CHAPTER 2

Glycosylation of Notch underlies signaling-dependent barrier function of brain endothelial cells

Melissa A. Lopes Pinheiro¹, Bert van het Hof¹, Dirk Geerts², Wendy W. Unger¹* and Helga E. de Vries¹*

*both authors contributed equally

¹ Department of Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam, the Netherlands

² Department of Pediatric Oncology/Hematology, Erasmus University Medical center, Rotterdam, the Netherlands

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Abstract
The blood-brain barrier (BBB) assures the optimal homeostasis of the brain through the specialized function of brain endothelial cells (BECs). Importantly, neurological disorders, such as multiple sclerosis (MS), are characterized by a disruption of the BBB due to inflammatory processes associated with the pathogenesis of the disease. Notch signaling has been shown essential for proper development of the vasculature; however, the role of this signaling pathway in BBB function in homeostatic and inflammatory conditions, has not been addressed. Here we demonstrate that under inflammatory condition, Notch signaling is reduced in BECs. Importantly, we demonstrate that Notch signaling mediates barrier function of BECs as shown by higher permeability and decreased transendothelial electrical resistance when BECs were treated with a Notch signaling inhibitor or upon specific knockdown of Notch. Furthermore, an altered expression of the glycosyltransferase Fringe is observed under inflammatory conditions, likely underlying barrier dysfunction observed in inflamed BECs. Therefore, we provide evidence for the functional role for Notch glycosylation in homeostatic and inflammatory conditions in the regulation of barrier function of BECs. Understanding how BEC function is regulated under these detrimental conditions is pivotal for the development of new anti-inflammatory therapies.
Introduction
The blood-brain barrier (BBB) is composed of specialized endothelial cells, which maintain a highly impermeable barrier in order to ensure adequate brain homeostasis. Dysfunction of the BBB is a prominent feature of a number of neurological diseases such as multiple sclerosis (MS), Alzheimer's disease, brain trauma, among others. In particular MS, a chronic inflammatory demyelinating disorder of the central nervous system (CNS) is marked by a disrupted and inflamed BBB, allowing immune cell entry into the brain. Importantly, altered endothelial-endothelial cell contacts have been suggested to result in the leakage of serum proteins in the brain of MS patients in areas of active demyelination, a phenomenon related to the pathogenesis of the disease. Therefore, understanding the underlying mechanisms of altered barrier function under inflammatory conditions will open new avenues for the development of possibly more specific therapeutic targets to dampen neuro-inflammatory events affecting the BBB.

An important pathway involved in cell-cell communication is the highly conserved Notch pathway. Notch signaling is initiated by binding of Notch receptors (in mammals Notch 1-4) on a signal receiving cell to a Notch ligand of the Delta-like family (Dll1, -2 and -4) or the Jagged family (Jagged1 and Jagged2) on a signal sending cell. Upon ligand binding, Notch receptors undergo two cleavages, resulting in the release of the Notch-intracellular domain (NICD) and its translocation to the nucleus where it induces transcription of downstream genes, such as Hes1. Notch receptors are glycosylated, which serves to fine-tune the receptors signaling properties. Glycosylation of Notch starts in the endoplasmic reticulum with the enzyme POFUT1 adding an O-fucose to the EGF domain of Notch. In the Golgi apparatus this O-fucose moiety is extended with N-acetylglucosamine (GlcNAc) by Fringe glycosyltransferases. In mammals, three different Fringes exist: Radical Fringe (Rfng), Manic Fringe (Mfng) and Lunatic Fringe (Lfng). Notch glycosylation by Lfng has been shown to inhibit the binding and signaling mediated via Jagged1 while potentiating the binding and signaling via DLL1. Notch signaling is shown to be essential for proper vascular development. Mice deficient for Notch1 or both Notch1/Notch4 display cardiovascular abnormalities and severe vascular defects, leading to lethality at early stages of development. These phenomena highlighted the importance of Notch signaling in vascular formation. However, the exact role of Notch signaling in the function of the adult vasculature and during inflammatory processes remains elusive. Furthermore, there is currently no evidence if the Notch signaling pathway or its glycosylation patterns are also involved in the specialized barrier function of brain endothelial cells (BECs).
In this study, we demonstrate for the first time a critical role of Notch in the function of the BBB. We provide evidence that inflammation alters glycosylation of Notch via reduced Lfng expression, resulting in impaired Notch signaling and, consequently, disrupted barrier function of BECs. Our data demonstrate the functional importance of this conserved signaling pathway in mediating barrier function of the brain endothelium and most importantly demonstrate how glycosylation of Notch regulates its signaling and function in the adult vasculature in both health and disease.

