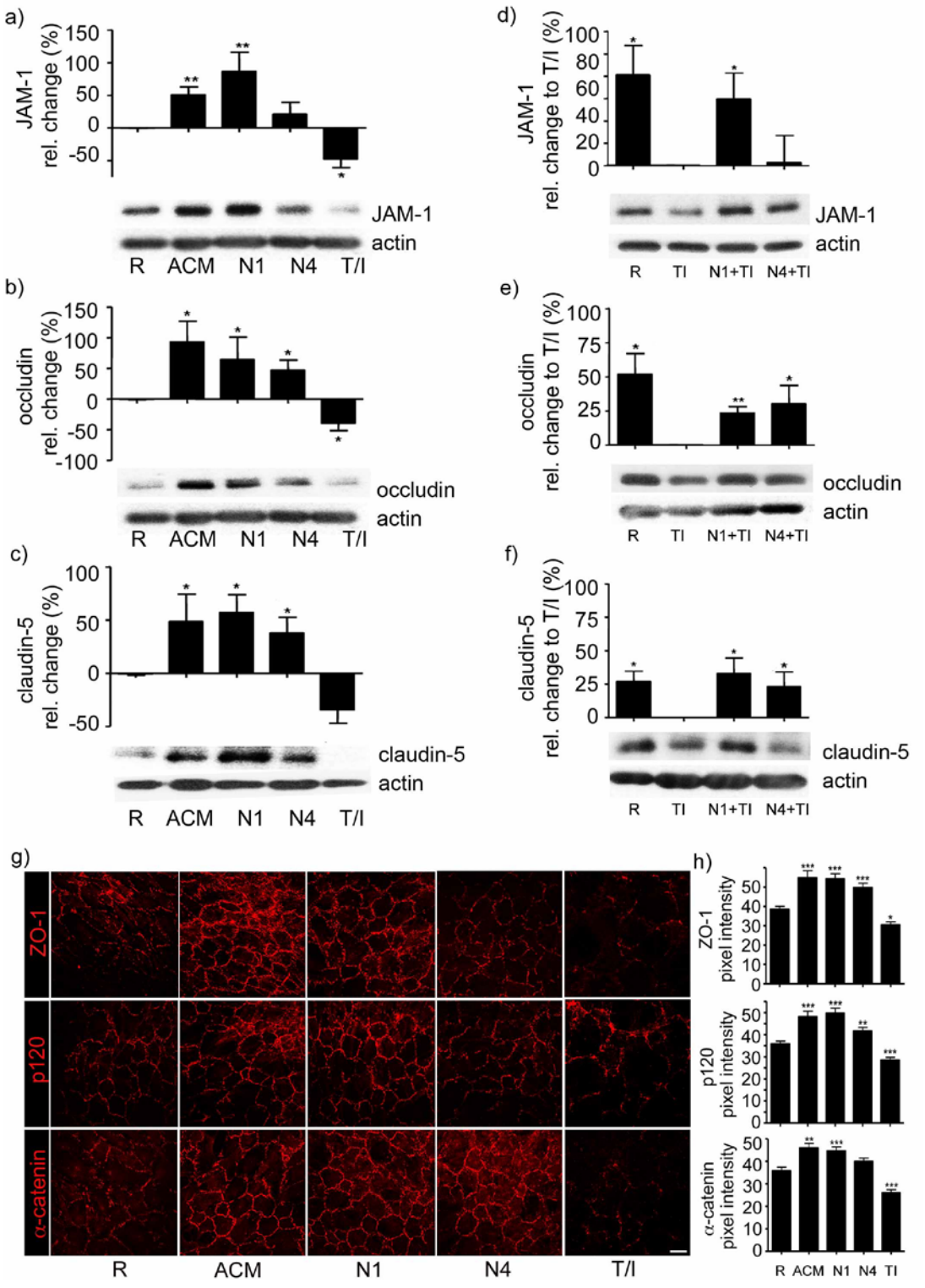

a)
JAM-1 rel. change (%)
150
100
50
0
-50
JAM-1
actin
R ACM N1 N4 T/I
b)
occludin rel. change (%)
150
100
50
0
-50
-100
occludin
actin
R ACM N1 N4 T/I
c)
claudin-5 rel. change (%)
100
50
0
-50
claudin-5
actin
R ACM N1 N4 T/I
d)
JAM-1 rel. change to T/I (%)
100
80
60
40
20
0
JAM-1
actin
R TI N1+TI N4+TI
e)
occludin rel. change to T/I (%)
100
75
50
25
0
occludin
actin
R TI N1+TI N4+TI
f)
claudin-5 rel. change to T/I (%)
100
75
50
25
0
claudin-5
actin
R TI N1+TI N4+TI
g)
ZO-1
p120
α-catenin
R ACM N1 N4 T/I
h)
ZO-1 pixel intensity
p120 pixel intensity
α-catenin pixel intensity
50
40
30
20
10
0
R ACM N1 N4 TI

Figure 9: Netrin-1 enriches tight junction proteins in lipid rafts. Lipid raft fractions were isolated by sucrose density ultra-centrifugation and were identified by quantification of protein (a) and cholesterol (b) content of each of the 12 fractions harvested. The cholesterol-dense fractions 4 and 5 were confirmed to be lipid raft fractions as we detected the presence of lipid raft markers GM1 and CD59 and the absence of TfR by western blot analysis (c). Western blot photographs show increased levels of JAM-1 (d) and occludin (f) within lipid rafts from either ACM or netrin-1 treated, over untreated cells. Quantification of JAM-1 (e) and occludin (g) revealed that occluding significantly increased in raft fractions 4 and 5, in presence of netrin-1, relative to the protein content in pellet fractions 10 to 12. (R = untreated, ACM = astrocyte conditioned media, N1 = netrin-1, N4 = netrin-4). n=4 BBB-EC preparations and individual lipid raft fraction analyses.

Figure 9

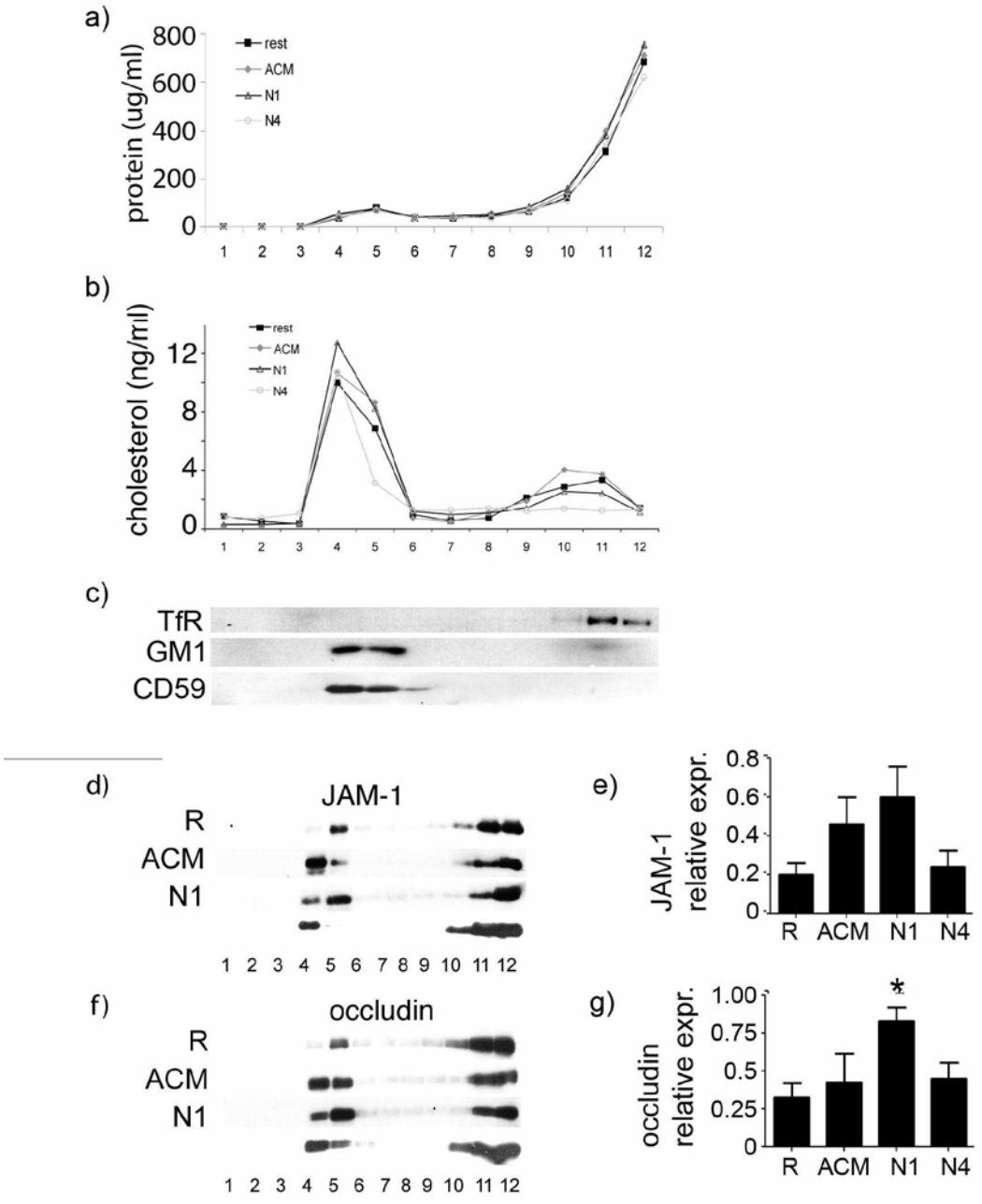

a)
protein (ug/ml)
800
600
400
200
0
rest
ACM
N1
N4
1 2 3 4 5 6 7 8 9 10 11 12
b)
cholesterol (ng/ml)
12
8
4
0
rest
ACM
N1
N4
1 2 3 4 5 6 7 8 9 10 11 12
c)
TfR
GM1
CD59
d)
JAM-1
R
ACM
N1
1 2 3 4 5 6 7 8 9 10 11 12
e)
JAM-1 relative expr.
0.8
0.6
0.4
0.2
0
R ACM N1 N4
f)
occludin
R
ACM
N1
1 2 3 4 5 6 7 8 9 10 11 12
g)
occludin relative expr.
1.00
0.75
0.5
0.25
0
*
R ACM N1 N4

Figure 10: Primary human BBB-ECs express netrin receptors. The netrin receptors neogenin (a, b), unc5B (c, d) and unc5C (e, f) are expressed by BBB-ECs, as analyzed by quantitative PCR (a, c, e, g) and by western blot (b, d, f). Neogenin expression is up-regulated by ACM and down-regulated by inflammation (T/I), similar to expression patterns that we have seen for netrin-4. Unc5B and Unc5C are expressed at low levels and remain unchanged throughout the differential treatment conditions. DCC, Unc5A, C, and D, and netrin-3 were undetectable in primary human BBB-ECs, while fetal brain tissue expressed these transcripts and served as a positive control (g). mRNA levels (a, c, e, and g) are presented as the difference in cycle threshold (ΔCt) of the respective receptor relative to actin. To represent the percentile change in protein expression relative to the expression levels of untreated BBB-ECs (at 0 %) we measured western blot protein-band intensities and normalized them to actin (b, d, and f). (R = untreated, ACM = astrocyte conditioned media, T/I = TNFα/IFNγ). n>3 BBB-EC preparations and n=2 human fetal brain preparations in n=3 qPCR or western blot experiments.

Figure 10

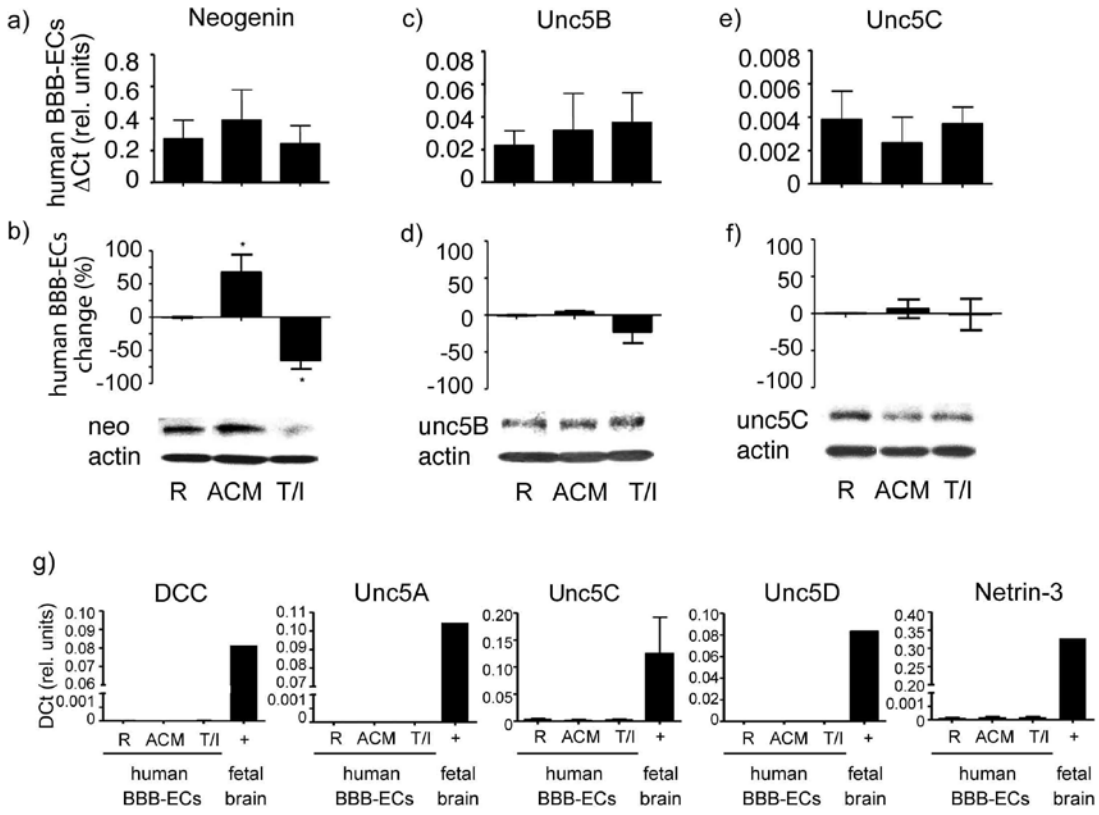

a)
Neogenin
human BBB-ECs ΔCt (rel. units)
0.8
0.6
0.4
0.2
0
c)
Unc5B
0.08
0.06
0.04
0.02
0
e)
Unc5C
0.008
0.006
0.004
0.002
0
b)
human BBB-ECs change (%)
100
50
0
-50
-100
neo
actin
R ACM T/I
d)
100
50
0
-50
-100
unc5B
actin
R ACM T/I
f)
100
50
0
-50
-100
unc5C
actin
R ACM T/I
g)
DCC
Unc5A
Unc5C
Unc5D
Netrin-3
DCt (rel. units)
R ACM T/I +
human BBB-ECs
fetal brain

Figure 11: Human microvessels in MS brain tissue express netrins. Both netrin-1 (red) (a) and netrin-4 (red) (b) were co-stained with the endothelial marker caveolin (green) and the nuclear marker TOPRO (blue) in human brain sections. Netrin expression co-localized to blood vessels in the brain (overlay). Whereas netrin-1 was absent (a, upper row) and netrin-4 was present (b, upper row) in the microvasculature of NAWM, blood vessels within lesioned and infiltrated areas of brains from MS patients exhibited an increased expression of netrin-1 (a, lower row) but decreased or absent expression of netrin-4 (b, lower row).

Levels of netrin-1 were elevated in blood serum from MS-patients (~4500 pg), compared to healthy blood donors (~2500 pg) by ELISA-analysis (c). No significant difference in levels of netrin-1 was detected in CSF-samples from MS-patients and patients with other neurological conditions (d). Scale bars: 10 μm (a, b, e). Rat IgG2a and goat IgG antibodies were used as isotype controls (e). (NAWM = normal appearing white matter, MS = Multiple Sclerosis). n=3 human tissue donors, n>7 human serum samples from each healthy donors or MS patients, n>4 human CSF from each healthy donors or MS patients.

