The Role of Wnt/β-catenin Signaling in Blood-Brain Barrier Integrity in Experimental Autoimmune Encephalomyelitis (Multiple Sclerosis)

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Abstract

Compromised blood-brain barrier (BBB) integrity – a defining feature of Multiple Sclerosis (MS) – leads to increased immune cell infiltration, inflammation, and symptomatic expression of MS. There is no current cure for this autoimmune disease. One area of interest for treatment is targeting the leaky BBB, which can be explored using the accepted disease animal model, experimental autoimmune encephalomyelitis (EAE). Wnt/β-catenin signaling is essential for BBB formation and vascularization during development. However, early lab findings revealed hyperactivated Wnt/β-catenin signaling in EAE resulted in exacerbated MS symptoms.

To explore the disease progression observed during increased Wnt signaling, a Wnt reporter mouse was used to evaluate the presence of (1) BBB formation transcription factors necessary and (2) neoangiogenesis markers during EAE. Spinal cord cross-sections were collected and stained for confocal microscopy. During peak EAE, one BBB transcription factor *Zic3* was absent or at low levels compared to expression in developing healthy mice ($p < .05$). Preliminary results suggested that neoangiogenesis, indicated by *Egfl7* expression, was at higher levels during peak EAE compared to healthy adult mice. This study suggests the lack of *Zic3* and increased neoangiogenesis are possible explanations for leakage in the BBB and increased inflammation when Wnt/β-catenin signaling is activated in EAE. Future research should continue to explore the underlying mechanisms of the BBB degradation as a target for therapeutics to restore the BBB.

Introduction

Multiple Sclerosis

Multiple Sclerosis (MS) is the most prevalent, nonfatal neurological disability among young adults (Dendrou, Fugger, & Friese, 2015; Wu & Alvarez, 2011). It is characterized by demyelination and inflammation of the central nervous system (CNS) (Dendrou et al., 2015; Wu & Alvarez, 2011). The most common form is relapsing-remitting MS in which patients have periods of remission between flares of symptoms (Dendrou et al., 2015). Unfortunately, there is no current cure for MS (Dendrou et al., 2015; Wu & Alvarez, 2011). One area of interest for treatment, research, and development is the blood-brain barrier (BBB) (Minagar & Alexander, 2003; Weiss, Miller, Cazaubon, & Couraud, 2009). The loss of BBB integrity during MS contributes to the entrance of immune cells into the central nervous system (CNS) which impairs

neurotransmission and is associated with MS symptoms including numbness, fatigue, and paralysis (Dendrou et al., 2015; Minagar & Alexander, 2003). Current drugs provide symptomatic relief. Understanding the repair mechanisms for a leaky BBB presents an opportunity to reverse disease progression.

Blood-Brain Barrier

The BBB is a semi-permeable membrane that selectively restricts the blood-to-brain transport of substances across the epithelium preventing injury to the CNS (Begley, 2006; Dobbing, 1961; Palmela, Brites, & Brito, 2012). The BBB is formed by a network of complex tight junction proteins between the endothelial cells (Begley, 2006). The BBB properties and integrity depend on the presence of tight junction proteins between adjacent cells (Begley, 2006; Dobbing, 1961; Palmela et al., 2012). In the BBB, tight junction proteins restrict the entry of immune cells and peripheral inflammatory mediators, including cytokines and antibodies, between adjacent endothelial cells (Begley, 2006; Dobbing, 1961). This selective restriction is necessary for cerebral homeostasis and proper neuronal function (Weiss et al., 2009).

A common feature of MS is compromised BBB integrity. Disrupted tight junction organization or degraded tight junction proteins lead to a leaky BBB and an increase of immune cell infiltration. Immune cells attack the nervous system along the brain, spinal cord, and optic nerve, resulting in inflammation (Frischer et al., 2009; Glass, Saijo, Winner, Marchetto, & Gage, 2010; Sospedra & Martin, 2016). Due to inflammation, the myelin surrounding nerve fibers becomes damaged, known as demyelination. Demyelination slows, and even stops, nerve impulses (Lubetzki & Stankoff, 2014). Immune cell infiltration, implicated in BBB degradation, results in the symptomatic expression of MS (Glass et al., 2010; Sospedra & Martin, 2016).