**Materials and Methods**

**Cell culture and treatments**

The human brain endothelial cell (BEC) line hCMEC/D3 was kindly provided by Dr. Couraud (Institute Cochin, Universite Paris Descartes, Paris, France). BECs were grown in EBM-2 medium supplemented with hEGF, hydrocortisone, GA-1000, VEGF, hFGF-B, R3-IGF-1, ascorbic acid and 2.5% fetal calf serum (Lonza, Basel, Switzerland). When stated, BECs were treated with 10µM DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester, Sigma Aldrich, Zwijndrecht, Netherlands) or DMSO as vehicle control for 24h followed by 24h with 5ng/ml rhTNFα (Peprotech). Human peripheral blood lymphocytes were recovered after monocyte isolation from buffy coats (remaining fraction) and subsequently activated with 1mg/ml phytohaemagglutinin (PHA-L: Sigma Aldrich, Zwijndrecht, the Netherlands) and 10ng/ml IL-2 (MACS Miltenyi Biotech, Germany) for 48 h. HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, in a 37°C incubator with 5% CO2. OP9 stromal cells transduced with hDLL4 or a control vector (designated as OP9-hDLL4 and OP9-ctrl respectively, a kind gift from Dr. T. Taghon, Gent, Belgium) were cultured in MEMα medium supplemented with 20% FCS. To determine DLL4 induced binding and/or signaling in BECs, resting or TNFα activated BECs were added to a confluent monolayer of OP9 cells in 24 well plates. 24h later, BECs were isolated to analyze gene expression.

**Immunohistochemistry**

For immunohistochemical analysis, 5µm cryosections of brain tissue from non-neurological controls were fixed in acetone and incubated with an antibody against Lunatic Fringe (Abcam, Cambridge, United Kingdom) o/n at 4°C in PBS containing 1% bovine serum albumin (BSA, Boehringer-Mannheim, Germany). Subsequently, sections were incubated with EnVision Dual Link-HRP (DAKO, Glostrup, Denmark) for 30min at
RT. Diaminobenzidine tetrachloride (DAB; DAKO, Glostrup, Denmark) was used as the chromogen. Between incubation steps, sections were thoroughly washed with phosphate-buffered saline (PBS). After a short rinse in tap water, sections were stained with haematoxylin for 1 min and extensively washed with tap water for 10 min. Finally, sections were dehydrated with ethanol followed by xylol and mounted with Entellan (Merck, Darmstadt, Germany).

**Lentivirus-mediated delivery of shRNA**

For knockdown of NOTCH1, NOTCH2, NOTCH4 or LFNG we used a vector-based shRNA delivery system. Twenty shRNA expression plasmids from the TRC library targeting human NOTCH1, NOTCH2, NOTCH4 or LFNG (5 each) were used to produce recombinant lentiviruses. To this end, subconfluent human embryonic kidney (HEK) 293T cells were co-transfected with the shRNA lentivirus expression plasmid, packaging plasmids (pMDLg/pRRE and pRSV Rev), and the envelope vector pMD2.G using calcium phosphate as a transfection reagent. Infectious lentiviruses were collected 48 h after transfection and the supernatant was centrifuged to remove cell debris. BECs were transduced with the shRNA-expressing lentivirus. Forty-eight hours after infection, stable cell lines were selected by puromycin treatment (2 µg/ml). The knockdown efficiency of all 5 constructs per gene was tested, and the most effective construct used in subsequent experiments: TRCN350330, encoding CCGGGACATCAGGATCATAT targeting nucleotides 6258-6278 of the NOTCH1 RefSeq NM_017617.3; TRCN282339 encoding TGGAGGTCTCAGTGGATATAA targeting nucleotides 2502-2522 of NOTCH2 NM_024408.3; TRCN426949, encoding GCTCTGGAAAGAGGGTTAAG targeting nucleotides 6325-6345 of NOTCH4 NM_004557.3; TRCN151442 encoding GCATTTAATCTCCTCTCCAAA targeting nucleotides 1874-1894 of LFNG NM_001040167.1. The TRC SHC002 vector containing a non-targeting (NTC) sequence was used as a negative control.