Figure 11

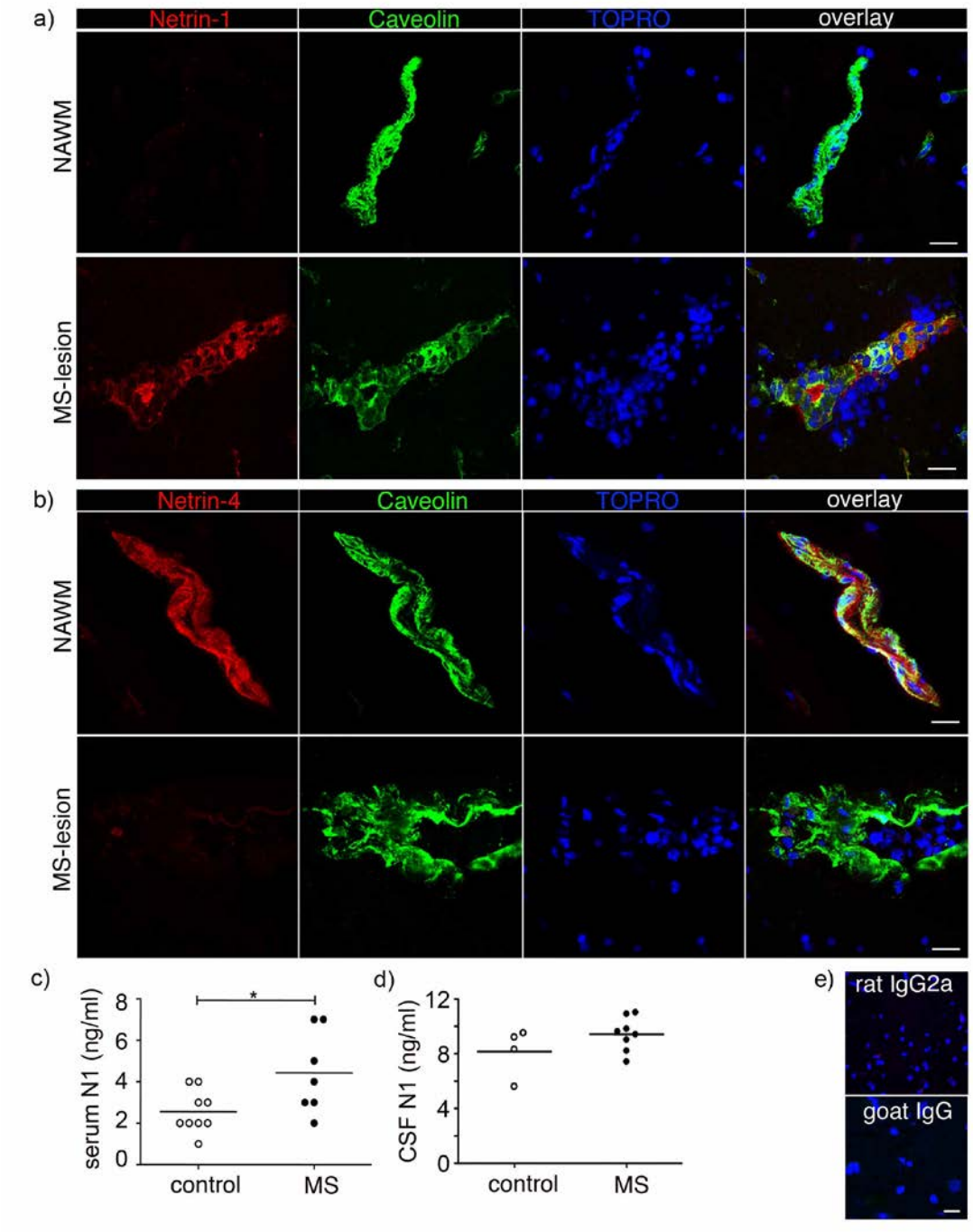

a)
Netrin-1
Caveolin
TOPRO
overlay
NAWM
MS-lesion
b)
Netrin-4
Caveolin
TOPRO
overlay
NAWM
MS-lesion
c)
serum N1 (ng/ml)
8
6
4
2
0
*
control
MS
d)
CSF N1 (ng/ml)
12
8
4
0
control
MS
e)
rat IgG2a
goat IgG

Figure 12: Brain-vessels in healthy and EAE mice express netrins. Brain sections of control (a,b, upper panels) or EAE mice (a,b, lower panels) were labeled for the endothelial marker caveolin (green) and the nuclear marker TOPRO (blue) together with specific antibodies against either netrin-1 (red, a) or netrin-4 (red, b). While netrin-1 was absent and netrin-4 was present in the microvasculature of the control mice, blood vessels from the brains of mice with EAE at sites of immune cell infiltration exhibit increased expression of netrin-1 (overlay) and decreased or absent expression of netrin-4 (overlay). Application of isotype controls for the netrin-1 and netrin-4 antibodies showed no signal. Scale bars: 10 μm. n=4 healthy control mice, n=4 sick EAE mice, n>3 immunohistofluorescent staining analyses.

Figure 12

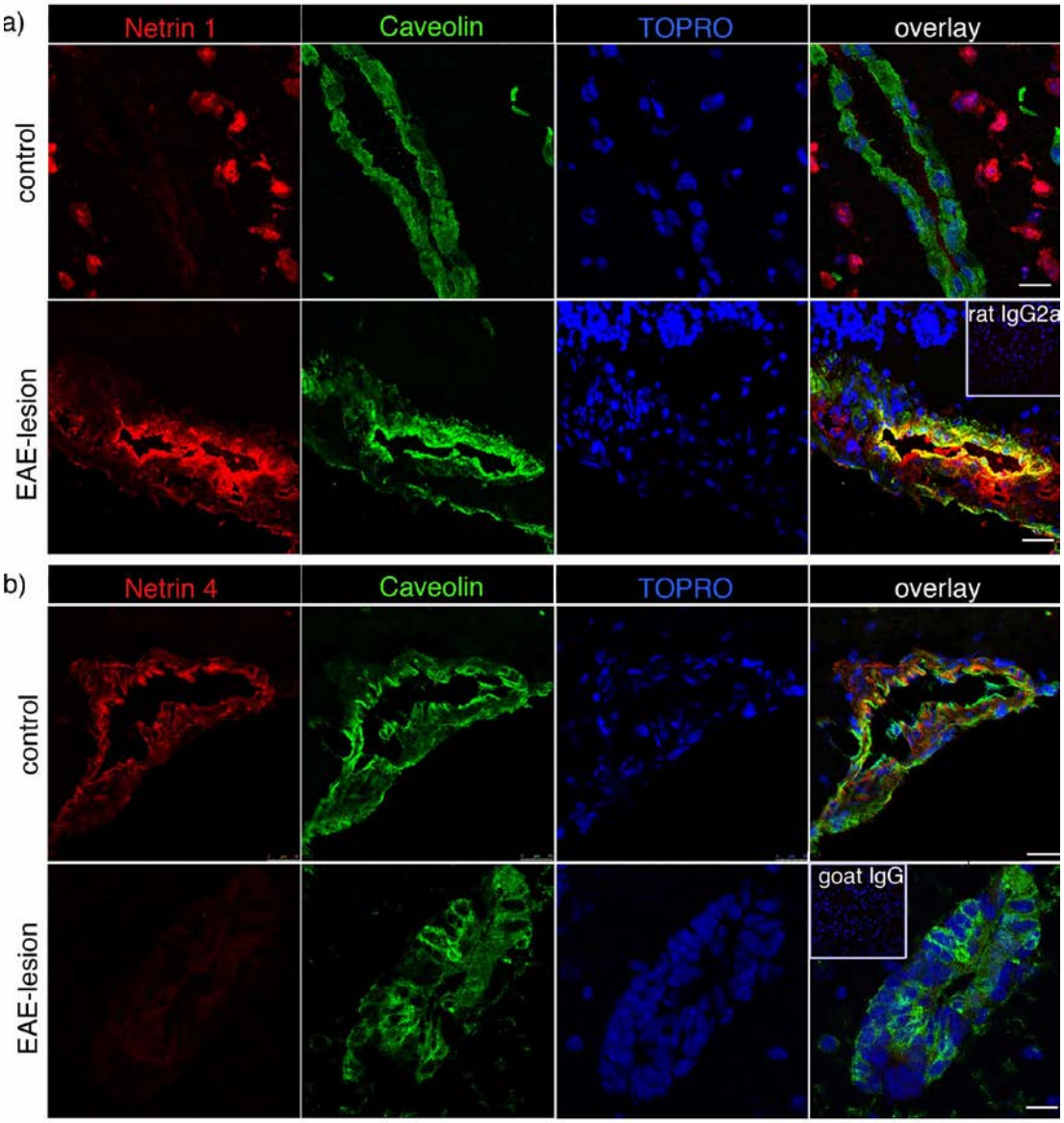
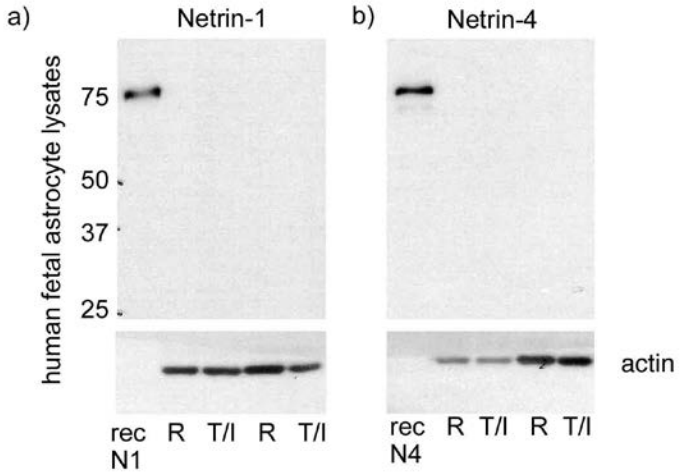

a)
Netrin 1
Caveolin
TOPRO
overlay
control
EAE-lesion
rat IgG2a
b)
Netrin 4
Caveolin
TOPRO
overlay
control
EAE-lesion
goat IgG

Figure 13: Astrocytes do not express netrins *in vitro* and *in vivo*. Cultured human foetal astrocytes did not express netrin-1 (a) or netrin-4 (b) by western blot analysis. Immunofluorescent staining of mouse brain sections for the astrocytic marker GFAP (red), nuclei (blue) and netrin-1 (green) or netrin-4 (green) did not detect netrin expression by astrocytes (c). In the healthy brain (left panel), netrin-1 is expressed by neurons and netrin-4 by blood vessels. In the inflamed brain (right panel), netrin-1 staining is up-regulated in the blood vessels and netrin-4 expression reduced in endothelial blood vessels. Scale bars: 10 μm (R=untreated, ACM=astrocyte conditioned media, rec N1 = recombinant netrin-1, rec N4 = recombinant netrin-4, T/I=TNFα/IFNγ). n=2 human fetal astrocyte supernatants for western blot analysis, n=2 immunohistofluorescent analysis of each healthy and EAE mouse brain tissues.

Figure 13


a)
Netrin-1
b)
Netrin-4
human fetal astrocyte lysates
75
50
37
25
actin
rec
N1
R
T/I
R
T/I
rec
N4
R
T/I
R
T/I

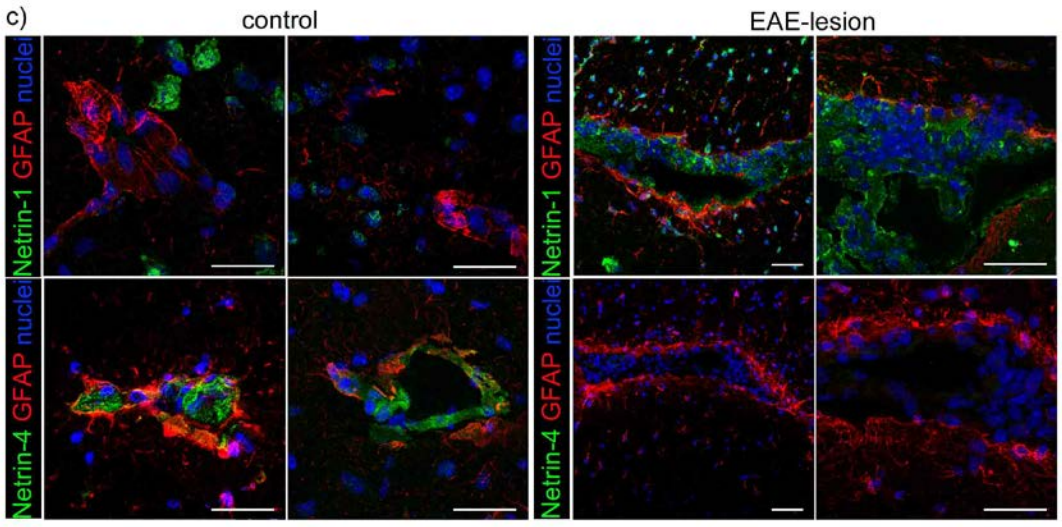
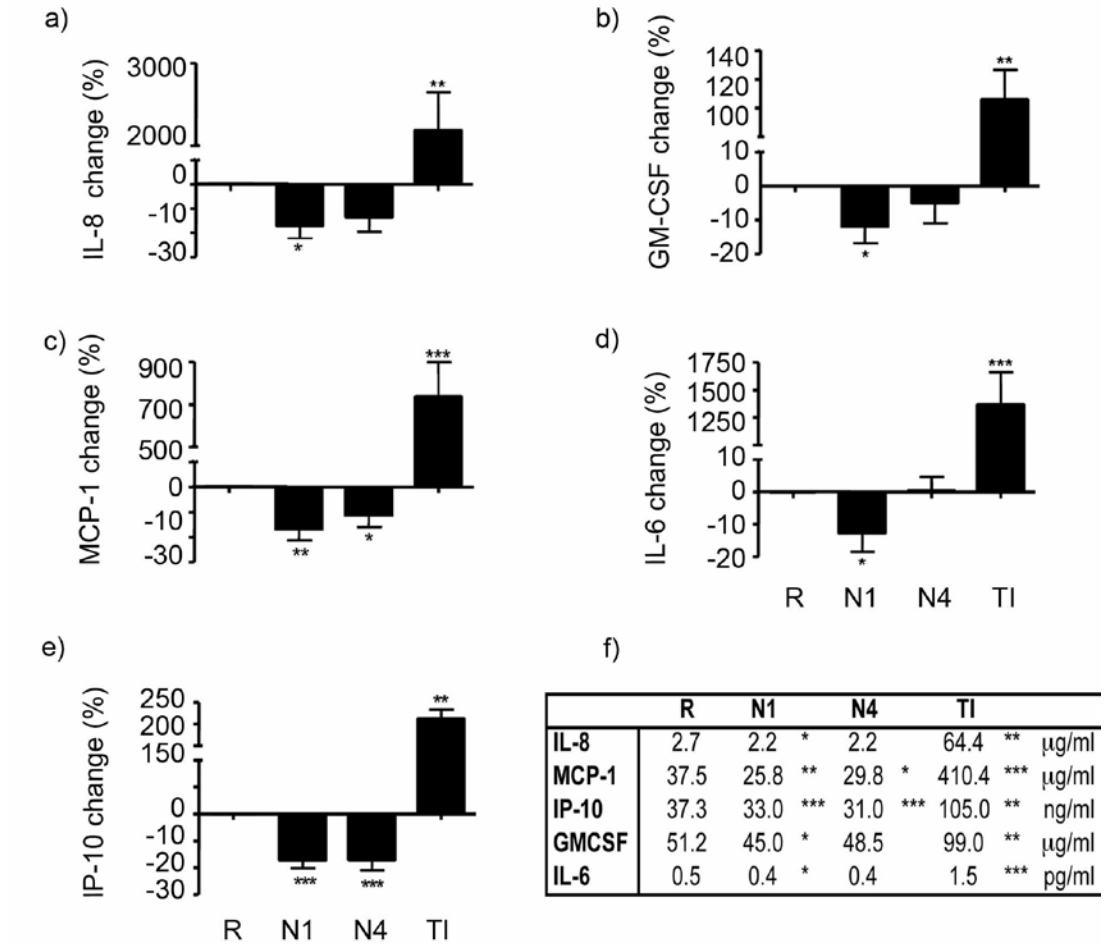

c)
control
EAE-lesion
Netrin-1 GFAP nuclei
Netrin-4 GFAP nuclei

Figure 14: Netrins reduce cytokine and chemokine secretion by BBB-ECs. By ELISA analysis, we tested netrins' anti-inflammatory potential on endothelial chemokine (a, c, e) and cytokine (b, d) expression and plotted them as a relative percentile change over untreated BBB-EC's. Netrin-1 reduced the expression of IL-8, MCP-1, IP-10, GM-CSF, and IL-6. Netrin-4 on the other hand, reduced the secretion of chemokines MCP-1 and IP-10. The table (f) shows the average concentrations of cytokines and chemokines, secreted by BBB-ECs under the different treatment conditions. n=5 ELISA experiments with supernatants of n>5 of BBB-EC preparations.

Figure 14



f)

	R	N1		N4		TI		
IL-8	2.7	2.2	*	2.2		64.4	**	µg/ml
MCP-1	37.5	25.8	**	29.8	*	410.4	***	µg/ml
IP-10	37.3	33.0	***	31.0	***	105.0	**	ng/ml
GMCSF	51.2	45.0	*	48.5		99.0	**	µg/ml
IL-6	0.5	0.4	*	0.4		1.5	***	pg/ml

Figure 15: Netrins do not affect levels of cell adhesion molecule expression nor T cell trans-endothelial migration. To screen BBB-ECs for CAM expression after exposure to netrin we used flow cytometry analysis (a-f). Treatment of resting (a,b) BBB-endothelial cells did not alter expression of cell adhesion molecules ICAM-1 and MCAM, which are normally up regulated under inflammatory conditions (T/I) in order to help immune cells to cross the endothelium. Netrins also did not affect ICAM-1, MCAM, and ALCAM expression when BBB-ECs were activated (c-e). Netrin-1 and -4 had no effect on the expression of antigen-presenting complex HLA-ABC by activated BBB-ECs (f).

To study T cell transendothelial migration we performed Boyden chamber migration assays (g, h). Numbers of CD4 or CD8 T cells that migrated through a BBB-EC monolayer in presence of netrin remained the same compared to untreated BBB-ECs (g, h). (R=untreated, N1= recombinant netrin-1, N4= recombinant netrin-4, T/I=TNFα/IFNγ). n=3 flow cytometry experiments with n=3 BBB-EC preparations, n>6 migration experiments with n=6 T cell donors (each CD4 and CD8) $_{\text{and}}$ n>3 BBB-EC preparations.