Compared to healthy subjects, MS patients show significantly more leakage of T-cells, indicated by increased gadolinium (Gd) magnetic resonance imaging (MRI) enhancement (Cramer, Simonsen, Frederiksen, Rostrup, & Larsson, 2014; Floris et al., 2004; Lund et al., 2013; Vos et al., 2005). Additionally, MRIs have revealed that disruption of the BBB precedes other MRI abnormalities in MS patients, signifying that it is an early and possibly crucial event in the pathogenesis of MS (Cramer, Simonsen, Frederiksen, Rostrup, & Larsson, 2014; Floris et al., 2004; Lund et al., 2013; Vos et al., 2005; Kermode et al., 1990). Due to the infiltration of

immune cells manifesting as MS symptoms, there is an interest in addressing the leakiness of the BBB through specific pathways.

Experimental Autoimmune Encephalomyelitis (EAE)

To explore the mechanisms of the BBB, experimental autoimmune encephalomyelitis (EAE) is a widely accepted disease animal model with pathological and clinical characteristics highly relevant to the study of MS (Constantinescu, Farooqi, O'Brien, & Gran, 2011a, 2011b; Miller, Karpus, & Davidson, 2010). EAE is an antigen-driven autoimmune model rather than a genetic model. EAE is induced through myelin oligodendrocyte glycoprotein 35-55 (MOG_{35-55}) which causes the infiltration of T cells into the CNS leading to myelin destruction, emulating MS disease pathology (Miller et al., 2010).

The degradation of tight junction proteins that are associated with damaged tissue, or lesions, occur in EAE to a similar degree as that seen in MS patients (Constantinescu et al., 2011a; Miller et al., 2010). To evaluate BBB integrity, tight junction proteins are assessed at the level of blood vessels. Endothelial cells lining each blood vessel contribute to the BBB between the blood and the rest of the body tissues. The integrity of tight junction proteins between endothelial cells prevents immune cell infiltration. Therefore, the permeability of the BBB is directly correlated with disease severity in EAE. With increased permeability, worse clinical outcomes are most likely attributed to increased infiltration of inflammatory cells (Constantinescu et al., 2011b).

Wnt/β-catenin Signaling

A potential repair mechanism for the BBB in MS is the Wnt/β-catenin signaling pathway. Throughout embryonic and postnatal development, Wnt signaling contributes to both blood-brain barrier formation and angiogenesis, which is the development of new blood vessels (Daneman et al., 2009; Liebner et al., 2008; Stenman et al., 2008; Zhou et al., 2014). In healthy adults, Wnt/β-catenin signaling is reduced to low levels which is still necessary to maintain BBB integrity (Liebner et al., 2008; Suryawanshi et al., 2015). Dysregulated activation of Wnt signaling has been related to the pathology of many autoimmune diseases, including rheumatoid arthritis (RA) and inflammatory bowel disease (IBD). Understanding Wnt signaling in

autoimmune diseases may provide a new diagnostic marker and therapeutic target for developing novel treatments through targeting the Wnt signaling pathways (Shi et al., 2016).

Using the mouse model of MS, EAE, previous studies have explored the effects of inhibiting Wnt/β-catenin signaling on the BBB and MS symptoms. In healthy humans, Wnt signaling is reduced to low levels during adulthood. This led to an interest in the complete inhibition of Wnt signaling in adult EAE mice. In an experiment when Wnt was inhibited in the CNS endothelium before the onset of disease, there was an observed clinical exacerbation of EAE. EAE mice with inhibited Wnt signaling exhibited more severe clinical EAE scores and increased mortality rates. Additionally, results indicated an increase of molecules that promote endothelial transcytosis, the movement of materials across the BBB from one side of an endothelial cell to the other side (J. E. Lengfeld et al., 2017). Increased endothelial transcytosis led to increased CD4+ T-cell infiltration and demyelination (Lengfeld et al., 2017; Lutz et al., 2017; Nag, 2003). This suggests that inhibited Wnt signaling contributed to increased EAE clinical scores which was not surprising since Wnt signaling helps in maintaining BBB integrity.

Hyperactivated Wnt/β-catenin Signaling

The observed worse clinical outcomes may be a result of compromised BBB integrity caused by decreased Wnt/β-catenin signaling. My mentor's lab initially hypothesized that the upregulation of Wnt signaling would help maintain BBB integrity and improve EAE disease progression. A mouse model with upregulated Wnt/β-catenin signaling was used to further understand the role of Wnt signaling during EAE. Apcdd1 is an inhibitor of Wnt/β-catenin signaling, thus my mentor's lab created an Apcdd1 knockout model to explore hyperactivated Wnt signaling in EAE (Mazzoni et al., 2017; Shimomura et al., 2010). EAE mice that were induced with Apcdd1$^{-/-}$ were expected to be protected against EAE due to the increase in Wnt signaling. Unexpectedly, the Apcdd1$^{-/-}$ mice exhibited a significant increase in peak clinical scores compared to wild-type mice. These results indicated that worse EAE neurological outcomes were associated with the hyperactivation of Wnt/β-catenin signaling.