**Electric cell-substrate impedance sensing (ECIS)**

BECs (1x10^5/well) were seeded on collagen-coated 8W10+ ECIS arrays (Ibidi GmbH, Munchen, Germany). Transendothelial electric resistance (TEER) of BECs was measured at multiple frequencies in real-time with ECIS™ Model 1600R (Applied BioPhysics, New York, USA). When maximum barrier resistance was achieved, cells were treated with DAPT or DMSO prior to TNFα stimulation. The ECIS measurements were further analyzed and subjected to a mathematical modeling to calculate the barrier resistance (Rb) at each time point measured.
Permeability assay
BECs were cultured on collagen-coated 0.4µm pore size transwells (Corning, Amsterdam, The Netherlands). Paracellular permeability to FITC-dextran (70kDa, Sigma Aldrich, Zwijndrecht, Netherlands) in the luminal to abluminal direction was assessed in the presence or absence of TNFα, after pre-treatment with DAPT or vehicle. FITC-dextran was added to the upper chamber at final concentration of 500µg/ml and samples were collected from the lower chamber. Fluorescence intensity was determined using a FLUOstar Galaxy microplate reader (BMG Labtechnologies), excitation 485nm and emission 520nm.

Flow cytometry
Cells were stained with anti-VE-cadherin (BD Bioscience, New Jersey, USA) or anti-VCAM-1 antibodies diluted in PBS/2%FCS for 20min on ice. Alternatively, BECs were incubated with APC-labeled anti-Notch1 (clone MHN1-519; eBiosciences, San Diego, CA) or biotin-labeled anti-Notch2 (clone MHN2-25; Miltenyi, Auburn, CA) antibodies. Non-specific binding was blocked with 5% normal goat serum (NGS). Goat anti-mouse Alexa 488 (Molecular Probes, Eugene, OR) or streptavidin-APC (BD Pharmingen, San Diego, CA) were used as secondary antibodies. Fluorescence intensity was measured using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). Analysis was conducted using FlowJo software.

Immunocytochemistry
BECs were seeded in 8 well µ-slides (Ibidi, GmbH, Munchen, Germany) and pre-treated with DAPT or vehicle followed by activation with TNFα. Cells were fixated with 4% formaldehyde (Sigma, St Louis, MO, USA) and non-specific binding was blocked with 5% NGS. Subsequently, cells were incubated with mouse anti-VE-cadherin (BD Bioscience, New Jersey, USA) which was detected with goat anti-mouse Alexa 488 (Molecular Probes, Eugene OR, USA). Hoechst (Molecular Probes, Eugene OR, USA) was used for nuclear staining. Staining was analyzed using a Zeiss microscope.

Migration assay
BECs were grown to confluence in 96-well plates and activated with TNFα. To test the role of Notch inhibitor on T cell migration, 10µM of DAPT (Sigma Aldrich) or vehicle were added to the BECs 1h prior to the migration experiment. Human T cells (7.5x10⁵ cells/ml) were added to EC monolayers and the number of migrated T cells was assessed after 4h. To monitor T cell migration, co-cultures were placed in an inverted
phase-contrast microscope (Nikon Eclipse TE300, Lijnden, the Netherlands) housed in a temperature-controlled (37°C), 5% CO₂ gassed chamber. A field (220x220µm) was randomly selected and recorded for 10min for 50 times by using a digital video camera using Cell F imaging software (Olympus, Heidelberg, Germany). Diapedesis was assessed by enumerating the number of T cells within the field that had either adhered or migrated through the monolayer. Transmigrated cells (phase-dark) could be readily distinguished from those remaining on the cell surface by their highly refractive (phase-bright) morphology.