Figure 15

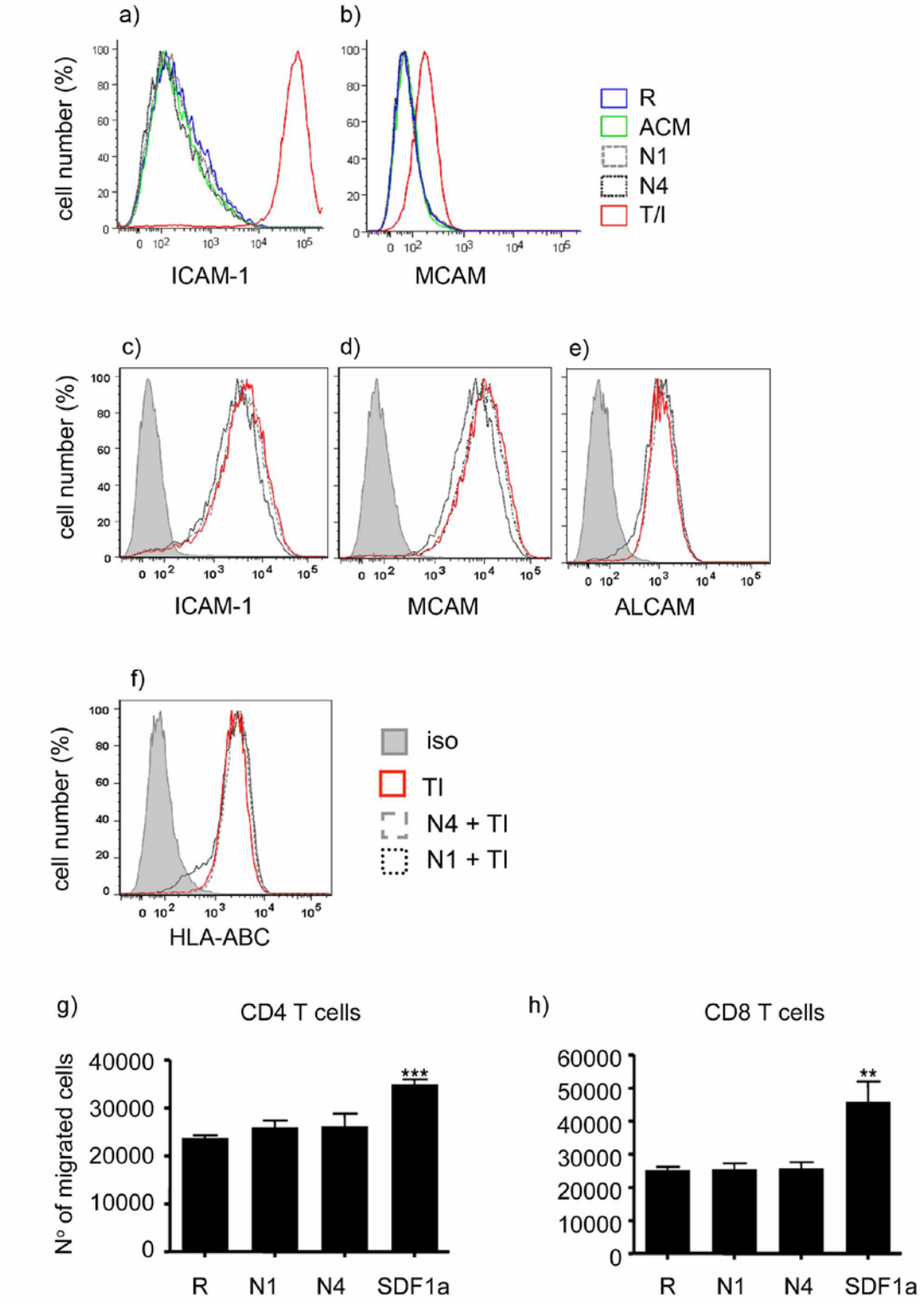

a)
b)
cell number (%)
ICAM-1
MCAM
R
ACM
N1
N4
T/I
c)
d)
e)
ICAM-1
MCAM
ALCAM
f)
HLA-ABC
iso
TI
N4 + TI
N1 + TI
g)
CD4 T cells
Nº of migrated cells
40000
30000
20000
10000
0
***
R
N1
N4
SDF1a
h)
CD8 T cells
60000
50000
40000
30000
20000
10000
0
**
R
N1
N4
SDF1a

Figure 16: T cells express netrin receptors, while mature antigen presenting cells express netrin-1. Monocytes, when differentiated to immature and mature (LPS-activated) dendritic cells, increase their level of netrin and netrin-receptor expression by qPCR (a, b, c). Monocytes do not express any netrin or netrin receptor, but when we differentiated them to dendritic cells they up-regulated neogenin and Unc5B. Only in the activated state, as mature antigen presenting cell, dendritic cells expressed netrin-1. *Ex vivo* and *in vitro* CD4 and CD8 T cells on the other hand expressed only netrin-4, neogenin and Unc5B by qPCR (d-f). (Mono = monocytes, iDC = immature dendritic cells, mDCs = mature dendritic cells). n>3 qPCR experiments, n=4 myeloid cells, n=6 T cells.

Figure 16

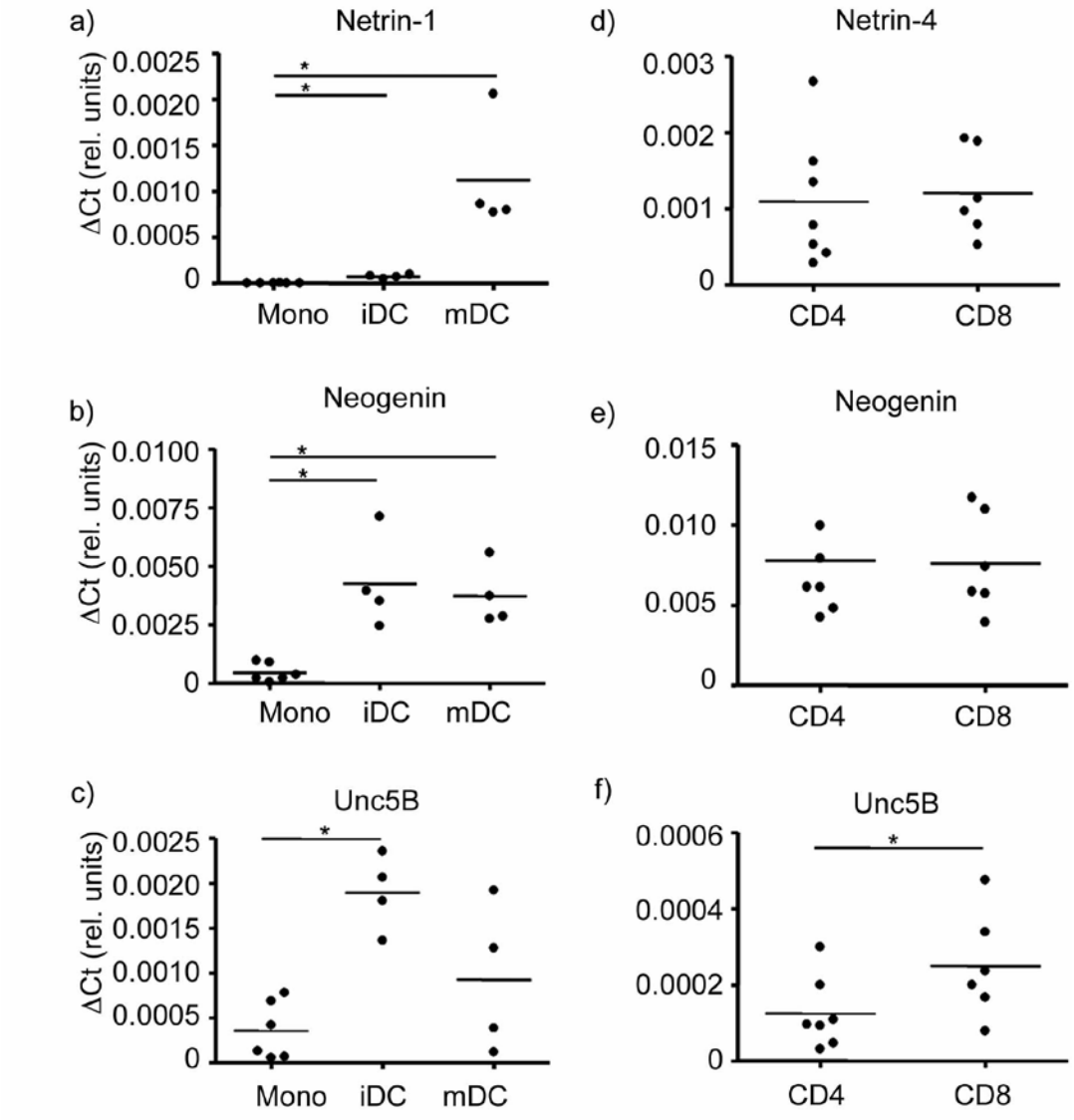

a)
Netrin-1
ΔCt (rel. units)
0.0025
0.0020
0.0015
0.0010
0.0005
0
*
*
Mono
iDC
mDC
d)
Netrin-4
0.003
0.002
0.001
0
CD4
CD8
b)
Neogenin
ΔCt (rel. units)
0.0100
0.0075
0.0050
0.0025
0
*
*
Mono
iDC
mDC
e)
Neogenin
0.015
0.010
0.005
0
CD4
CD8
c)
Unc5B
ΔCt (rel. units)
0.0025
0.0020
0.0015
0.0010
0.0005
0
*
Mono
iDC
mDC
f)
Unc5B
0.0006
0.0004
0.0002
0
*
CD4
CD8

Figure 17: T cells adhere to netrin-1, but not to netrin-4. In a cell adhesion assay, we added T cells to different substrates. Both, CD4 and CD8 T cells adhered strongly on netrin-1 coated surfaces, more than to the fibronectin positive control, but not to surfaces coated with netrin-4 or to BSA, as a negative control substrate (a,b). We abolished this adhesive effect by adding a netrin-1 function blocking antibody, compared to an isotype control antibody (c, e). Although T cells express neogenin and Unc5B, the addition of neogenin-FC or Unc5B-Fc receptor constructs, which competitively bind netrin-1, did not reverse netrin's adhesive effect (d). Neither did antibody-mediated function blocking antibodies against beta-1 integrins (d). We also observed that T cells adhered to netrin-1 coated surfaces by video-microscopy (f). While T cells explored a control surface with extracellular matrix proteins and actively changed their cell shape during crawling, in the presence of netrin-1, T cells obtained a round shape and no longer migrated on these matrix proteins. (BSA = bovine serum albumin, N1 = recombinant netrin-1, N4 = recombinant netrin-4, FIBR. = fibronectin, α N1= rabbit anti netrin function blocking serum, NeoFc = neogenin receptor Fc construct, α Neo = goat anti neogenin function blocking antibody, α integrin = hamster anti pan beta-1 integrin function blocking antibody, g/ha IgG = goat/hamster IgG isotype control, ECM = extracellular matrix proteins). n>2 for netrin or receptor blockade experiments with n>2 different T cell donors, n=3 videomicroscopy with n=3 different T cells donors.

Figure 17

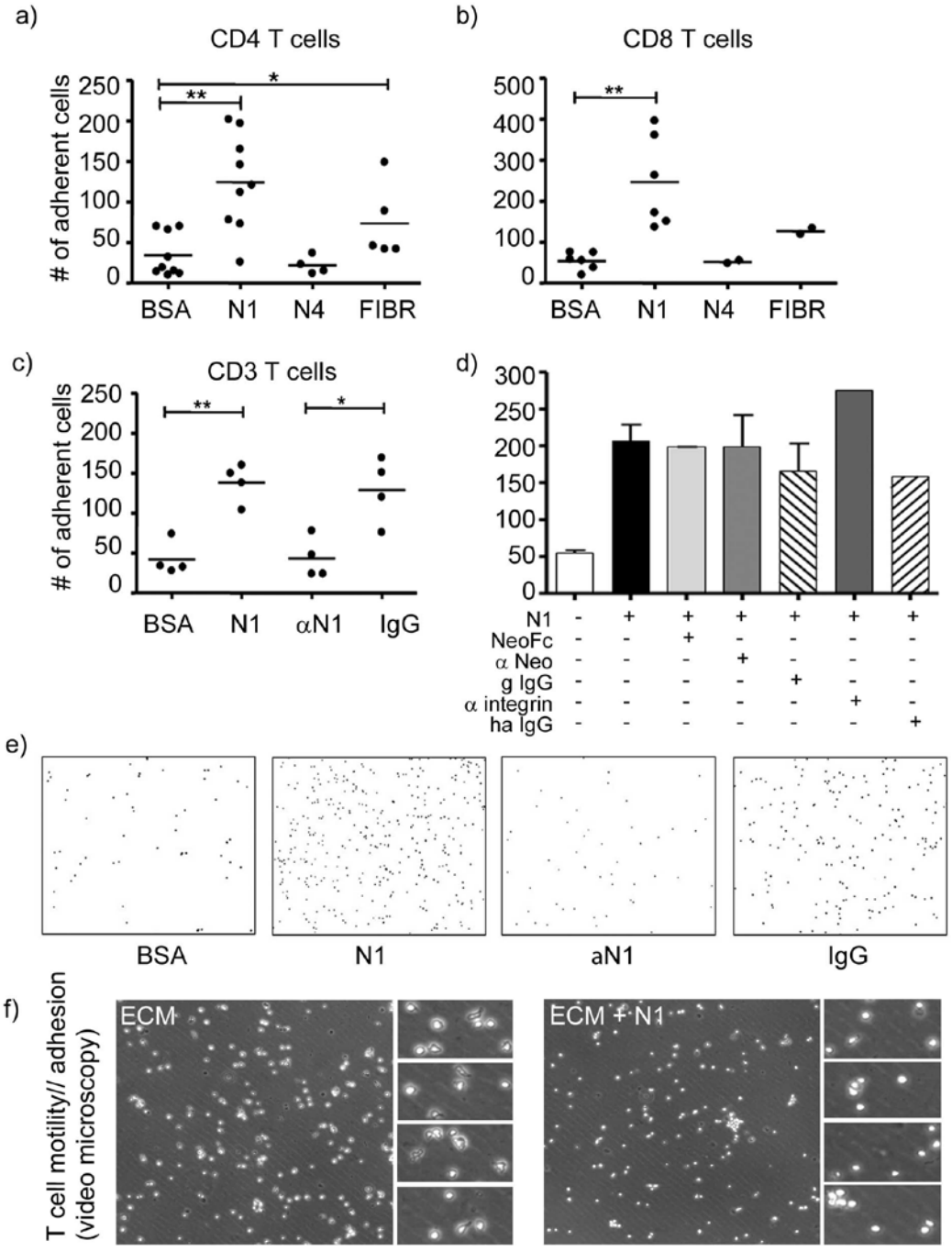

a)
CD4 T cells
# of adherent cells
250
200
150
100
50
0
**
*
BSA
N1
N4
FIBR
b)
CD8 T cells
500
400
300
200
100
0
**
BSA
N1
N4
FIBR
c)
CD3 T cells
# of adherent cells
250
200
150
100
50
0
**
*
BSA
N1
αN1
IgG
d)
300
250
200
150
100
50
0
N1 - + + + + + +
NeoFc - - + - - - -
α Neo - - - + - - -
g IgG - - - - + - -
α integrin - - - - - + -
ha IgG - - - - - - +
e)
BSA
N1
aN1
IgG
f)
T cell motility// adhesion (video microscopy)
ECM
ECM + N1

Figure 18: Netrin-1 does not affect T cell polarization. We evaluated netrin-1's potential to change T cell skewing, by adding different cytokine cocktails to monocytes and T cells, which consequently polarize to Th1, Th2, Th17, or Th0 cells (a). We first tested whether netrin-1 influences the secretion of chemokines IL-8 and MCP-1 by monocytes pooled with anti-TCR activated Th0 cells (c), but did not detect any effect. Adding monocytes to effector (CD45+CD4+) Th cells with cell type stimulating cocktails did not change number of IFNγ producing Th1, IL-4 producing Th2, or IL-17 producing Th17 cells in presence of netrin-1 (d), as plotted in graphs (b).