These early lab findings reveal that the activation of Wnt signaling in CNS endothelial cells fails to maintain BBB integrity in EAE. The explanation for why Wnt/β-catenin activation fails to restore/repair BBB function in EAE remains unknown. This study explores novel molecular pathological mechanisms to help explain the dysfunctional BBB in EAE and reasons

for worsened disease progression. The goal of this study was to better explain the initial unanticipated findings of increased (worse) clinical EAE scores with activated Wnt signaling in EAE observed by my mentor in a previous experiment.

Transcription Factors for BBB Formation

For this exploration of BBB integrity, I selected three transcription factors for observation: *Zic3, FoxQ1, FoxF2*. Along with Wnt/β-catenin signaling during development, several transcription factors induce the unique BBB properties of CNS blood vessels. *Zic3, FoxQ1, FoxF2* are three transcription factors that have been identified to play a role in BBB formation in brain endothelial cells at distinct stages of development. These transcription factors were shown to increase the expression of genes encoding markers of brain endothelial cell differentiation, so they are considered important in BBB formation (Hupe et al., 2017). After the activation of the Wnt pathway, these transcription factors begin to transcribe DNA to form the BBB, which makes them downstream targets of the Wnt pathway (Hupe et al., 2017). During development, these transcription factors are specifically enriched in brain endothelial cells and possess domains that bind to the DNA-regulatory sequences to regulate the rate of gene transcription of BBB-specific genes (Weksler et al., 2005). Thus, understanding the presence of these factors during EAE may better explain the role and mechanisms of Wnt/β-catenin signaling in maintaining BBB integrity.

Neoangiogenesis

Another factor I was interested in exploring in relation to the worse clinical scores was angiogenesis, the growth of new blood vessels from existing vasculature. Wnt/β-catenin signaling is responsible for CNS vascularization during development (Daneman et al., 2009; Dejana, 2010; J. Lengfeld, Cutforth, & Agalliu, 2014). In MS, inflammatory cells cause the migration and proliferation of blood vessel endothelial cells into areas where blood vessels do not belong (Moore, 2002). This abnormal process is known as neoangiogenesis. Typically, neoangiogenesis produces poorly made blood vessels with abnormal vascular organization (Moore, 2002). MRIs have indicated increased blood vessel density due to increased local blood flow and blood volume during MS (Girolamo, Coppola, Ribatti, & Trojano, 2014; J. Lengfeld et

al., 2014). This increased neoangiogenesis contributes to supplying nutrients to inflamed lesions, exacerbating disease progression (Girolamo et al., 2014; J. Lengfeld et al., 2014).

Wnt signaling has been found to play a role in angiogenesis during development (Daneman et al., 2009; Dejana, 2010; J. Lengfeld et al., 2014). I wondered if the upregulated Wnt in the Wnt reporter mouse model was contributing to increased neoangiogenesis in EAE, which might explain worsened clinical progression. Thus, to observe neoangiogenesis in EAE, I selected the marker epidermal growth factor-like 7 (*Egfl7*). *Egfl7* is a unique secreted angiogenic signaling molecule because it is almost exclusively expressed by endothelial cells (Fitch, Campagnolo, Kuhnert, & Stuhlmann, 2004; Nichol & Stuhlmann, 2012; M. H. H. Schmidt et al., 2009; M. Schmidt et al., 2007). This is different from other angiogenic markers, such as vascular endothelial growth factor (VEGF), which are also expressed in non-endothelial cell types (Nichol & Stuhlmann, 2012). *Egfl7* expression is the highest when the endothelium is in an active, proliferating state which shows neoangiogenesis. In exploring worsened clinical scores associated with increased Wnt signaling, neoangiogenesis during EAE can be better understood by observing marker of *Egfl7*.

Statement of Purpose

Early and persistent BBB damage plays an important role in the infiltration of immune cells during EAE, leading to pathological outcomes (Kermode et al., 1990; Minagar & Alexander, 2003; Palmela et al., 2012). Similarly, neoangiogenesis generates leaky blood vessels that may exacerbate EAE pathogenesis. The goal of this study was to understand the association of higher EAE clinical scores with upregulated Wnt signaling. The Wnt reporter mouse model was used to upregulate Wnt/β-catenin signaling in order to investigate the expression of BBB-inducing transcription factors along with the expression of neoangiogenesis during EAE and activated Wnt/β-catenin signaling.