Quantitative PCR
RNA was isolated using the TRIzol® method (Life Technologies, Bleiswijk, the Netherlands) and cDNA was synthesized with the Reverse Transcription System kit (Promega, Leiden, the Netherlands). The following primer sequences were used: GAPDH forward 5’- ccagtgtgctcatggttg-3’, reverse 5’- gttgctaatgcctttgttg-3’; Notch1 forward 5’- atcagcaaccccgttaacg-3’, reverse 5’- gcactcatccagctcttg-3'; Notch2 forward 5’- accctcacctgctactg-3’, reverse 5’- acacacccctcactgac-3’; Notch4 forward 5’- agtgcctttagctcctc-3’, reverse 5’- gccttgctttctccttac-3’; Hes1 forward 5’- aagccggacattctgaat-3’, reverse 5’- tcaactgcgtccactc-3’; Rfng forward 5’- agcagacgtttaaccttacg-3’, reverse 5’- aacttgctatccacggac-3’; Mfng forward 5’- gggaaactcaacgattaaac-3’, reverse 5’- agcagttcaggattccag-3’. Oligonucleotides were synthesized by Invitrogen (Bleiswijk, the Netherlands). Quantitative PCR (qPCR) reactions were performed in an ABI7900HT sequence detection system using the SYBR Green method (Applied Biosystems, New York, USA). Expression levels were normalized to GAPDH expression levels.

Western blot
Cells were washed with ice-cold PBS and lysed on ice with lysis buffer (Cell Lysis buffer, Cell Signaling Technology, Boston, MA, USA) containing protease inhibitor cocktail (Roche, Almere, the Netherlands) for 30min following manufacturer's instructions. Protein lysates were taken up in sample buffer (100mM Tris-HCl pH 6.8, 20% glycerol, 2% bromophenol blue) and heated to 95°C for 3min. Lysates were analyzed on a 10% gel by SDS-PAGE, proteins were transferred to a PVDF membrane and blocked for 1h at room temperature with blocking buffer (LI-COR, Lincoln, USA). Subsequently, membranes were incubated with antibodies against Lfng (Abcam, Cambridge, United Kingdom) and actin (clone AC-15, Sigma Aldrich), which were detected and quantified by incubation with secondary antibodies IRDye using the Odyssey infrared imaging
system (LI-COR, Lincoln, USA). Actin quantification was used to correct for protein loading.

Statistical analysis
Results are shown as mean values with standard error of the mean. Statistical analysis was performed using GraphPad Prism software (v5.01 GraphPad Software, La Jolla, CA, USA) using either unpaired Student t-test or one-way ANOVA followed by posthoc Bonferroni correction. All statistical tests are described in the figure legends.

Results
Inflammation reduces Notch signaling in brain endothelial cells
Notch signaling has been extensively studied in the context of vascular development. However, its importance in adult vascular homeostasis and in particular in barrier function of BEC remains elusive. Therefore, we first determined the expression levels of Notch receptors in BECs and assessed whether this is altered by inflammation. Under homeostatic conditions, the human BEC cell line hCMEC/D3 expresses Notch1, Notch2 and Notch4 mRNA, as determined by qRT-PCR. Under inflammatory conditions, Notch2 transcripts were significantly upregulated, whereas Notch1 and Notch4 mRNA levels were not significantly altered (fig. 1A). Interestingly, the expression of Hes1, a target gene of canonical Notch signaling was downregulated in inflamed endothelial cells (fig. 1B). Thus, these data indicate that Notch signaling is impaired in BEC under inflammatory conditions.

Notch signaling mediates barrier function of brain endothelial cells
To determine the role of Notch signaling in BECs function, we used a pharmacological gamma secretase inhibitor, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), which inhibits the release of the intracellular domain of Notch and thus decreases its signaling capacity. In homeostatic conditions, significantly lower Hes1 mRNA levels were present in BECs treated with DAPT when compared to vehicle-treated BECs (fig. 2A), illustrating reduced Notch signaling; resulting in a phenotype that resembles inflamed BECs (fig. 1B). Importantly, expression of Hes1 was further decreased when DAPT treatment was combined with TNFα (fig. 2A).
To determine whether Notch signaling is important for BEC function, we determined the permeability of BEC monolayers for FITC-dextran. We observed that under homeostatic conditions, inhibition of Notch signaling resulted in a significant increase in permeability of the BEC monolayer (fig. 2B). The permeability of the BBB in vitro was further increased when Notch was inhibited under inflammatory conditions, suggesting a cross-talk between the Notch and TNFα pathways (fig. 2B). Furthermore, we observed a decrease in the transendothelial electrical resistance (TEER) of the barrier under homeostatic conditions when BECs were treated with Notch inhibitor DAPT (fig. 2C). Interestingly, inflamed BECs achieved a value of TEER comparable to BECs treated with DAPT. This result demonstrates that BECs in which Notch is inhibited functionally resemble inflamed BECs. Notably, barrier function was further reduced when DAPT preceded TNFα treatment (fig. 2C). Therefore, we can conclude that inhibition of Notch under inflammatory conditions further reduced barrier function of BECs. These results