Figure 18

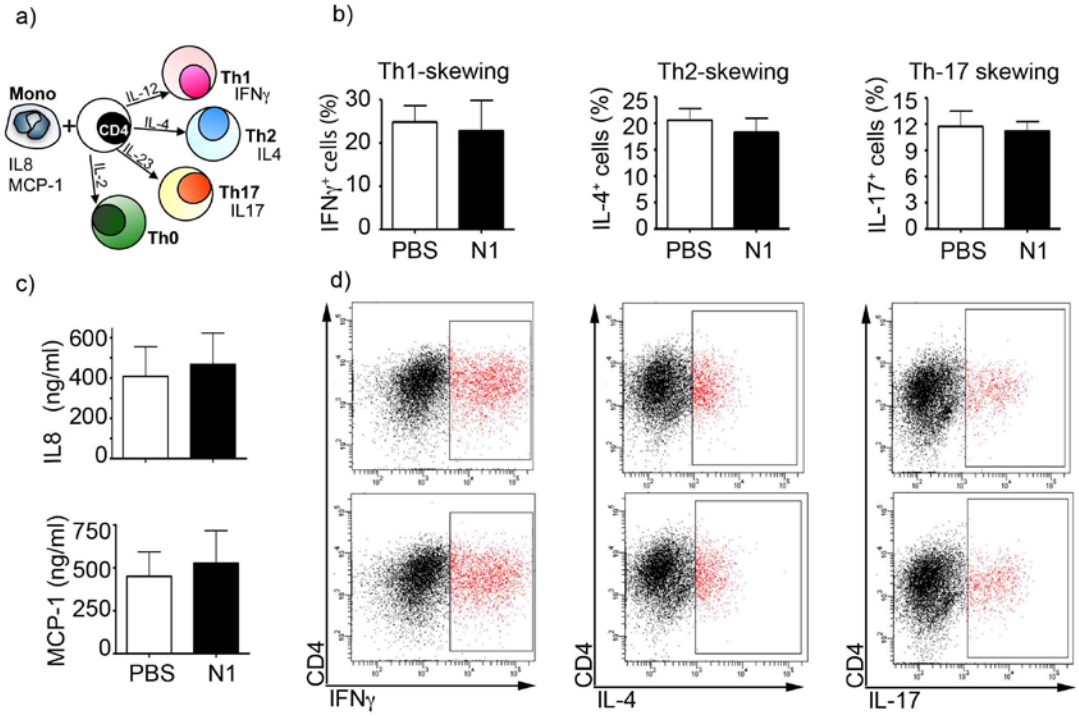
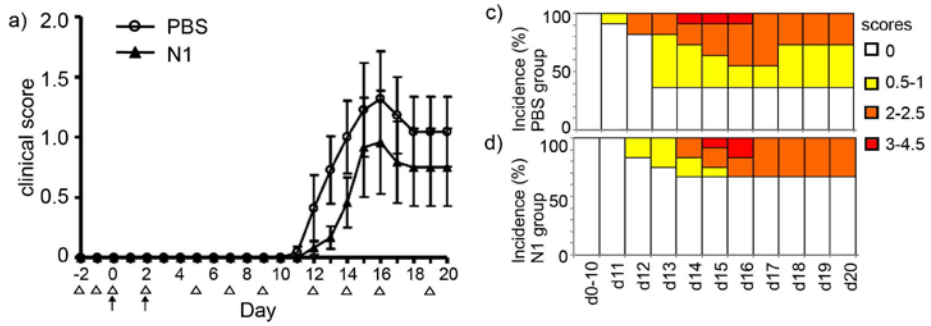

a)
Mono
IL8
MCP-1
CD4
IL-12
IL-4
IL-23
IL-2
Th1
IFNγ
Th2
IL4
Th17
IL17
Th0
b)
Th1-skewing
IFNγ+ cells (%)
30
20
10
0
PBS
N1
Th2-skewing
IL-4+ cells (%)
25
20
15
10
5
0
PBS
N1
Th-17 skewing
IL-17+ cells (%)
15
12
9
6
3
0
PBS
N1
c)
IL8 (ng/ml)
600
400
200
0
MCP-1 (ng/ml)
750
500
250
0
PBS
N1
d)
CD4
IFNγ
CD4
IL-4
CD4
IL-17

Figure 19: Netrin-1 therapy has dual functions in EAE. Netrin-1 therapy reduces disease incidence and severity and delays EAE onset, however, during the chronic disease phase, netrin-1 has no more beneficial effects but rather promotes additional symptoms of ataxia and spasticity (a-d). We assessed disease severity by scoring the degree of paralysis: from no disease symptoms (score 0) to half a floppy tail (score 0.5) progressing to complete hind limb paralysis (score 4), and being moribund (score 5). When we analyzed the immune cell populations within the CNS (e) by flow cytometry, we distinguished infiltrated leukocytes, gated for the CD45high population (f) and CD11b+ CNS resident microglia, found in the CD45low, CD11b+ gate (h). With netrin-1 treatment, we found fewer microglia (h) and fewer infiltrated myeloid cells (i) in the CNS at day 14, but on day 20 we saw higher numbers of CD4 Th (j) and CD8 Tc (k) cells in the CNS, compared to PBS treated mice. Additionally, infiltrated leukocytes appeared to be more activated (g). (Empty arrow heads = i.v. injections of netrin-1 or PBS, black arrows = EAE induction [MOG35-55 CFA, PTX, day 0] and immune stimulation [PTX day 2]). n=4 total EAE experiments, n=2 EAE experiments analyzed by immunohistofluorescence and flow cytometry analysis with each n=12 PBS-treated EAE control mice, n=12 netrin-1 treated EAE mice, n=3 (per group) for mice sacrificed on day 14 (acute disease phase), n= 6 (per group) for mice sacrificed on day 20 (chronic disease phase).

Note: In the appendix (Figure A1) netrin-1 i.p injections lead to an even greater anti-inflammatory effect.

Figure 19



b) Table 1 Clinical scores of B57BL/6 mice immunized with MOG(35-55)

		Clinical Score									
Treatment	Incid. (%)	Days 0-10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Day 17	Day 18	Day 19
PBS	64	0 ± 0	0,1 ± 0,0	0,4 ± 0,3	0,7 ± 0,3	1,0 ± 0,3	1,2 ± 0,4	1,3 ± 0,4	1,2 ± 0,3	1,1 ± 0,3	1,1 ± 0,3
Netrin-1	33	0 ± 0	0,0 ± 0,0	0,1 ± 0,1	0,2 ± 0,1	0,4 ± 0,2	0,8 ± 0,4	0,8 ± 0,4	0,6 ± 0,3	0,6 ± 0,3	0,6 ± 0,3

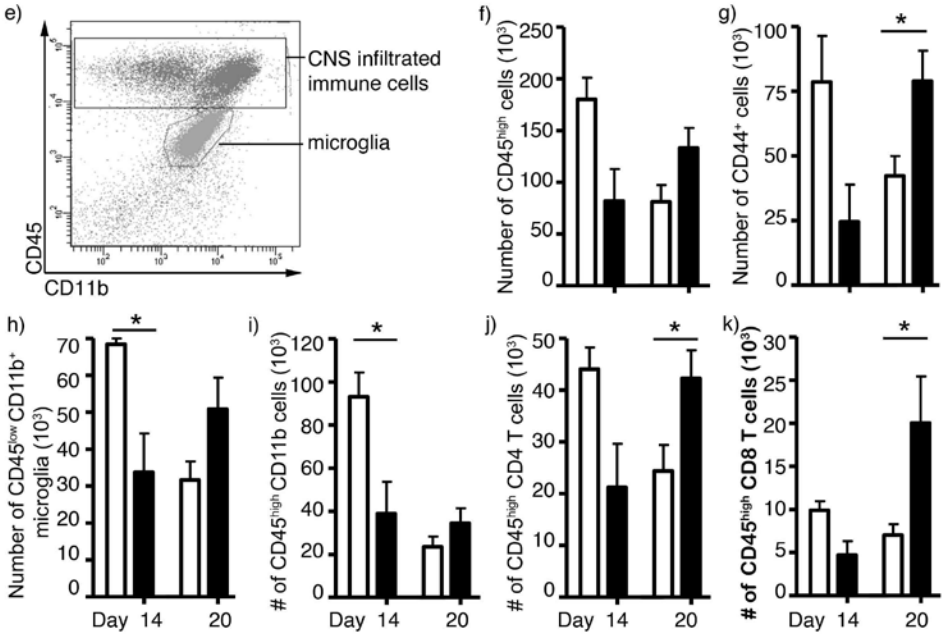


Figure 20: Netrin-1 influences immune cell presence in the CNS during EAE. To visualize CNS lesions, we performed an H&E staining (a) of spinal cord and brain sections and identified large predominantly cortical lesions in spinal cord sections from control mice but not in netrin-treated mice on day 14. On day 20, lesions started to clear of immune cells in the control mice but consisted in the netrin group. This correlated with lower numbers of infiltrated T cell (CD3) and myeloid (CD11b) cells at the onset of disease, and increased levels of immune cells in netrin treated mice at the chronic phase of the disease, compared to control mice at these time points (b). We confirmed these findings with immunofluorescence staining for macrophages (red, F4/80), Th cells (red, CD4), or Tc cells (red, CD8), together with blood vessels (green, tomato-lectin) and nuclei (blue, TOPRO) (c). At disease onset (2 panel columns to the left), in control mice, we found large infiltrates, consisting of predominantly macrophages and few T cells, while we hardly detected any immune cells in spinal cords from netrin-1 treated mice. On day 20 (2 panel columns to the right), we observed more macrophages and particularly high levels of CD4 and CD8 T cells in spinal cord sections from netrin-1 mice, compared to control mice. Scale bars: 30 μm. n=3 (per group) for mice sacrificed on day 14 (acute disease phase), n= 6 (per group) for mice sacrificed on day 20 (chronic disease phase).

Figure 20

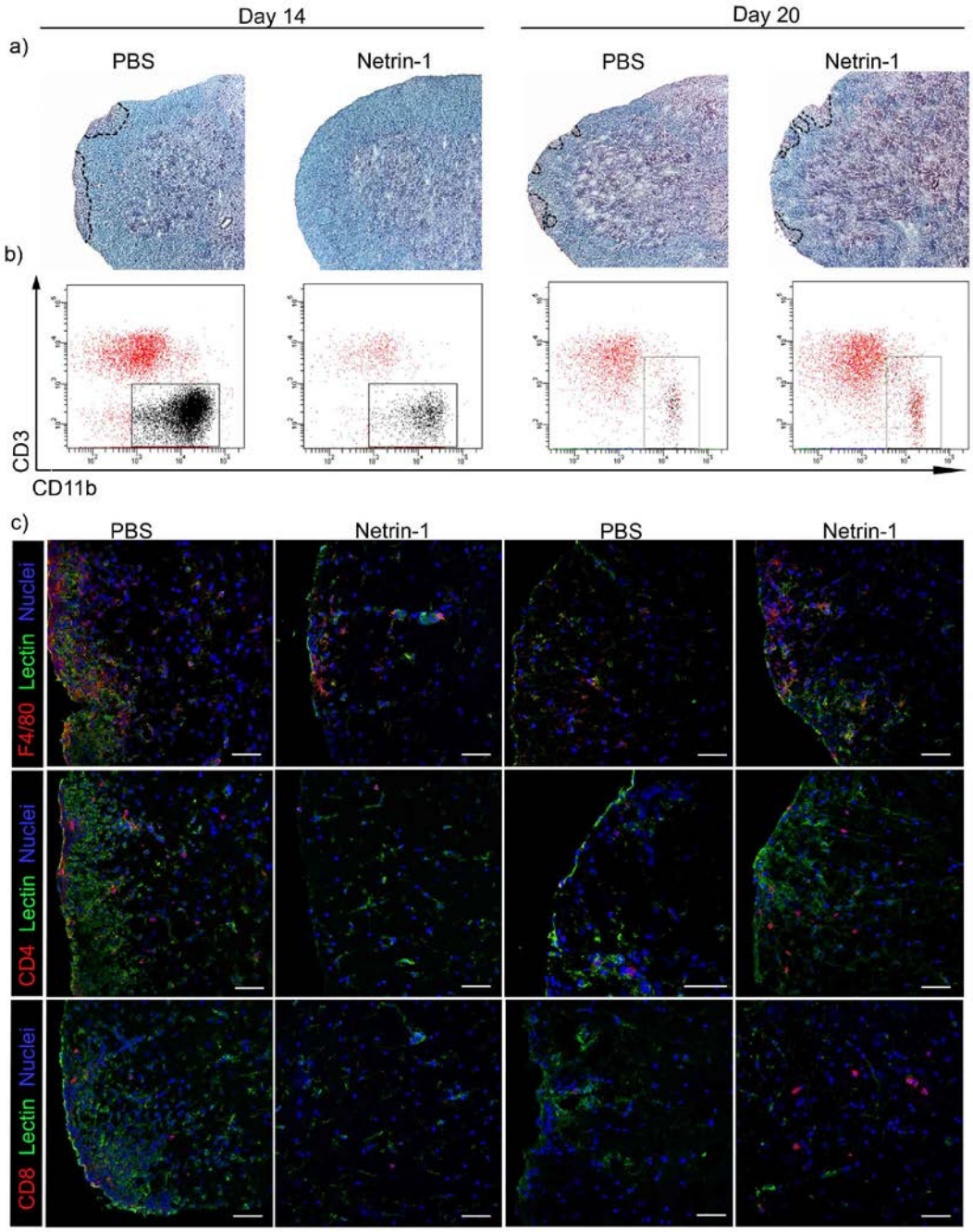

Day 14
Day 20
a)
PBS
Netrin-1
PBS
Netrin-1
b)
CD3
CD11b
c)
PBS
Netrin-1
PBS
Netrin-1
F4/80 Lectin Nuclei
CD4 Lectin Nuclei
CD8 Lectin Nuclei

Figure 21: Netrin-1 changes CD8 T cell phenotypes during EAE. To investigate netrins' effect on T cell phenotype polarization, we measured the total amount of IL-17 or IFNγ producing Th (CD4) or Tc (CD8) lymphocytes in EAE mice treated with PBS or with netrin-1. In the CNS (a-d), spleen (e-h), and lymph nodes (i-l), we did not detect any difference in IFNγ (a, e, i) or IL-17 (b, f, j) producing Th cells nor in IFNγ producing Tc cells (c, g, k) on both day 14 (d14) and day 20 (d20). However, we observed a decrease in total numbers of IL-17 producing CD8 Tc cells in the CNS (d) and spleen (h) in netrin-1 treated mice compared to the control group on day 14. Gating: IL-17 and IFNγ producing T cell subpopulations were identified within the following gates: CD45high, CD3+, selected for either CD4 or CD8 T cells. n=3 (per group) for mice sacrificed on day 14 (acute disease phase), n= 6 (per group) for mice sacrificed on day 20 (chronic disease phase).

Figure 21

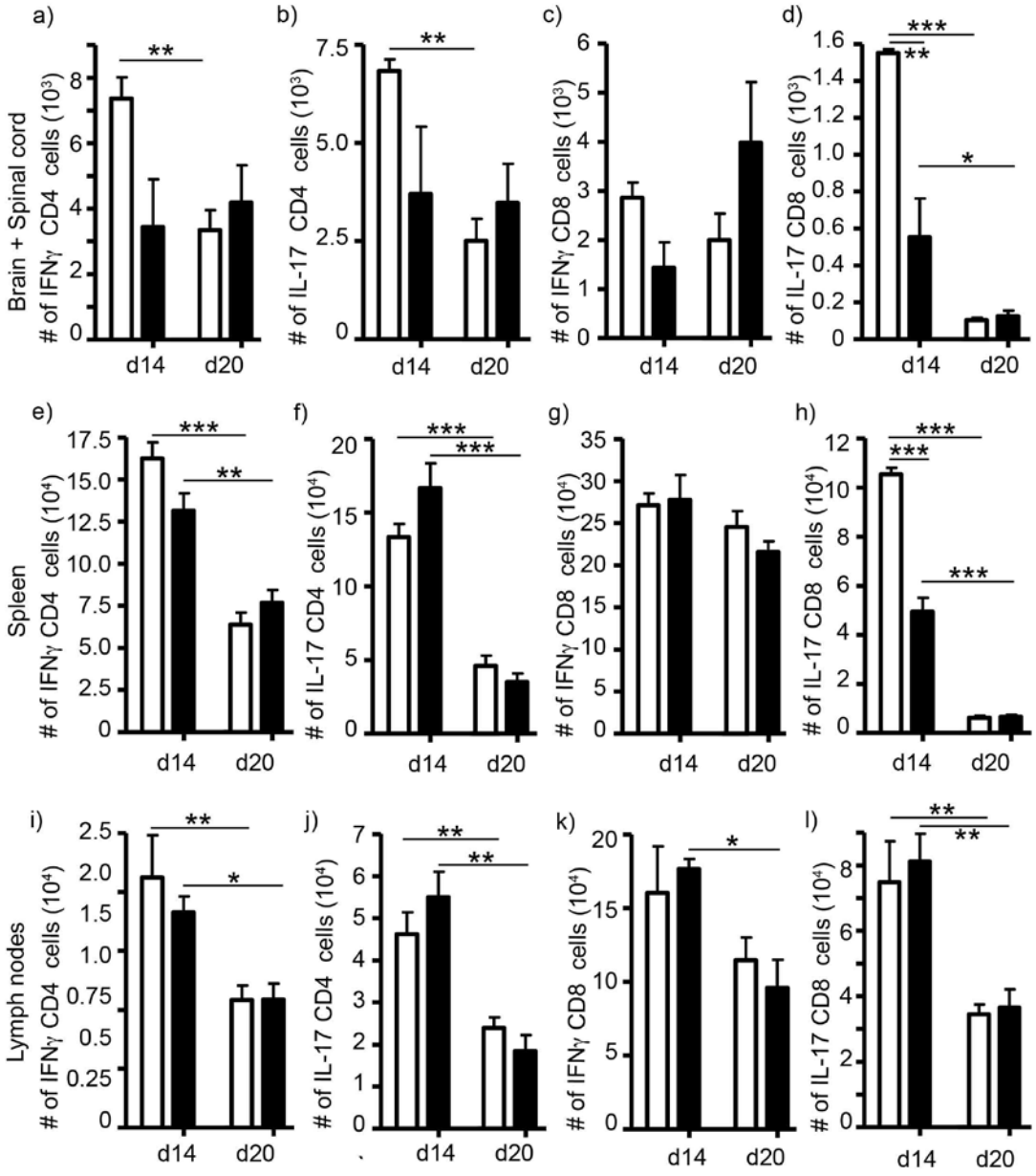
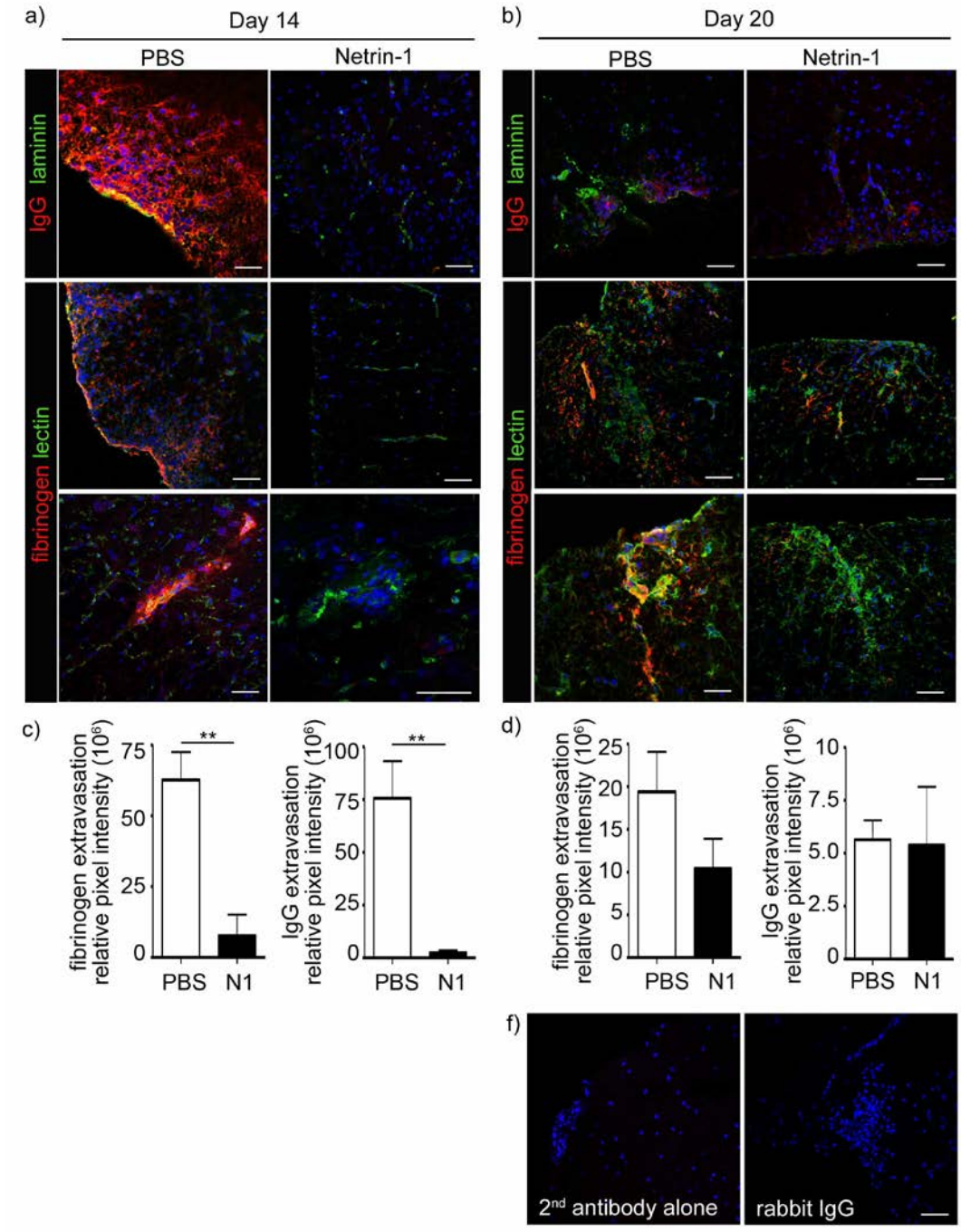