The first research question aimed to understand activated Wnt and BBB repair: How does the hyperactivation of Wnt/β-catenin signaling in CNS endothelial cells in EAE affect the presence of transcription factors that act downstream of Wnt/β-catenin signaling to promote BBB formation? I hypothesized that the expression of several key transcription factors necessary for BBB maintenance would be reduced or absent in EAE.

The second part of this study explored the relationship between Wnt signaling and neoangiogenesis. How does the hyperactivation of Wnt/β-catenin signaling in endothelial cells worsen affect neoangiogenesis, measured by *Egfl7*, leading to worse EAE neurological outcomes? I hypothesized that neoangiogenesis is upregulated during peak EAE.

Studying the role of the Wnt/β-catenin signaling pathway in EAE addresses the long-term goal of this research: to develop effective treatment strategies for MS. A leaky BBB leads to immune cell infiltration, and therefore, inflammation. Thus, maintaining BBB integrity can be a preventive measure against MS. Understanding the signaling pathway and the molecular mechanisms that induce CNS vascularization in EAE, and ultimately MS, may allow us to develop novel treatments that aim to repair BBB function and reduce pathogenic neoangiogenesis.

Methodology

Induction of EAE and Clinical Observations (Performed by Mentor)

To upregulate Wnt/β-catenin signaling, the mice used in this experiment were *Tcf/Lef1*::H2B-eGFP Wnt reporter mice. EAE was induced in ten to twelve-week old Wnt-reporter mice, which were observed for clinical symptoms for sixteen days after EAE induction. All experimental procedures involving live animals were performed by the supervising scientists and were approved by the IACUC at the research institution. EAE was induced in the mice by subcutaneous injection of 100μg of myelin oligodendrocyte glycoprotein 35-55 (MOG_{35-55}, MEVGWYRSPFSRVVHLYRNGK-COOH) in complete Freund's adjuvant (CFA) containing 200μg heat killed mycobacterium tuberculosis in order to sensitize the immune system against the attack of myelin. Intravenous injections of 400ng of Bordetella pertussis toxin were administered at day 0 and day 2 post-immunization. Pertussis toxin breaks down the BBB and allows immune cells access to the CNS tissue. This immunization leads to multiple areas of demyelination in the spinal cord and the onset of clinical symptoms. The cerebellum is also easily susceptible to the breakdown of the BBB in EAE (Zhao, Nelson, Betsholtz, & Zlokovic, 2015). Clinical expression of EAE was evaluated on a standard scale: score 0 for no observed effects, score 1 for flaccid tail, score 2 for hind limb paresis, score 3 for bilateral hindlimb paralysis, score 4 for tetraplegia, and score 5 for moribund.

Collection of Tissues (Performed by Mentor)

Tissue samples were collected from four experimental groups of Wnt reporter mice. For P3 mice, cerebellum tissue samples were collected at day 3 from healthy, developmental mice. Since Wnt/β-catenin signaling is very high at embryonic and early postnatal stages, P3 mice served as a positive control. For healthy adult mice, spinal cord tissue samples were collected at day 90. The healthy adult mice served as a negative control because Wnt/β-catenin signaling is reduced in late postnatal and adult stages. For acute EAE mice, spinal cord samples were collected at day 14 with an EAE clinical score of 2.5. For chronic EAE mice, spinal cord samples were collected at day 74 with an EAE clinical score of 2.

The lumbar, thoracic, and cervical regions of the spinal cord, along with the cerebellum, were removed after perfusion with PBS solution and 4% PFA, postfixed overnight, and then transferred into 20% sucrose in PBS solution. The samples were then sectioned using superfrost plus slides. The cross-sections were fixed in 4% PFA at room temperature, washed with PBS, treated with Proteinase K solution, fixed in 4% PFA, washed with PBS, treated with acetylate, and washed again with PBS. Then, 1000 mL of hybridization buffer was placed on each slide and incubated overnight in a 5x saline-sodium citrate (SSC) humidified chamber.

Evaluation of Transcription Factors (Student Experiment)

To understand where and when neoangiogenesis exists in cells, fluorescence in situ hybridization (FISH) was performed to label the transcription factors I selected for their BBB-forming properties: *Zic3, Foxq1, and Foxf2*. FISH is a technique that uses fluorescent probes that are nucleic acid labeled with fluorescent groups and can bind to specific DNA/RNA sequences.

The collected cross sections of the spinal cord and brain were obtained from acute EAE, chronic EAE, P3, and healthy mice (n=3) and were stained using FISH.