Figure 1: Expression of Notch receptors is altered in activated BECs. (A) BECs were stimulated with 5ng/ml rhTNFα for 24h and RNA was isolated using the Trizol method. The expression of Notch1, Notch2 and Notch4 was determined by rt-PCR (B) as well as the downstream effector of the Notch signaling Hes1. Gene expression was normalized using GAPDH. Data presented are the means of triplicate values ± SEM of at least three independent experiments. Statistical analysis was carried out using student t-test where *p<0.05, **p<0.01, ***p<0.001.
demonstrate the importance of Notch signaling for barrier maintenance in homeostatic conditions and its potential anti-inflammatory effects.

**Figure 2: Notch signaling mediates BEC function.** (A) To determine the inhibitory effect of the gamma-secretase inhibitor DAPT, the expression levels of Hes1 were determined in BECs treated with DAPT (10µM) for 24h, followed by 5ng/ml TNFα stimulation. (B) The functional effect of DAPT treatment was assessed by measuring the leakage of the BEC monolayers to 70kDa fluorescent dextran in resting or TNFα-activated BECs. (C) Barrier resistance was measured as cells reached confluence. Cells were then stimulated for 24h with DAPT after maximum Rb was reached. After DAPT treatment, BECs were stimulated with 5ng/ml TNFα and barrier resistance was determined after 24h. Results shown represent the maximum Rb value for each treatment measured after maximum barrier was reached (set as 100%). (D) T lymphocytes were isolated from buffy coats and cultured with PHAL/IL2 for 48h. The effect of DAPT on T cell migration was determined by assessing the number of migrating T cells in the presence of DAPT and after TNFα stimulation of BECs for 48h by time-lapse microscopy. Data presented are mean triplicate values ± SEM of at least three independent experiments. Statistical analysis was carried out using one-way ANOVA where *p<0.05, **p<0.01, ***p<0.001 with post-hoc Bonferroni correction.
As in MS T cell trafficking across the inflamed and leaky BBB into the brain accounts for disease progression, we hypothesized that the reduced Notch signaling in inflamed BECs facilitates this process. However, treatment of BECs with DAPT resulted in a similar number of migrated T cells through the endothelial monolayer as when using vehicle treated BECs (fig. 2D). Together, these results suggest that impaired Notch signaling in BECs leads to enhanced permeability of the BBB without affecting T cell migration.

Figure 3: BECs with decreased Notch receptor expression show decreased barrier function. Notch silencing was accomplished by lentiviral knockdown of the receptors in BECs. (A) Surface expression of Notch1 and Notch2 was determined by flow cytometry and expression of Notch4 by rt-PCR after cells reached confluence. (B) Notch-deficient cell lines were seeded in 0.4µm pore size transwells and permeability of the monolayers was determined by adding 500µg/ml of FITC-dextran to the upper chamber and collecting samples in the lower chamber after 24h. (C) Besides increased permeability, Notch knockdown cell lines also showed decreased TEER of the monolayers measured by ECIS. The maximum Rb from non-template control (NTC) transduced cells was set as 100% to which the maximum Rb value from the different Notch-deficient cell lines was compared. Data presented are mean of triplicate values ± SEM of at least three independent experiments. Statistical analysis was carried out using one-way ANOVA where *p<0.05, **p<0.01, ***p<0.001 with post-hoc Bonferroni correction.
To further investigate the role of individual Notch receptors in this process, we generated knockdown cell lines of Notch-1, -2 and -4 using shRNA (fig. 3A) and assessed whether barrier function was affected. Using this approach, we observed a significant increase in permeability of the endothelial monolayer (fig. 3B) and decreased TEER (fig. 3C) features of a disrupted barrier, in particular when Notch1 or Notch2 expression was decreased. These data confirm our findings in inflamed BECs or those treated with the gamma-secretase inhibitor DAPT (fig. 2) and show that Notch signaling underlies barrier function of BECs.