a)
b)
c)
d)
Brain + Spinal cord
# of IFNγ CD4 cells (10³)
# of IL-17 CD4 cells (10³)
# of IFNγ CD8 cells (10³)
# of IL-17 CD8 cells (10³)
e)
f)
g)
h)
Spleen
# of IFNγ CD4 cells (10⁴)
# of IL-17 CD4 cells (10⁴)
# of IFNγ CD8 cells (10⁴)
# of IL-17 CD8 cells (10⁴)
i)
j)
k)
l)
Lymph nodes
# of IFNγ CD4 cells (10⁴)
# of IL-17 CD4 cells (10⁴)
# of IFNγ CD8 cells (10⁴)
# of IL-17 CD8 cells (10⁴)
d14
d20

Figure 22: Netrin-1 reduces plasma protein leakage across the BBB during EAE. When we immunofluorescently labeled immunoglobulins (red, IgG) and fibrinogen (red) in spinal cord sections of PBS treated mice, we saw high levels of these plasma proteins on day 14 (a). We quantified the area of leakage and fluorescence intensity and plotted this as relative pixel intensity (c). Serum proteins extravasated mainly in and beyond infiltrated lesioned areas, indicating massive BBB breakdown. In contrast, we barely observed plasma proteins outside of blood vessels in netrin-1 treated animals on day 14, even in the presence of immune cell infiltrates.

On day 20, we still detected serum protein extravasation from blood vessels in both the control and netrin group (b). Although lesions in netrin-1 treated mice had more immune infiltrates, levels of extravasting IgG remained similar to control mice and levels of fibrinogen appeared to be even lower. Nevertheless, when we quantified total serum protein extravasation, regardless of the extent of accumulated immune cells, we saw no difference between the netrin-1 and control group (d). Scale bars: 20 μm. (N1 = netrin-1). n=3 (per group) for mice sacrificed on day 14 (acute disease phase), n= 6 (per group) for mice sacrificed on day 20 (chronic disease phase).

Note: In the appendix (Figure A1), brain microvessels remain more impermeable to serum protein leakage at both acute and chronic disease stages with netrin-1 i.p injections.

Figure 22


a)
Day 14
PBS
Netrin-1
IgG laminin
fibrinogen lectin
b)
Day 20
PBS
Netrin-1
IgG laminin
fibrinogen lectin
c)
fibrinogen extravasation relative pixel intensity (10^6)
75
50
25
0
**
PBS
N1
IgG extravasation relative pixel intensity (10^6)
100
75
50
25
0
**
PBS
N1
d)
fibrinogen extravasation relative pixel intensity (10^6)
25
20
15
10
5
0
PBS
N1
IgG extravasation relative pixel intensity (10^6)
10
7.5
5.0
2.5
0
PBS
N1
f)
2nd antibody alone
rabbit IgG

DISCUSSION

Netrins enhance the blood-brain barrier by up regulating tight and adherens junction molecule expression and decreasing barrier permeability in the developing and adult BBB. At the injured BBB, netrin-1 increased TJ protein expression recovering them back to baseline expression levels. Additionally, netrins had anti-inflammatory effects on the BBB *in vitro*, by decreasing endothelial cytokine and chemokine secretion. During the neuroinflammatory disease EAE, netrin-1 therapy reduced plasma protein diffusion through the BBB, which is indicative of a more stable BBB. However, we observed a dual effect of netrin-1 on myeloid and lymphoid immune cell recruitment. At disease onset, it reduced myeloid cell recruitment into the CNS, delaying and diminishing EAE disease severity. However, at later disease stages, we observed that in addition to paralysis, netrin-1 treated mice had more atypical disease symptoms, such as ataxia and spasticity. This correlated with a large number of infiltrated and more activated CD4 and CD8 T lymphocytes within the CNS. Thus, netrin-1 protects and maintains the BBB and inhibits myeloid cell recruitment to the CNS during early stages of EAE but changes T cell behavior over a longer period of time.

1. Netrins increase blood-brain barrier stability

From the literature, we know that neural guidance cues such as slits, semaphorins, and ephrins play similar roles in regulating the vascular network [152,281-289]. Among these factors, researchers also identified netrin as a factor to regulate vascular growth and arborization. Here, we propose a model wherein netrin-1 and netrin-4 maintain, protect, and repair the BBB. They enhance the BBB's barrier properties during development, adulthood and during neuro-inflammatory disease. Netrin-4 maintains the healthy barrier with moderate effects on TJ and AJ molecule expression. However, when the barrier becomes inflamed, netrin-4 becomes down-regulated and its more effective paralogue netrin-1 up-regulated. Netrin-1 then functions to repair the barrier by promoting and recovering TJ molecule expression and recruitment into functional membrane microdomains. When

applied to animals prior to EAE induction, netrin-1 reduces the extent of plasma protein extravasation across the BBB during early and late stages of the disease. Even in the presence of perivascular immune cell infiltrates, the barrier remained more impermeable compared to control mice. In consequence, netrin-1 helps to maintain the integrity of the barrier, which decreases extravasation of immune cells and other blood derivatives.

We believe that netrin's barrier stabilizing effect may be valuable to other experimental neurobiologists for two reasons. Firstly, netrin's potential to protect and stabilize the BBB may apply to other neurological pathologies as many of them exhibit a breach in BBB function. This becomes even more important as we know only a few factors able to regulate and strengthen this crucial barrier [77,277]. Angiopoietin-1 (via Tie-2), angiotensin II (via type 1 angiotensin receptor), and the neural guidance cue Slit-2 (via Robo-4) [284,285] for example increase endothelial barrier stability. In addition, our group recently discovered the developmental morphogen hedgehog to promote BBB integrity [84], which interestingly, also induces netrin-1 expression [221,222,271].

Shaking perceptions: Netrins role in angiogenesis versus endothelial permeability

We also support the argument that netrins reduce barrier permeability despite their reported angiogenic properties, which are often associated with a more leaky endothelial barrier. However, angiogenesis does not always result in hyperpermeability. Ang-1 for example stabilizes the endothelial barrier during VEGF-mediated vascular growth [76,290,291]. In the CNS, Netrin-4 also promotes angiogenesis without affecting vascular permeability [219]. In our model, without underlying oxygen or nutrient deprivation that would otherwise create the need for angiogenesis, netrin had no effect on BBB-endothelial angiogenesis *in vitro*, and *in vivo* during development and immune disease. On the contrary, during brain development we observed thickening of the meningeal vasculature in netrin-1 knockout mice. Two different mechanisms may explain this. If netrin-1 normally inhibits sprouting angiogenesis in meningeal vessels, then a lack of

netrin-1 would no longer provide this growth inhibiting signal and the meningeal layer could grow more thick and complex. On the other hand, if netrin functioned as an adhesive cue that supports adhesion among cells or between cells and extracellular matrix components, then this could for example help to integrate endothelial cells to the glia limiting basement membrane. Thus, absence of netrin-1 would result in less compacted cell structures, similar to the more loose and detached structures that we see at the paranode in netrin-1 knockout mice [171]. We also need to consider that when we compare different disease models or different tissues such as the vasculature from the periphery and from the CNS, netrin-response mechanisms may differ, because netrin responses depend on the ratio and availability of netrin receptors and the physiological or therapeutic concentration of netrin. Netrin-1 may act differently on parenchymal vessels, which are tightly embedded within the neurovascular unit versus the meningeal vessels that are embedded in a more fibrous network. These different microenvironments could potentially decide whether netrin-1 is efficiently captured, and presented, or made available to the surrounding cells. Thus the microenvironment would affect how cells can respond to netrin. If netrin-1 were to integrate and bind more efficiently to the meningeal network for example, it may enhance cell growth, migration and/or adhesion, which in turn would contribute to the meningeal architecture.

Responding to netrin: Endogenous netrin sources and netrin receptors

In the adult, endothelial cells express both netrin-1 and netrin-4. Netrin-4 is only present under normal conditions and netrin-1 only becomes up-regulated in inflamed endothelial cells. Similarly, BBB-endothelial cells from newborn (P0) wild type and knockout mice, did not express netrin-1, nevertheless, numerous types of neurons and oligodendrocytes express it [203]. In fact, many researchers reported netrin-1 expression throughout development and adulthood, however, netrin expression patterns change during development and pathology [171,292,293]. For example, oligodendrocyte precursor cells do not express netrin-1 and are repelled by netrin-1 via unc5 homologues to migrate away from the midline.

When they mature into oligodendrocytes, they become a source of netrin-1 themselves. Netrin-1 then promotes oligodendrocyte process extension and myelin sheet formation that efficiently contact axons via DCC. On the other hand, we know that several epithelial and endothelial cell types express netrin-4 during adulthood, which then accumulates in basement membranes, but we know little about its expression patterns during development. We noticed that only larger meningeal type vessels expressed netrin-4 in newborn mouse brain.

To compare netrins' barrier-enhancing functions in the developing and adult CNS, we also need to consider that netrin receptor expression shifts as development progresses toward adulthood. DCC and neogenin are gradually being down regulated and unc5 homologues up regulated [216]. This in turn changes cellular responses to netrin-1.

Adult brain-derived endothelial cells express the adhesive netrin receptor neogenin and low levels of unc5B and unc5C. However, we did not identify through which receptor(s) netrin mediates its barrier tightening effect. We showed that competitive neogenin and unc5B receptor constructs successfully sequester netrin-1 and reverse its effect on endothelial barrier tightening. However, we do not know through which receptor netrin signals and what type of downstream mechanisms it engages to regulate the BBB. In fact, we also know little about the receptors involved in regulating vascular growth [168,211,254]. Some researchers report that DCC mediates vascular branching [210,237,219], while others suggest that unc5B directly [195] or indirectly stimulates angiogenesis by promoting endothelial cell survival [165,215 194,195,211].

Taking a closer look at netrins barrier strengthening abilities in the CNS

We discovered that netrins increase adhesion between endothelial cells indirectly. At the adult BBB, netrins strengthen the endothelial barrier by increasing JAM-1, occludin, claudin-5, ZO-1, p120, and α-catenin expression. In the developing CNS, netrin-1 also helps to establish a tighter BBB. Mice lacking netrin-1 show increased BBB permeability and have altered levels of junctional proteins, such

that endothelial cells down-regulate occludin, p120, and JAM-1, but increase expression of ZO-1, probably to compensate for the reduced barrier function.

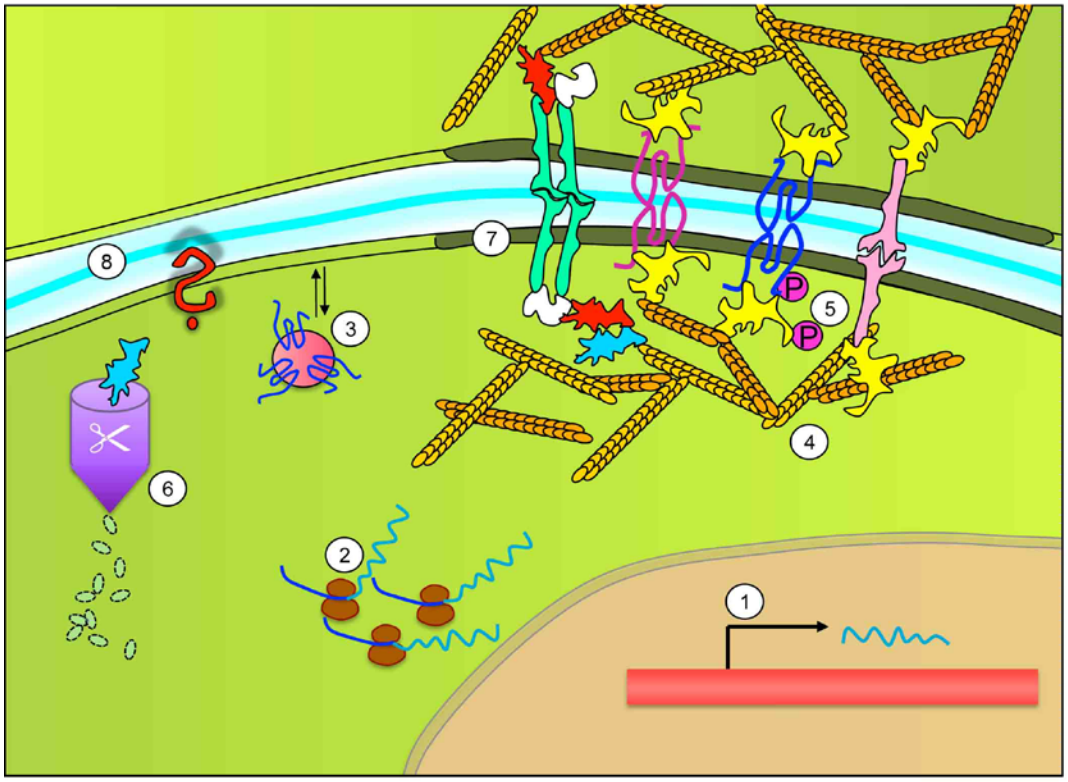
Several mechanisms exist to regulate inter-endothelial cell adhesion and vascular permeability. They include i) expression or degradation of tight and adherens junction molecules, ii) the supportive interplay among adhesion molecules within the junctional complex through their recruitment and enrichment in lipid rafts [77,102], and iii) their modulation, i.e. by phosphorylation that impacts functionality and assembly within the tight inter-cellular connections [114-116], and iv) endothelial cell contraction through cytoskeletal reorganization [133].

Netrins increase the expression of adhesive proteins at inter-endothelial junctions. And netrin-1 helps to enrich them in raft-like membrane domains that are important for these adhesive proteins to interact with each other to most efficiently link endothelial cells tightly together. Whether the augmented TJ protein levels are a result of protein stabilization or modification that reduce their degradation, or whether these high TJ levels are caused by an altered protein translation, or gene transcription, remains questionable. Previous studies showed that netrin-1 induces localized translation of β-actin [294] and that its receptor DCC also regulates ribosomal protein translation [295]. Using qPCR analysis for quantifying TJ and AJ transcripts in short-term (2-6 hours) netrin treated compared to unstimulated endothelial cells would answer whether netrin regulates gene transcription.

Although, our findings demonstrate that netrin supports the BBB indirectly by increasing levels of TJ proteins, the fact that netrin also enriches them in raft-like membrane microdomains supports the argument that besides regulating transcription or translation, netrin may modulate the actin cytoskeleton. We know from numerous studies on axonal guidance and growth cone motility that netrin-receptor mediated signaling targets the actin cytoskeleton [153,154]. Thus, it is possible that netrin increases levels of TJ proteins by modulating the actin cytoskeleton to support adhesive complexes [170-172,296] and facilitate the recruitment or interaction of adhesive factors among each other [154,297]. This in turn would stabilize proteins within the adhesive complex and protect them from degradation.

One further possibility through which netrin may additionally support inter-endothelial adhesion considers netrin itself as an adhesive factor. Previous studies reported that netrins possess a "sticky" character and remain attached to cell surfaces, rather than being freely soluble [203]. We also found that netrins remained associated with the endothelial cell membrane, which may promote inter-cellular or cell-matrix adhesiveness in several ways. Netrins may act as non-specific adhesive factors, potentially by associating with other extracellular matrix proteins [183] and creating an adhesive and supportive matrix for cells. This may indirectly help and adjust barrier-supporting proteins to be sufficiently proximal to one another to interact. Secondly, netrin may directly promote adhesive signaling either through classical netrin receptors [170,296], or alternatively through receptor complexes [164].

Figure vii



Netrin-1 increases inter-endothelial adhesion and decreases BBB permeability. We showed that netrin-1 increases TJ and AJ protein expression and enrichment in lipid raft microdomains. However, the underlying mechanism this remains unclear. Mechanisms that would increase junctional protein expression, could involve increased transcription (1), translation (2), and transport to the plasma membrane (3).Or netrin-1 could promote actin cytoskeleton reorganization (4), which supports protein interactions for functional modulation (5), stabilizes proteins and protect them from protein degradation (6) and recruitment into lipid rafts for more efficient protein interaction (7). Moreover, netrin-1 could act as a direct adhesive factor (8), similar to an extracellular matrix protein or mediated through yet to be identified receptor(s).

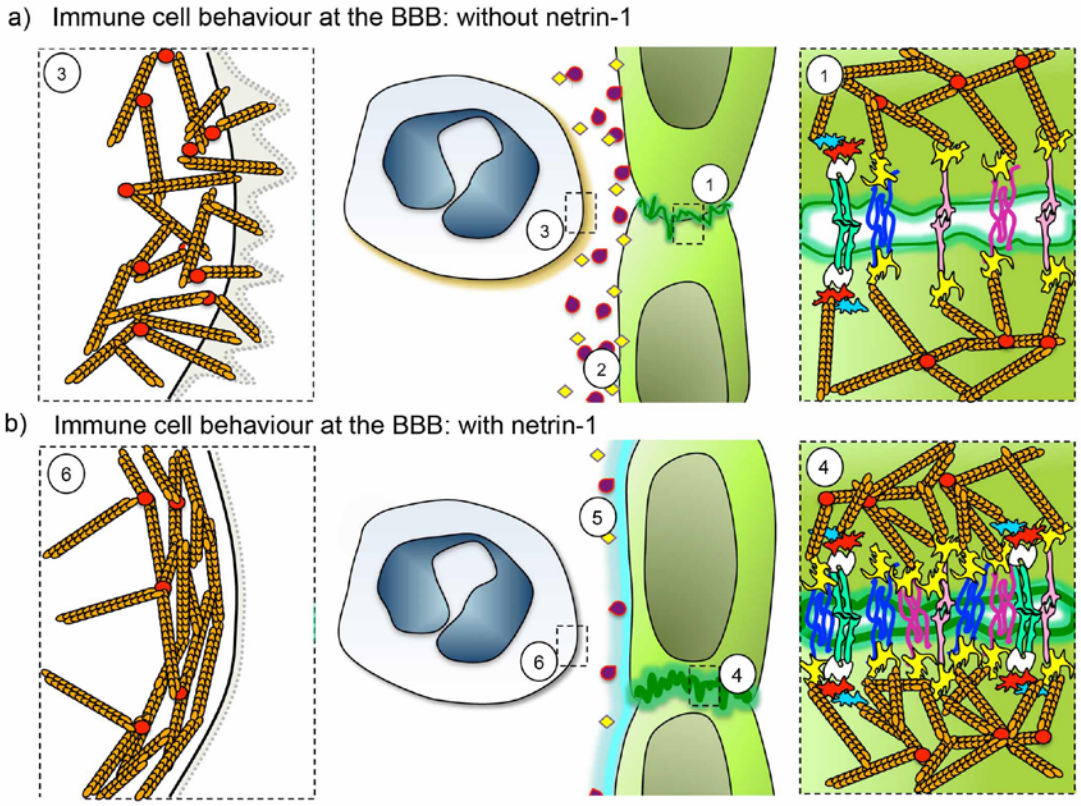
2. Netrin-1 has dual functions in the neuro-autoimmune disease EAE

In recent years, we recognized that it is not uncommon that neural-derived molecules function in the immune system and vice versa [298]. Besides their typical adhesive, chemotactic, or survival functions, neural molecules have potent immune regulatory functions. They suppress [299-302] or activate immune responses [303-307]. Netrins themselves exhibit sequence similarity to tissue inhibitors of metalloproteinases (TIMPS) [308] and the C3, C4, C5 molecules of the complement system [309,310] and have ascribed anti-inflammatory functions.

In accordance with previously published data on netrin-1's anti-inflammatory effect on myeloid cells, we observed that netrin reduced myeloid cell accumulation in the CNS during the onset phase of EAE. Peripheral leukocytes, especially of the myeloid type, infiltrated to a lesser extent into the CNS during this time. This can be the result of netrin's multiple effects (Figure viii). In our study we showed that netrin-1 increases BBB stability, which physically blocks immune cells from crossing the BBB. Netrin-1 also lowered levels of chemokines and cytokines by BBB endothelial cells, which leukocytes need to be recruited to the site of inflammation. From previous reports we know that netrin-1 directly inhibits myeloid cell migration. Regardless of the underlying mechanism, netrin-1 treatment causes fewer mice to get ill and delayed disease symptoms and severity.

At disease onset, we also noted fewer microglia in the CNS of netrin-1 treated mice. Microglia are the CNS-resident immune equivalent to peripheral myeloid cells. We think that netrin may have indirectly decreased microglia expansion in the CNS, because netrin-treatment reduced peripheral immune cell infiltration into the brain that would have otherwise promoted microglia activation. Alternatively, netrin may directly affect microglia, however, we do not know whether therapeutically applied netrin-1 crosses the BBB and reaches the brain parenchyma to act on CNS-residing cells during disease onset.

Figure viii



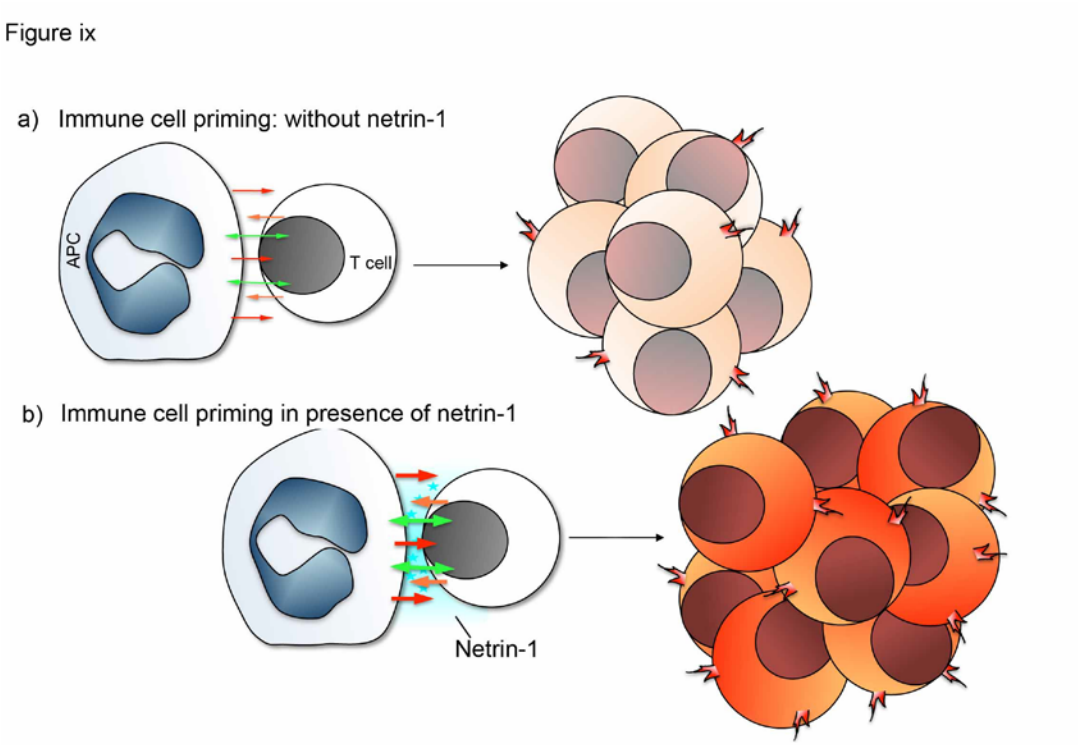
We propose several mechanisms through which netrin-1 reduces myeloid cell accumulation in the CNS. In absence of netrin-1, the BBB secretes chemokines and cytokines (2), which promote myeloid cell transendothelial migration across the BBB (a). During this process, myeloid cells have to actively protrude (3) and overcome the interendothelial connections (1). We found that intravenous netrin-1 application diminished myeloid accumulation in the CNS and suggest the following mechanisms (b). Firstly, netrin-1 enhances inter-endothelial adhesion by upregulating TJ and AJ protein expression (4). It also reduces endothelial cytokine and chemokine expression (5), and it reduces myeloid cell motility (6), so that they can no longer protrude (6) and advance across the endothelium.

Netrin-1: An adhesive communication factor for immune cells? - Staying in touch to learn more

Netrin's beneficial effect is lost during the chronic phase of disease, when the peak of the disease has passed, symptoms ameliorate, and inflammation slowly subsides but lesions persist with low-grade inflammation or the formation of scar tissue [53,311,312]. During this time, mice treated with netrin exhibit high levels of T lymphocytes in the CNS, which approximately doubled compared to control EAE mice, and compared to mice in the netrin group at day 14. On the

other hand, the number of CNS-infiltrated myeloid cells remained at levels similar to control mice and to netrin treated mice on day 14. Why do T lymphocytes respond differently than myeloid cells? Does netrin increase T cell migration across the BBB but inhibit motility of myeloid cells? In our *in vitro* migration experiments, netrins had no effect on T cell transendothelial migration. By using an *in vivo* model, we have to consider that cellular interactions are vast and complex. Rather then influencing T lymphocyte motility, we think that netrin-1 changes the way T cells communicate with antigen presenting cells during the priming and maturation period in the periphery. Although this data is preliminary and raises a series of questions, we propose that T cells, which strongly adhere to netrin, engage in longer or stronger communicative contact with antigen presenting cells (APCs) in the presence of netrin-1. In fact, we showed that only when matured and activated with a "danger-signal", APCs increase netrin-1 expression to its highest level, compared to immature APCs or undifferentiated cells. An exogenous source of netrin-1 may intensify or prolong this communication. This, in turn, could affect T cell activation and behavior. We also know that T cells readily cross the BBB when activated [112,117,119]. In our disease model, CNS-infiltrated peripheral immune cells, mainly T cells, were indeed more activated at later disease stages. While we did not detect any changes in the number pro-inflammatory cytokine producing Th cells, we detected more IFNγ-producing cytotoxic T cells in the CNS of netrin-1 treated mice. Additionally, we noted that during disease onset the number of IL-17 producing Tc cells decreased in both the CNS and in the spleen in netrin-1 treated mice. This demonstrates that netrin-1 certainly influences T cells behavior and activation.

Figure ix



During the process of T cell priming, an antigen presenting cell (APC) communicates environmental information to the T cell (colored arrows). Depending on the information the T cell receives during this interaction, the T cell responds accordingly, by clonally expanding, changing its activation status, and differentiating into a specific phenotype (a).Because matured and activated APCs up-regulate netrin-1, and T cells adhere to netrin-1, we hypothesize that netrin-1 strengthens or prolongs the adhesive contact between the APC and T cell which leads to more efficient cellular interaction (bold arrows), which changes T cell activation, polarization and behavior (b).

Is netrin-1 beneficial in both acute and chronic immune disease?

While netrin-1 clearly possesses anti-inflammatory functions in early acute immune responses, researchers are still unclear about its function during chronic conditions [138,231]. Here, we demonstrated that netrin-1 possesses dual functions during immune responses in the neuro-autoimmune disease EAE. During acute phases, it decreases disease severity by reducing myeloid recruitment into the CNS. However, in later chronic stages, netrin-1 interferes with remission and appears to alter T lymphocyte activity.

When we compare netrin's effect on acute and chronic disease, we need to consider that the predominant cell types during these phases may differ, and that during acute disease immune cells are actively recruited and during chronic disease phases immune responses persist or recur at the inflammatory site. Many

researchers agree that netrin-1 inhibits immune cell migration in acute disease. In most studies, they assessed infiltration of myeloid cells such as granulocytes and monocytes. These innate immune players are the first cells to be recruited in an acute inflammatory response and mount nonspecific immune attacks. Immune cells of lymphoid origin, T and B cells, however, are highly specialized immune cells that need to be primed first and usually respond after the first line of innate immune defense. We show first evidence, that netrin-1 increases T cell activation, which we believe is the result of a more adhesive contact between T cells and antigen presenting cells during T cell priming and maturation. In our model, the more important ("dangerous") information an antigen-presenting cell (APC) has to convey to a T cell, the more it must ensure that it remains in contact with it. We demonstrated that the more danger-signals an APC encounters the more netrin-1 it produces. Thus, netrin-1 may promote T cell attachment to the APC and thereby intensifying the communicating contact.

Two independent groups have studied netrin-1's effect on chronic disease, using atherosclerosis as a disease model. Atherosclerosis is a chronic disease characterized by inflammatory plaque formation in arterial walls. Recruitment and infiltration of monocytes and macrophages into activated vessel walls is a key component of the disease, promoting fat-laden macrophages to transform into gigantic foam cells that further contribute to and advance endothelial injury [144]. This can lead to artery thickening, hardening, or rupture, resulting in a decreased vessel-lumen, which in turn causes high blood pressure, a major health risk [313]. While Khan et al found netrin-1 gene-therapy to reduce vascular inflammation and plaque formation, because netrin-1 inhibited monocytes from migrating into the plaque [268], van Gils and colleagues discovered that activated macrophages secreted endogenous levels of netrin-1, which blocked macrophages from exiting the plaque, and thus further enhanced atherosclerotic plaque formation [138]. Interestingly, both authors observed that netrin-1 inhibited macrophage migration, however, when and where leukocytes were exposed to netrin changed the disease outcome. When netrin-1 was therapeutically overexpressed in aorta, before disease induction, it prevented monocytes from migrating into the plaque, and

consequently reduced macrophage accumulation within the aortic wall [268]. Van Gils demonstrated that macrophages that are already present inside the plaque become activated when they engulf oxidized lipids. This activation causes macrophages to secrete netrin-1, which inhibits their motility in an autocrine manner, so that they can no longer respond to chemokines and exit the plaque.

Although it is innate immune cell types that predominantly mediate artherosclerosis, several parallels exist to the chronic immune disease Multiple Sclerosis (MS) and its animal model EAE. During EAE and MS, microglia and macrophages within the CNS also engulf oxidized lipids in inflamed, lesioned areas [139], which could also induce netrin-1 expression [138]. Whether this stops microglia from leaving lesioned areas is still unknown. Interestingly, microglia and macrophages phagocytose the myelin debris from eroded oligodendrocytes, which may contain netrin-1 [171,203,293]. We noticed that during the chronic disease phases, the CNS of netrin-1 treated mice tended to contain more microglia, perhaps reflecting netrin's potential to promote microglia proliferation or survival.

How does netrin-1 change immune responses?

From the neural guidance literature, we know that in order to regulate cell adhesion and motility, netrins modulate and reorganize the actin-cytoskeleton, which requires precise process outgrowth, turning, and collapse [154,193,194,196,237,281,314,315]. When a cell migrates or extends towards a cue, its leading edge protrudes with large membrane ruffles or lamellipodia and finger-like extensions, called filopodia. While reaching out to the attracting cue, the cell establishes focal adhesions to anchor these protrusions along the surface on which the cell moves. The rear and of the cell, in contrast, has to detach and contract to allow for directional movement. These very dynamic cell shape changes result from rapid cytoskeletal reorganization that effectively regulate cell motility. We can speculate that netrin may inhibit immune cell motility by altering signaling to the actin-cytoskeleton. Unc5B is known to mediate netrin's repellent and retractive responses. Most immune cell types express unc5B [138,233,258] but not DCC [233] or neogenin [138]. In our study, both myeloid and lymphoid immune cells

express unc5B and neogenin. In response to netrin-1, they take on a rounded morphology and are unable to respond to chemokines [138,233], indicating that netrin-1 promotes cytoskeletal retraction in myeloid cells. In support of unc5B mediating this effect, some researchers reported that unc5B blockade reversed netrin-1's inhibitory effect on immune cell motility [138,233]. However, others found no evidence for this [186]. Instead, they claimed $A_{2B}AR$ to mediate netrin's inhibitory effects and studied a more complex transwell migration model, using transepithelial migration [186].

The quest of finding the receptors that mediate netrin's effect on immune cells becomes ever more complex, when we consider that immune cells can change their netrin receptor expression depending on the specific cell type or their state of activation or maturity. Our analysis showed that monocytes increased expression of neogenin and unc5B upon differentiation to mature antigen presenting dendritic cells. Additionally, CD8 Tc cells expressed higher levels of unc5B compared to CD4 Th cells. Others researchers demonstrated that activated and matured B cells up-regulate DCC expression, while T cells did not express DCC [316]. Furthermore, cancerous B cells such as B-lymphoid leukemia cells, Burkitt plasmacytoid lymphomas, and lymphoblastoid cell lines expressed these high DCC levels but T cell specific lymphomas and malignant myeloid cells did not [316].

3. A long way for netrin to become a therapeutic: Pros' and Cons' for netrin therapies

In recent years, several exciting high-profile studies have emerged and demonstrated netrins' versatile functions during various types of pathological conditions. They demonstrated that:

- Netrin-1 has anti-inflammatory properties in acute immune responses
- Both netrin-1 and netrin-4 promote angiogenesis during conditions of ischemia
- Netrin-1 stabilizes and protects the BBB during neuroinflammatory disease

However, netrins' can also have undesired effects:

- Netrin-1 and netrin-4 may increase the risk of developing cancer and metastasis by promoting cell survival, angiogenesis, and cell migration
- Netrin-1, as a myelin-associated inhibitor, may impair neural regeneration by promoting adult progenitor cell repulsion during neurodegenerative disease
- Inflammatory sites express increased levels of endogenous netrin-1, which reduces immune cell motility and increase their cell survival, which can exacerbate inflammation.
- Long-term application of netrin-1 may encourage pro-inflammatory responses, as this may influence T cell/APC communication, and immune cell priming, activation, or survival

Because of their ability to enhance vascular growth, and therefore increase nutrient and energy supply to growing tissues, netrins have the potential to promote cancer development. In our model, netrin did not promote brain vessel angiogenesis, however, netrins have been linked to various types of cancers of the lungs, breast, ovarian, kidney, colon, and immune cells [1]. Several researchers showed that netrins can affect tumor growth and dissipation by regulating not only angiogenesis [165,193,196,207,255,317] but also cell survival [225,318], and migration [207,296].