**Notch signaling impairment reduces membrane VE-Cadherin localization**

Barrier function and solute impermeability of BECs are properties highly dependent on proper formation of tight and adherence junctions (TJ and AJ)\(^{28,29}\). VE-cadherin is an AJ component and specifically expressed by the endothelium. Under homeostatic conditions, VE-cadherin is highly present at cell-cell contacts (fig. 4A). However, in inflamed conditions, a decreased localization of VE-cadherin at cell-cell contacts is observed. This loss of junctional localization is further enhanced when cells are treated with the Notch response inhibitor DAPT (fig. 4A). Quantitative analysis using flow cytometry revealed a decreased expression of VE-cadherin in activated BECs compared to resting conditions (TNF vs medium, respectively) (fig. 4B). Similar to our observations by immunocytochemistry, membrane VE-cadherin expression further decreased when BECs were activated and Notch signaling inhibited (fig. 4C and 4D). Thus Notch signaling regulates membrane VE-cadherin localization.

**Expression and activity of the glycosyltransferase Fringe is decreased by inflammation**

Our data thus far have demonstrated the importance of Notch signaling to preserve an adequate barrier function of BECs. Regulation of Notch signaling is dependent on its glycosylation status, a process known to be of importance for the binding and signaling of Notch via the different ligands\(^{17,18}\). We therefore hypothesized that altered glycosylation of Notch underlies the decreased Notch signaling in inflammatory conditions (fig. 1B). To study this, we analyzed the expression of different enzymes involved in Notch glycosylation in inflamed BECs.
Figure 4: Notch signaling inhibition reduces membrane VE-cadherin localization. (A) BECs were cultured in 8 wells µ-slides. After reaching confluence, cells were treated with 10µM DAPT for 24h treated and then activated with TNFα. Cells were washed and subsequently fixated with 4% formaldehyde and stained for VE-cadherin. (B) The expression of membrane VE-cadherin was quantified by flow cytometry in resting (medium) and inflamed (TNFα) BECs. Inflammatory stimulus reduced the membrane localization of VE-cadherin. (C) Furthermore, pre-treatment with DAPT further reduced VE-cadherin membrane localization when compared with TNFα treatment only. (D) Comparison of the mean fluorescence intensity (MFI) measured by flow cytometry of membrane VE-cadherin is shown for the different treatment conditions. Data presented are mean triplicate values ± SEM of at least three independent experiments. Statistical analysis was carried out using one-way ANOVA where *p<0.05, **p<0.01, ***p<0.001 with post-hoc Bonferroni correction.
The expression of the fucosyltransferase POFUT1 was not changed in BECs in the presence of inflammatory stimuli (fig. 5A). While Rfng mRNA levels were not altered by inflammation, the expression levels of Mfng and Lfng were significantly downregulated when BECs were exposed to TNFα (fig. 5B). The decreased expression of Lfng was confirmed at the protein level (fig. 5C). Furthermore, we confirmed its localization in the brain vasculature of port-mortem human brain tissue (supplementary figure 1). This data, therefore, demonstrates that Fringe expression is decreased in inflamed BECs. To determine if the altered glycosylation of Notch affects Notch-ligand induced signaling, we cultured resting and activated BECs on OP9 cells expressing human DLL4 and assessed Hes1 expression. In resting BECs, engagement of Notch by DLL4 induced significant Notch signaling as indicated by the increase in Hes1 expression compared to BECs co-cultured on OP9 control cells (fig. 5D).

![Figure 5: Inflammation alters expression and function of Lfng in BECs.](image)

- (A) BECs were stimulated with 5ng/ml rhTNFα for 24h and RNA was isolated using the Trizol method. The relative expression (RE) of the glycosyltransferases POFUT1, Radical Fringe, Manic Fringe and Lunatic Fringe was determined by rt-PCR. Expression values were normalized using GAPDH.
- (B) Lfng protein levels were quantified by western blot after TNFα stimulation (5ng/ml) for 24h.
- (D) Resting and activated BECs were co-cultured with OP9-expressing hDLL4 or OP9 control cells for 24h after which Hes1 expression was quantified by rt-PCR. Data presented are mean triplicate values ± SEM of at least three independent experiments. Statistical analysis was carried out using student t-test or one-way ANOVA where *p<0.05, **p<0.01, ***p<0.001 with post-hoc Bonferroni correction.
By contrast, DLL4 induced significantly less Notch signaling when binding Notch on inflamed BECs. These data suggest that the alteration in Lfng-mediated glycosylation of Notch during inflammation underlies the impaired signaling.