Netrin-4 overexpressing breast cancer cells for example enhanced lymphatic vessel growth, leading to increased tumor growth [206,207]. Silencing netrin-1 in pancreatic tumor cells reduced angiogenesis and lessened tumor cell invasion [206]. However, netrins can also inhibit vessel outgrowth, thus delaying and reducing tumor growth [165,193,196,255]. These paradoxical findings show how little we know about netrins' angiogenic functions or the underlying regulatory mechanisms, although we know that netrin responses differ with varying

[1] Netrins onocogenic effect was demonstrated through cancer incidence/ netrin epression correlation studies or in experimental models where netrins promoted tumor development and metastasis,

concentrations of netrin. Also, different cell types may be equipped with different sets and levels of netrin receptors, which can alter netrin function.

Similar to our results, showing that netrin-1 expression increases in endothelial cells and dendritic cells upon inflammatory activation, researchers found that chronically persisting gut inflammation up regulated netrin-1 expression in intestinal epithelial cells via NfkB. In this model of irritable bowl syndrome netrin-1 also promoted cancer development by helping intestinal epithelial cells to survive, as it inhibited apoptotic cell death [225]. Actually, some researchers suggest that netrins are dependence factors. In this role, when netrin-receptors, or dependence receptors, remain unbound of netrin, they trigger apoptosis through caspase activation and cleavage, which then leads to cell death. In the presence of netrin, cell survival is promoted by the inhibition of death signaling [319,320]. This harbors the potential for netrin to be an oncogene [319-321] or its receptors to be tumor-suppressors [322]. Although netrin-receptors encompass caspase binding and cleavage sites, several researchers do not agree with this theory and did not detect increased cell death in absence of netrin-1 [292,323,324]. However, another theory states that when cells lose their integrity, their adhesive contact to either a matrix or neighboring cells, they die through anoikis [325]. While netrin is proposed to promote cell survival in both models, the underlying mechanism is fundamentally different. This becomes important during tumor development and metastasis, where cells change their properties to survive, adhere and migrate [326]. If netrin simply suppressed apoptosis, tumors could develop in the absence of dependence receptors or abundance of netrin as both promote cell survival. On the other hand, if netrin functioned as an adhesive cue that helps to integrate cells, it would inhibit cell detachment and tumor dissemination [296,325]. This would distinguish netrin either as an oncogenic survival factor or a tumor-suppressing adhesive factor. We are left to say that netrins' implications in tumor genesis are complex and remain controversial and require further more detailed studies.

In regards to netrins inhibitory effect on leukocytes, many studies used netrin-1 as a preventative treatment to ameliorate acute inflammatory tissue

injury. However, the timing, route of netrin application, and type of disease might dramatically change netrins' therapeutic effect. So far, scientists repeatedly demonstrated netrins' anti-inflammatory effect during short-term treatment in early acute disease. In most cases netrin-1 was applied before disease induction through application routes that perhaps enhance netrin-1 coating on endothelial or mucosal surfaces (Table 3). We propose the following model for this successful application. When netrin-1 is applied early during disease, at sites where it can deposit to build a repellent or perhaps paralyzing wall, immune cells that are sensitive to netrin-1 can no longer cross this endothelial, epithelial or mucosal wall. This prevents immune cells from migrating into injured tissue, which consequently diminishes the immune attack. In addition, during early acute responses, cells of the innate immune system of myeloid origin are most prominent. These cells appear to be most susceptible to netrins' anti-inflammatory actions.

This scenario may differ completely, if netrin were applied at later time points, when myeloid cells already entered the injured tissue, or if netrin was added directly into the site of injury, where paralyzing cell motility may prolong their presence at the injury site [138]. What happens when we prescribe long-term netrin treatment? Adaptive immune cells, T and B lymphocytes, may be affected by this, because these cells are highly specialized and require longer time periods to be primed, to mature and differentiate. This occurs in lymphatic tissues and involves cell communication with antigen presenting cells, that we know produce netrin-1 as well. However, we do not know how or whether netrin partakes in any of these lymphocyte-initiating processes.

Certainly, we need to address many more fundamental and specific research questions to apply netrin safely and most effectively. We need to ask which receptors and signaling mechanisms do netrins use? With which other proteins do they interact? What are the kinetics or netrin's specific functions during complex, chronic, or lasting diseases? What other factors regulate netrin expression? Which cells does netrin target? What are the mechanisms through which cells respond?

CONCLUSION

Our findings demonstrate that netrins increase adhesion between endothelial cells at the blood-brain barrier during development, during adulthood and in inflammatory disease. We also found netrin-1 to be anti-inflammatory during acute disease phases where it targeted mostly innate immune cells and ameliorated inflammation and damage to the CNS. However, we also noted that netrin-1 has the potential to exacerbate inflammation in the CNS, when applied long term. This changes the behavior of adaptive immune cells, but we do not yet understand how. Many interesting questions remain to be answered. We hope that our work will encourage other scientists to address these questions and shed more light on the varied functions of netrin.

APPENDIX

Table A1 Netrins' role in angiogenesis

Netrin-1						
Main function	**Tissue,**	**Therapeutic effect**	***In vitro* effect**	**Endoge-nous expr.**	**Recep -tor**	**Author**
Pro-angiogenic	heart (A) m	Transplantation of Shh over-expressing MSCs or recombinant N1 increases blood vessel density in heart and increased blood flow after myocardial infarction.	Recombinant N1 increased vascular branching and tube formation in the heart.	↑ N1 via Shh signal	n.d.	Ahmed 2010
Pro-angiogenic + anti-apoptotic	Aorta, (A/D) c,z	Implantation of N1 containing cellulose disks increase vessel branching in CAM. Knockdown of netrin-1a in developing zebrafish results in the absence of PAV and abnormalities in intersegmental vessels.	N1 decreased HUVEC, HUAEC cell death. (similar response in venous, arterial ECs) Recombinant N1 increased vessel outgrowth in *ex vivo* aortic ring assay	N1 is not expressed by HUVECs or HUAECs	Unc5 B	Castets 2009
Pro-angiogenic	Heart (A) rat	Transplantation of N1 overexpressing heart stem cells decreases apoptosis, tissue scarring, and increased NO production and vessel density.	Conditioned media from N1 overexpressing heart stem cells increased HUVEC tube formation and branching, phosphorylation of Akt, and increased rat EC migration	n.d.	n.d.	Durrani 2011
Pro-angiogenic	Brain (A) m	Intracerebral injection of N1 (chick) encoding adenovirus resulted in vessel enlargement.	N1 increased proliferation, migration, and tube formation in HBEC and HASMC	n.d.	n.d.	Fan 2008 ☹
Pro-angiogenic	Hind-limbs (A) rat	Transplantation of MSCs and / or intramuscular injection of N1 increased VEGF serum levels and increased capillary density and arterial vessel formation during hind limb ischemia.	N1 increased HUVEC migration, and tube formation.	n.d.	n.d.	Li 2009
Pro-angiogenic	Retina (A) m,h	No netrin application! Oxygen induced retinopathy (m) Proliferative diabetic retinopathy (h)	n.d.	↑ N1 in se-rum, retina (m);↑ N1 in vitreous	n.d.	Liu 2011

				fluid (h)		
Pro-angiog.	Brain (A) m	Intracerebral injection of N1 containing adenovirus reduced infarct size, vessel density and behavioral recovery after transient focal ischemia.	n.d.	n.d.	n.d.	Lu 2012
Pro-angiog.	Placenta (A/D) m,z	Endothelial cell selective Tie2 promotor driven knockout of unc5B reduced placental artery growth, embryonic hypoxia, death and reversed blood flow. Unc5B silencing (z) inhibited PAV formation.	Recombinant N1 increased vessel outgrowth and sprouting from placental explants from unc5B wild type mice but not from unc5B knockouts mice.	n.d.	Unc5 B	Navank asattusa s 2008
Pro-angiog.	n.d.	n.d.	N1 increased *ex vivo* mouse aortic endothelial outgrowth. Bovine aortic EC proliferation increased as N1 activated DCC, and increased downstream phosphor. of ERK1/2 and eNOS, and NO levels.	n.d.	DCC	Nguyen 2006
Pro-angiog.	Retina (A) m,c	Implantation of N1 containing hydropellets into the corneal pocket increased vessel density. N1 containing sponges placed on the CAM, increased vessel vessel sprouting into the sponge. Venous and arterial cells respond similarly.	N1 increased migration, proliferation of HUVEC, HAEC, HMVEC, VSMC, and tube formation of HUVEC, HAMVEC. VSMC strongly adhered to N1 substrate via neogenin.	n.d.	No DCC, Neo in ECs	Park 2004
Pro-angiogenic	Placenta (A) h	n.d.	n.d.	Reduced N1 correlated with low weight fetus and placenta	n.d.	Qia-hua 2010 ☹
Pro-angiogenic	Brain (A) m	Intracerebral injection of N1 encoding adenovirus increased vessel density and improves motor function after experimental stroke	n.d.	n.d.	n.d.	Sun 2011 ☹
Pro-angioge	Retina (A) m	No netrin application! Oxygen-induced Retinopathy	n.d.	↑ N1 in retino-	n.d.	Tian 2011

nic				pathic retina		☹
Pro-angiog.	hind limb (A) m,z	i.m. injection of N1 gene constructs during diabetes or hindlimb ische-mia increased blood vessel density, blood flow, and nerve conduction velocity. Knockdown of netrin-1a in zebrafish led to absent PAVs.	N1 increased HUVEC and HUAEC proliferation, migration, and tube formation.	n.d.	Un-known netrin recept or	Wilson 2006
Pro-/anti-angiog.	Retina (A) rabbit	N1 application in a corneal micropocket assay increased retinal vessel outgrowth at a low dose and reduced it at a high dose.	At low doses (10-100 ng/ml) N1 increased proliferation, migration of HUVECs. At high doses (1.5 µg/ml) N1 decreased proliferation, migration, and vascular branching.	n.d.	Unc5B	Yang 2007
Anti-angiog.	Tumor (D) c	Application of N1 to the CAM increased plasma leakage of CAM vessels. Transplantation of N1 overexpressing tumor to embryos decreased vessel sprouting.	n.d.	n.d.	Unc5B	Bouvree 2008 ☹
Anti-angiog. Anti-inflam matory	Retina (A) rat	Topical application of rec N1 on alkali burned retinas showed less neovascularization and blood vessel degeneration. N1 decreased expression of VEGF.	n.d.	n.d.	n.d.	Han 2012
Anti-angiog.	Tumor (A) m	Implantation N1 overexpressing tumor cells delayed tumor angioge-nesis, decreased vessel density, and decreased tumor volume via unc5B. Unc5B expression occurred in arte-ries, but not in capillaries or veins.	n.d.	n.d.	Unc5B	Larrivee 2007 ☹
Anti-angioge nic	Embryo (D) z	Knockdown of unc5B or netrin-1a increased vascular branching.	HUAEC migrated less in response to N1 (1, 2.5 µg/ml). HUAEC express more unc5B than HUVEC.	n.d.	Unc5B	Lu 2004 ☹
Anti-apoptoti c	CAM (D) c	N1 siRNA treatment of CAM, implanted with tumor cells, decreased tumor cell invasion.	N1 decreased HUVEC and pancreatic cancer cell line proliferation, apoptosis, while increasing their adhesion	n.d.	n.d.	Dumarti n 2009 ☹

			to an immobilized N1 substrate.			
Anti-apoptotic	Brain (A) m	Intracerebral implantation of N1 containing osmotic pumps before ischemic stroke reduced apoptosis.	N1 increased neural progenitor migration towards N1 (chemoattractive).	↑ Unc5B	n.d.	Wu 2008
Anti-apoptotic	Heart (A) m	*Ex vivo* injection of N1 into aorta before myocardial infarction reduced infarct size and apoptosis, and increased NO production.	n.d.	n.d.	DCC	Zhang 2010

Netrin-4

Function	**Tissue**	**Therapeutic effect**	***In vitro* effect**	**End. exp.**	**Receptor**	**Author**
Pro-angiogenic + anti-apoptotic	Brain (A) m	Intra ventricular N4 containing osmotic pump implantation increased blood vessel density and promoted behavioral recovery, but did not affect BBB permeability.	n.d.	↑ N4 on vessels + astrocytic processes in ischemic core	n.d.	Hoang 2009
Pro-angiogenic	Embryo (D) z	Knockdown of N4 caused absence and defect of inter-segmental vessels .	Rec N4 decreases cell death in serum-starved HUAEC and even more in HUVEC Rec N4 increases HUVEC proliferation, tube formation, and outgrowth via phosphorylation of FAK, Akt, ERK1/2, JNK,	N4 expressed by blood vessels		Lambert 2012
Pro-angiogenic Pro-tumorigenic	Lymphatic vessels Tumor (A) m	Selective overexpression of N4 in keratocytes increased lymphatic vessel density, and promoted a redder skin. Without directly affecting tumor cell proliferation, transplantation of N4 overexpressing breast cancer cells increased lymphangiogene-sis and vascular permeability. This	N4 increased lymphatic HMVEC proliferation, migration, tube formation, and adhesion to a N4 substrate via Akt, ErK1/2, and ribosomal protein S6 phosphorylation. It reduced apoptosis and reduced TEER via increased levels of RhoA	n.d.	Not by Unc5B or neogenin	Larrieu-Lahargue 2010

		enhanced tumor development and lung and lymph node metastasis.	GTP and increased phosphorylation of SFK and reduction of TJ proteins			
Pro-angiogenic	hind limb (A) m,z	I.m. injection of N4 gene constructs during diabetes or hindlimb ischemia increased blood vessel density, blood flow, and nerve conduction velocity. Knockdown of netrin-1a in zebrafish led to absence of PAV	N4 increased HUVEC and HUAEC proliferation, migration, and tube formation.	n.d.	Unknown netrin receptor	Wilson 2006
Anti-angiogenic, Anti-tumorigenic	Tumor (A) m	Transplanted N4 overexpressing tumor cells decreased tumor proliferation, growth, and vascularization, without affecting VEGF-induced vasc. permeability.	N4 (10 μg/ml) decreased HUVEC tube formation.	n.d.	Neogenin	Eveno 2011
Anti-angiogenic Anti-tumorigenic	Tumor (A) m	Subcutaneous injection of N4 overexpressing tumor cells reduces tumor vascularization and growth.	N4 decreases angiogenic effects of VEGF on HUAEC tubule formation, migration, and FAK phosphorylation	n.d.	Neogenin, Unc5B	Lejmi 2008
Anti-angiogenic	n.d.	n.d.	N4 (25-100 μg) reduced HMVEC migration, prolife-ration, tube formation, increased apoptosis, and reduced tumor cell line proliferation by inhibiting Akt and Jnk phosphorylation	N4 in HMVEC, HUVEC, but less in tumor cell lines, Not peric.	n.d.	Nacht 2009 ☹

Table A2 Netrins' role during immune pathologies

↓ = decrease of
↑ = increase of
= numbers of

IL-6 = interleukin-6
IL-8 = interleukin-8 / CXCL-8
IFNg = interferon gamma
MCP-1 = Monocyte chemotactic protein-1 / CCL-2
MIP-1a = Macrophage inflammatory molecule-1alpha / CCL-3
TNFa = tumor necrosis factor alpha
TGFb = transforming growth factor beta
MPO = myeloperoxidase

Mo = monocytes
MΦ = macrophages
PMN = polymorphonuclear cells / granulocytes
Th1 cells = IFNg-secreting T helper
Th2 = IL-5-secreting T helper cells
Th17 cells = Th17-secreting T helper
Treg = CD4+CD25+FoxP3+

regulatory T cells
EC = endothelial cells

h = human
m = mouse
p = pork
r = rat
z = zebrafish

i.v. = intravenous injection of
i.p. = intraperitoneal injection of
inh. = inhalation of
i.m. = intramuscular injection of
i.c. = intracardial injection of
i.ce. = intracerebral injection of
icv.i. = intracerebroventricular injection of osmotic pump containing
s.c.o. =subcutaneous osmotic pump containing
gen. = gene-delivered overexpression of
Shh = sonic hedgehog
MSCs = mesenchymal stem cells
tMCAO = transient middle cerebral artery occlusion
AAV =adeno-associated viral vector

N1 = netrin-1
N4 = netrin-4

NG1 = netrin-G1
NG2 = netrin-G2

DSCAM = Down Syndrome Cell Adhesion Molecule

h = hours
d = days
w = weeks
m = months

N1 $^{+/-}$ = netrin-1 heterozygous mice (in comparison to wild type mice)
A2BAR $^{-/-}$= A2BAR knockout mice (in comparison to wild type mice)
LDL-R $^{-/-}$= low density lipoprotein receptor knockout
apoE $^{-/-}$= apolipoprotein E knockout

LPS = lipopolysaccharide
fMLP = f-formyl-methionine-leucine-phenylalanine
ZyA = Zymosan A

Disease model	**Affected organ**	**Endogenous expression in disease**	**Netrin-application and overall effect after … time**	**Netrin-mediated effect in disease model**	**Receptor mediating netrin-effects**	**Author**
Hypoxia in mucosal surfaces	**Lung** (m), Intestine (m)	↑ N1 in CaCo2 ↑ N1 in T84 ↑ N1 in HMECs upon hypoxia	i.v. N1, N1 $^{+/-}$, A2BAR $^{+/-}$ Anti-inflammatory Tissue-protective 4h, 8h, 24, 48 h	↓ TNFa, ↓ IL6, ↓ IL1b, ↓ MIP-1a, ↓ # immune cells in lung (mainly neutrophils), ↓ MPO	A2BAR	Rosen-berger 2009 [186]
LPS or mechanically induced acute lung injury	Lung (m)	↓ N1 in HMEC, A549 post TNFa via NfkB ↓ N1 in lung epi- and endothelium post LPS	Inh. or i.v. N1, N1 $^{+/-}$, A2BAR $^{+/-}$ Anti-inflammatory Tissue-protective 4h	↓ TNFa, ↓ IL6, ↓ MIP-1a, ↓ KC/IL8, ↓ # immune cells in lung (granulocytes), ↓ MPO, ↓ protein in infiltrate	A2BAR	Mirakaj 2010 [187]
LPS induced acute lung injury	Lung (p)	n.d.	inh. or i.v. N1 Anti-	↓ TNFa, ↓ IL6, ↓IL1b, ↓IL8, ↓ #	A2BAR	Mutz 2010 [226]

			inflammatory Tissue-protective 6h	immune cells in lung, ↓ Serum TNFa, ↓ serum IL6		
Septic pneumonia	Lung (m)	↓ N1 with TNFa or IFNg on HUVEC ↓ N1 in lung vasculature	i.v. N1 Anti-inflammatory 6h, 12h, 24h, 48h	↓ Migratory response to chemokines *in vitro*	In vitro Unc5B on CD45+ leucocytes	Ly 2005 [233]
Irritable bowl syndrome / inflammation tumor progression	**Intestine** (m,h)	↑ N1 in epithelium of IBD cancers	Anti-apoptotic, cancer-promoting 10 weeks	In vitro disruption of N1 via DCC-fbn promotes ↑ caspase 3, no impact on inflammation	DCC	Paradisi 2009 [225]
IBD / DSS-induced colitis	Intestine (m)	↑ N1 in colon and epi- and endothelial cell lines (6h post TNFα, IFNγ, IL1β)	s.c.o. N1 N1 $^{+/-}$, A2BAR $^{+/-}$ Anti-inflammatory 0,1,3,5,7d	↓ Disease severity, ↑ body weight, ↑ colon length, ↓ granulocyte infiltration, ↓ TNFα, ↓ IL1β,	A2BAR, Unc5B	Aherne 2011 [256]
fMLP-induced peritonitis	**Perito-neum** (m)	n.d.	i.p. N1 Anti-inflammatory 4h	↓ # immune cells in affected organ	*In vitro* Unc5B	Ly 2005 [233]
ZyA-induced peritonitis	Perito-neum (m)	↓ N1 in colon post ZyA ↓ N1 mRNA in Caco-2 cells post TNFa or IL-6	i.p. N1, N1 $^{+/-}$, A2BAR $^{+/-}$ Anti-inflammatory	↓ TNFa, IL6, IL1b, IL8, ↓ # immune cells in perineum and mesenterial fat tissue, ↓ MPO	A2BAR	Mirakaj 2011 [188]
right nephrectomy, with 30 minute left renal artery ischemia	**Kidney** (m)	↑ N1 in HK-2 renal epithelial cells 8,24 h post hypoxia ↑ N1 in urine	i.v. N1 prior to injury, N1 $^{+/-}$ 24h	↓ TNFa, ↓ IL6, ↑ glomerular filtration rate, ↑ serum creatinine clearance , ↓ plasma creatinine, ↓ urinary K^+ excretion, ↑ IL10, ↓ MPO↓ #	n.d.	Grenz 2011 [227]

				neutrophil in kidney		
Mechanically induced Ischemia-reperfusion	Kidney (m)	↓ N1 in serum	i.v. N1 Anti-inflammatory 6h, 24h, 48h, 72h	↓ # neutrophil, ↓ # monocyte, ↓serum creatininine, ↓necrosis, ↓ apoptosis, ↓Th polarization	Unc5B	Tadagavadi 2010 [258]
Mechanically induced Ischemia-reperfusion	Kidney (m)	↑ N1 in interstitium and endothelium, ↑ N1 in RMEC with hypoxia, ↓ N1 TKPTS, ↓ N4 in epithelial basal membrane, ↓ Unc5B	i.v. N1 or N4 N1 Protective N4 no effect 3h, 6h, 24h, 48h, 72h	↓ blood urea nitrogen, ↓ serum creatinine, ↓ # neutrophil in kidney, ↓ MCP-1	n.d.	Wang 2008 [228]
Mechanically induced Ischemia-reperfusion	Kidney (m)	Transgenic N1 in tubular epithelial cells	Transgenic N1 protective, Anti-apoptotic 6, 24h	↓ Necrosis, ↓ apoptosis, ↓ caspase 3, ↓ serum creatinine, ↓ blood urea nitrogen, ↓ MCP1, ↓ IL10, ↓ IL6, ↓ oxidative stress	n.d.	Wang 2009 [259]
Mechanically induced Ischemia-reperfusion; acute kidney injury Chronic kidney disease	Kidney (m,h)	↑ N1 in urine and epithelium (↓ after 24h)	1h, 3h, 6h, 24h, 48h, 72h	N1 is a early acute marker in urine of kidney injury (post cisplatin, folic acid and LPS administration or ischemia reperfusion)	n.d.	Reeves 2008 [230]
Cisplatin nephrotoxicity	Kidney (m)	n.d.	N1 overexpression in tubular	↓ serum creatinine, ↓ MCP-1, ↓ MIG, ↓	n.d.	Rajasundari 2011

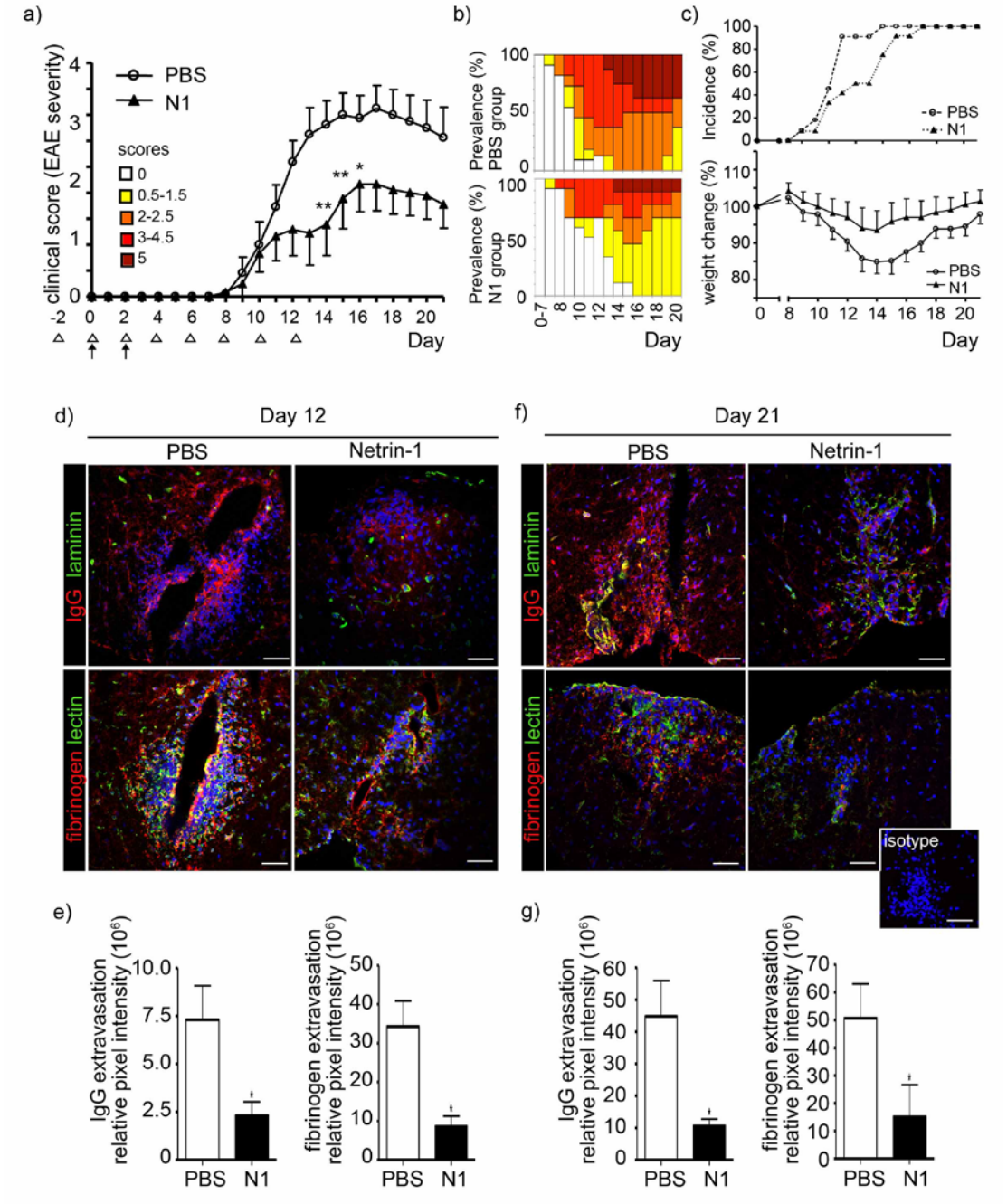
			epithelial cells 24, 48, 72h anti-apoptotic, anti-inflammatory	IL-8, ↓ RANTES ↓ complement C3, ↓ osteopontin, ↓ ICAM, ↑ IL-4, ↑ IL-13, ↓ IL-10, ↓ TNFa, ↓IL-1b, ↓ Il-18, ↓ apoptosis		
Diabetes , hindlimb ischemia	**Vascular-ture** (m) (z)	n.d.	i.m. N1 or N4 gene construct delivery 7,14,21,28 d angiogenic	↑ parachordal vessel formation, ↑ EC migration, ↑ proliferation, ↑ nerve conduction velocity	Unknown netrin receptor	Wilson 2006 [211]
Diabetic and oxygen-induced retinopathy	Retinal vasculature (m,h)	↑ N1 in serum, retina, vitreous fluid	n.d.	n.d.	n.d.	Liu 2011 [238]
Oxygen-induced Retinopathy	Retinal vasculature (m)	↑ N1 in retina	n.d. 1,3,5 d	n.d.	n.d.	Tian 2011 [251]
Ischemia-reperfusion myocardial infarction	Heart vascular-ture (m)	n.d.	Ex vivo into aorta N1, Within 1-2 h, Protective, anti-apoptotic	↓ infarct size, ↑ NO production, ↓ apoptosis	DCC	Zhang 2010 [223]
Diet induced atherosclerosis in LDL-R KO	aorta vascular-ture (m)	n.d.	Gen. N1 Anti-inflammatory Protective 3 weeks	↓ # MΦ, ↓ CD86 mRNA, ↓ROS, ↑ FOXP3 and CD25 mRNA (Tregs), ↓ lipid deposition/ plaque formation, ↑ vessel diameter, ↓ blood velocity	n.d.	Khan 2010 [231]
Diet induced atherosclerosis in LDL-R $^{-/-}$, aopE $^{-/-}$	Aorta (m)	↑ N1 expressed and secreted by CD36-activated macrophages (via NfkB)	Liver cell transplantation from N1 $^{-/-}$ mice to irradiated LDL-R $^{-/-}$, mice	↑ # MΦ in plaques, ↑ lesion area size, ↑ disease stage advancement	Unc5B (MΦ) Neogenin (arterial SMC)	Van Gils 2012

			Enhances chronic inflammation by blocking macrophage egress from plaques, recruits smooth muscle cells into the plaque			
Myocardial infarct (permanent ligation of coronary artery)	heart vascular-ture (r)	↑ N1 expression in response to Shh overexpression in MSCs	Transplantation of Shh over-expressing MSCs 10 d post infarct, analysis 4 d later, or i.c. N1 Angiogenic, protective	↑ tubular network structures, ↑ blood vessel density in heart, ↑ blood flow	n.d.	Ahmed 2010 [221]
Hind limb ischemia (ligation of right femoral artery)	Hindlimb vascular-ture (m)	n.d.	Transplantation of MSCs and / or i.m. N1 0,7,14,28 d Angiogenic, protective	↑ VEGF, ↑ EC migration, ↑ tube formation, ↑ vessel density, ↑ reperfusion	n.d.	Li 2009 [252]
Cerebral ischemia	CNS Vascula-ture (m)	↑ N4 on vessels + astrocytic processes within ischemic core, ↑ DCC in peri-infarct zone	icv.i. N4 1 -14 d angiogenic, restorative	↑ blood vessel density, ↑ behavioural recovery	n.d.	Hoang 2008 [219]
Lymphangiogenesis	Lympha-tic vascu-lature (m)	n.d.	Gen. N4 mitogenic, cancer-promoting	↑ EC proliferation, ↑ migration, ↑ tube formation, ↑ vessel density, ↓ apoptosis, ↑ metastasis, ↑ lymphatic vessel permeability	n.d.	Larrieu-Lahargue 2010 [207]
Mechanical	CNS	↑ N1 in	n.d.	n.d.	n.d.	Tsuchiya

induced brain ischemia	vasculature (r)	neurons, ↑ DCC in neurons and astrocytes 1d, 7d, 24d, 28d				2007 [327]
Ischemic stroke	CNS vasculature (m)	↑ Unc5B	icv.i N1 1,2,3,7,14 d Anti-apoptotic	Reduction of infarct area reduced neuronal apoptosis chemoattractant for neural progenitors	n.d. (unc5B suggested)	Wu 2008 [328]
Experimental stroke	CNS vasculature (m)		i.ce. AVV -N1 (chick)	↓ infarct size, ↑ vessel density, ↑ EC proliferation, ↑ long-term behavioural recovery		Sun 2011
Transient focal ischemia (tMCAO)	CNS vasculature (m)	n.d.	i.ce. AVV -N1 (chick) infection of astrocytes and neurons 1-4 weeks angiogenic	↓ infarct size, ↑ vessel density, ↑ EC proliferation, ↑ long-term behavioural recovery	n.d.	Lu 2012
No disease model	CNS vasculature (m)	n.d.	i.ce. AVV -N1 (chick) infection of astrocytes and neurons 1,3,5 weeks angiogenic	↑ HBEC and HASMC proliferation, migration, tube formation *In vitro*, ↑ vessel enlargement	n.d.	Fan 2008
Rheumatoid arthritis	Joints (h)	↑Unc5B and unc5C in synovial fibroblasts	N1 in vitro	↓ Migration of synovial fibroblast in vitro	n.d.	Schubert 2009 [329]
Osteoarthritis	Joints (h)	↑ DCC in chondrocytes	N1 *in vitro*	↑ Migration of chondrocytes *in vitro*	DCC in vitro	Schubert 2009 [330]
Diabetes , hindlimb ischemia	Vascular-ture (m) (z)	n.d.	i.m. N1 or N4 gene construct delivery	↑ parachordal vessel forma-tion, ↑ EC migra-	Unknown netrin receptor	Wilson 2006

			7,14,21,28 d angiogenic	tion, ↑, prolife-ration, ↑ nerve cond. velocity		
Oxygen-induced Retinopathy	Retinal vasculature (m)	↑ N1 in retina	n.d. 1,3,5 d	n.d.	n.d.	Tian 2011
Diabetic and oxygen-induced retinopathy	Retinal vasculature (m,h)	↑ N1 in serum, retina, vitreous fluid	n.d.	n.d.	n.d.	Liu 2011
Rheumatoid arthritis	Joints	↑Unc5B in osteoclasts and	n.d.	n.d.	n.d.	Schubert 2009
Osteoarthritis	Joints (h)	↑in chondrocytes	n.d.	n.d.	n.d.	Schubert 2009
EAN	Sciatic nerve (r)	↑N1 and DCC in macrophag., Schwann cells	n.d.	n.d.	n.d.	Moon 2006 [273]
EAE	Spinal cord (r)	↑ N1 and DCC in macrophages, astrocytes	n.d.	n.d.	n.d.	Moon 2010 [272]
Mechanical Spinal cord injury	Spinal cord (r)	↓ N1, DCC and to a lesser extent unc5Bh post injury	n.d. 33, 6, 14, 28 , 40 d; 7 m	n.d.	n.d.	Manitt 2006 [331]
Spinal cord injury	Spinal cord (r)	↓ N1 transiently post injury ↓ DCC, unc5Bh post injury	Icv.i. N1-expressing fibroblasts 1,2,4 ,5 w 3 m Inhibiting axonal regeneration	↓ neurofilament density and length	Unc5B (in vitro only)	Loew 2008 [332]
Spinal cord injury	Spinal cord (m)	n.d.	Organotypic slice cultures of N1 $^{+/-}$, N1 $^{-/-}$ N1 si RNA, Inibitor of regeneration	Repulsion of adult spinal cord progenitor cells	DCC (in vitro only)	Petit 2007 [333]
Alzheimers disease	Brain (m)	n.d.	N1 $^{+/-}$ improving memory function 12.5 d	↓ A-beta production	APP	Lourenco 2009 [334]

Figure A1


a)
clinical score (EAE severity)
PBS
N1
scores
0
0.5-1.5
2-2.5
3-4.5
5
Day
b)
Prevalence (%) PBS group
Prevalence (%) N1 group
Day
c)
Incidence (%)
PBS
N1
weight change (%)
Day
d)
Day 12
PBS
Netrin-1
IgG laminin
fibrinogen lectin
f)
Day 21
isotype
e)
IgG extravasation relative pixel intensity (10⁶)
fibrinogen extravasation relative pixel intensity (10⁶)
g)

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