**Disturbed Notch glycosylation is detrimental for barrier function**

To further demonstrate that Lfng-mediated Notch glycosylation is essential for Notch signaling and its consequent functional effect, we used a lentiviral approach to reduce the expression of Lfng in BECs.

![Figure 6: Decreased Notch glycosylation is detrimental for barrier function.](image)

Knockdown of Lfng expression was achieved by lentiviral delivery of shRNA. (A) Lfng expression was measured in knockdown and control cells by rt-PCR. (B) Surface expression of Notch1 and Notch2 was determined by flow cytometry in control cells (black line) and in Lfng-deficient cells (filled histogram). (C) Hes1 expression in Lfng-deficient and control cells was measured by rt-PCR. (D) Lfng-deficient cells were seeded in 0.4 µm pore size transwells and permeability of the monolayers was determined by adding 500 µg/ml of FITC-dextran to the upper chamber and collecting samples in the lower chamber after 24h. (E) Besides increased permeability, Lfng-deficient cells also showed decreased resistance measured by ECIS when compared to control cells. Expression values were normalized using GAPDH (A and C). Data presented are mean triplicate values ± SEM of at least three independent experiments. Statistical analysis was carried out using student t-test or one-way ANOVA where *p<0.05, **p<0.01, ***p<0.001 with post-hoc Bonferroni correction.
We confirmed that expression of Lfng was significantly decreased in the Lfng-deficient BECs, compared to cells BECs transduced with a non-targeting control shRNA (fig. 6A). Importantly, Notch1 and Notch2 expression levels were unaffected in Lfng-deficient BECs (fig. 6B). Interestingly, the expression of Hes1 was significantly decreased in Lfng-deficient BECs (fig. 6C), underlining the importance of glycosylation of Notch for its signaling capacity. Moreover, we observed a significant increase in the permeability to fluorescently labeled dextrans in Lfng-deficient BECs compared to control BECs (p<0.05, fig. 6D). Furthermore, Lfng-deficient BECs showed a significant reduction in barrier resistance (p<0.001, fig. 6E). Taken together, these results demonstrate that Notch glycosylation by Lfng is crucial for proper BEC function.

**Discussion**

Here we demonstrate the importance of Notch signaling in the adult brain vasculature under both homeostatic and inflammatory conditions. Increased Notch2 receptor expression and altered glycosylation of Notch receptors were observed under inflammation, resulting in reduced Notch signaling. Using two different approaches to inhibit Notch signaling, a gamma-secretase inhibitor and by genetic targeting of Notch receptors, we demonstrate that proper Notch signaling is crucial for barrier function of the human BBB *in vitro*. In this process, Lfng plays a crucial role. Our data reveal that in inflammatory conditions Lfng-mediated glycosylation of Notch is reduced, which likely underlies barrier dysfunction by affecting Notch ligand binding and/or induced signaling.

In this study, we show that inflammation by TNFα regulates Notch expression since a significant increase in Notch2 mRNA is observed in inflamed BECs. Our results on BECs are in line with previous studies using endothelial cells from other vascular beds. TNFα has previously been shown to induce upregulation of Notch2 and downregulation of Notch4 in peripheral endothelial cells\(^{30,31}\). More importantly, we extend these observations by showing for the first time that inflammation decreases Notch signaling in BECs, as demonstrated by decreased expression of Hes1. The modulation of Notch activity by inflammatory cytokines has been described in endothelial cells from other vascular beds\(^{30,31}\). Importantly, it has been suggested that a basal Notch activity is essential for the maintenance of arterial endothelial cell quiescence, since Notch4 or Hes1 downregulation induced endothelial activation and apoptosis\(^{30}\). These data, together with our own findings, suggest that Notch signaling is essential for BBB homeostasis.
Our data also show the essential role of Notch signaling for proper barrier function of BECs, since silencing of Notch receptors or inhibition of Notch signaling led to a dysfunctional barrier. So far only one report described the effects of inflammation on Notch expression on rat BECs. In this study rat BECs were exposed to oxidative stress, which resulted in decreased expression of Notch4. This effect was also correlated with decreased expression of the tight junction protein ZO-1. However, whether altered Notch4 expression affected BBB function was not addressed. We extend these observations by showing that inhibiting Notch signaling in BECs is sufficient to alter its barrier function capacity. Importantly, inhibition of Notch signaling reduced barrier function to the same extent as caused by TNFα treatment. Thus, inhibiting Notch signaling provides BECs with a signature that phenotypically resembles inflamed BECs. These results show the importance of Notch signaling in homeostatic conditions. Furthermore, we also demonstrate that Notch signaling in BECs is important in their response to inflammation, since inhibition of Notch signaling under inflammatory conditions lead to an even greater barrier dysfunction. Notch signaling seems to be necessary to preserve BEC function in resting conditions and upon inflammatory stimulus this regulation is lost, probably due to a cross-talk between Notch and TNFα signaling pathways. In fact, it has been shown that TNFα inhibits Notch signaling in a p65-dependent increase in nuclear localization of Notch repressors resulting in decreased Hes1 expression. Furthermore, signaling via Notch1 has been shown to inhibit NF-kB-dependent gene expression. Importantly, these reports go in line with our findings, since when Notch is inhibited under inflammatory conditions, barrier dysfunction is aggravated. This data demonstrates a cross-talk between these two signaling pathways. Barrier function of BECs is highly dependent on junctional complexes which maintain the solute impermeability characteristic of the BBB. Importantly, vascular permeability has been shown to be highly dependent on VE-cadherin function at AJs. Upon Notch signaling blockade, we observed an altered distribution of VE-cadherin to the endothelial cell membrane. A recent report has shown that in sprouting angiogenesis, low Notch signaling activity was correlated with increased VE-cadherin turnover and mobility from junctions. This finding is in line with our results, since we observe decreased membrane VE-cadherin when Notch signaling is inhibited. The displacement of VE-cadherin we observed could be a bystander effect due to the decreased Notch-ligand interaction between cells that could ultimately lead to a decreased endothelial-endothelial contact, rendering the junctional complex unstable. In diseases as MS, disruption of the junctions has been associated with increased leakage of
serum proteins into the brain in areas of active demyelination\textsuperscript{2,3}. Taken together, our data further reinforce the importance of BBB dysfunction in the pathogenesis of MS and lesion formation.

Regulation of Notch signaling can be achieved by glycosylation of EGF repeats on the external domain of the Notch receptor. We provide evidence that inflammation alters the expression and function of Lfng in BECs resulting in impaired Notch signaling and, consequently, in barrier dysfunction. Previous studies showed that Lfng-mediated glycosylation of Notch1 increasedDll1-mediated signaling and inhibits Jagged1-induced signaling using human embryonic kidney (293T) and fibroblast (3T3) cells\textsuperscript{17,18}. On the other hand, Lfng glycosylation of Notch2 was shown to potentiate both Jagged1- and Delta1-mediated signaling in mouse myoblast (C2C12) cell lines\textsuperscript{18}. Importantly, these studies demonstrate that Notch regulation by glycosylation could be cell specific. Furthermore, these \textit{in vitro} systems determine signaling under homeostatic conditions, which might be altered during inflammatory events as suggested by our results.

Although we demonstrate an important role for Lfng in Notch glycosylation and function, we cannot dismiss the functional importance of Mfng, which was also downregulated by inflammation in BECs. In has been previously described that Mfng also inhibits the signaling via Jagged1 from Notch1 and Notch2 receptors\textsuperscript{17-19}. Although both Lfng and Mfng were found to potentiate the signaling via DLL1 while inhibiting Jagged1-mediated signaling, Lfng was shown to be a stronger inducer of DLL1 signaling compared with Mfng\textsuperscript{17}, which was suggested to be due to its increased efficiency\textsuperscript{19,37}.

In conclusion, in this study we demonstrate the importance of Notch glycosylation in regulating Notch signaling and function in BECs. We not only show that Notch signaling is essential for proper barrier homeostasis, but is also important for BECs to cope with inflammation. This is an important aspect, since in neuroinflammatory diseases of the brain, as MS, inflammatory processes are present and account for disease pathogenesis. This study elucidates how inflammation alters BBB function, which will help pave the way for the understanding of disease processes and development of new therapies for vascular dysfunction.
References