On the first day, 1 mL DIG-labeled probe was mixed with 1 mL FITC-labeled probe and put in 100 mL of hybridization solution in a tube at 80°C, then immediately put on ice to denature any secondary structure that may have formed in the RNA. The hybridization buffer was poured off from the slide, and 100 mL of the hybridization solution with the probe was placed on each slide. The slides were hybrid slipped, then placed in a 72°C oven overnight to ensure hybridization.

To block free membrane sites prior to the primary antibody, the following day 50 mL of the blocking reagent (TNB) was prepared, placed in a 72°C water bath to dissolve and then filter sterilized. The slide rack was placed in a container with 5x SSC buffer at 72°C water bath and coverslips were removed. The slide rack was then transferred into a container with 0.2x SSC in a 72°C water bath and washed for 1 hour. Then, the rack was transferred to 0.2x SSC at room temperature for 10 minutes and then to B1 buffer for 10 minutes. Then, 1 mL of blocking reagent was placed onto the slides for 1 hour at room temperature. After being washed with TNT buffer, 1 mL of anti-FITC-POD was placed on each slide and incubated overnight in a humidified chamber at 4°C. On the third day, the slides were washed for 10 minutes with TNT buffer. The FITC tyramide tube was thawed, and then spun down to remove precipitate. Then, 250 µL of FITC tyramide was placed on each slide and incubated for 10 minutes. Parafilm was placed carefully on top of each slide. Again, the slides were washed 3 times with TNT buffer. 1 mL of blocking reagent was placed on the slides for 1 hour at room temperature. 1 mL of anti-DIG-POD was placed on each slide and incubated overnight in the humidified chamber at 4°C. On the fourth day, the slides were washed with TNT buffer. The Cy3 tyramide tube was spinned down, then 250 µL of Cy3 tyramide was placed on each slide and incubated for 10 minutes. The slides were rinsed in TNT buffer. An immunofluorescence with Anti-Cav-1 antibody was used to label blood vessels. Lastly, slides were mounted using VectaShield with DAPI (Vector Labs) and viewed using a confocal laser scanning microscope (LSM 510 Zeiss, Germany).

The same process to perform FISH was repeated to stain for each transcription factor, along with an immunofluorescence with Anti-Cav-1 antibody to label blood vessels. Slides were mounted using VectaShield with DAPI (Vector Labs) and viewed using a confocal laser scanning microscope (LSM 510 Zeiss, Germany).

Additional Staining for Zic3 (Student Experiment)

To further investigate the expression of *Zic3*, immunofluorescence was performed. Cross sections from the spinal cord and brain were obtained from P3 (three-days old) and acute EAE mice (n=3). The lumbar, thoracic, and cervical regions of the spinal cord, along with the cerebellum, were removed after perfusion with PBS solution and 4% PFA, postfixed overnight, and then transferred into 20% sucrose in PBS solution. The samples were then sectioned using superfrost plus slides.

On the first day, the slides were washed with PBS, and then 1 mL of PBT (PBS with 0.1% Triton X-100) and 10% Bovine Serum Albumin (BSA) were placed on horizontal slides for 1 hour. at room temperature to block nonspecific binding of antibodies. These slides were then washed and replaced with 1 mL of solution containing PBT with 1% BSA and diluted primary antibody at the specified dilution, then place on humidified chamber at 4C.

On the second day, the slides were washed with PBT. Then, 1 mL of solution containing PBT with 1% BSA and diluted secondary antibody at the specified dilution was placed on the slides. The slides were then incubated for 2 hours at room temperature in the dark. After being washed with PBT, immunofluorescence with Anti-Cav-1 antibody was used to label blood vessels, and slides were mounted using VectaShield with DAPI (Vector Labs) to view with a confocal laser scanning microscope (LSM 510 Zeiss, Germany).

Evaluation of Neoangiogenesis (Student Experiment)

To understand where and when neoangiogenesis exists in cells, FISH was performed to label *Egfl7* (a marker of neoangiogenesis). The obtained cross sections from acute EAE, chronic EAE, P3, and healthy mice (n=3) were stained for *Egfl7*. The same process to perform FISH was repeated, along with an immunofluorescence with Anti-Cav-1 antibody to label blood vessels. Slides were mounted using VectaShield with DAPI (Vector Labs) and viewed using a confocal laser scanning microscope (LSM 510 Zeiss, Germany).

Confocal Microscopy (Performed by the Student)

For laser scanning confocal microscopy, labeled sections were examined using an LSM 510 Zeiss, Germany. Gain and contrast levels were set according to procedures standardized to ensure that the image collected showed a full range of grey level values from black (0-pixel intensity level) to peak white (254-pixel intensity level). All sections were initially surveyed at low magnification at various levels so that overall patterns of blood vessels could be compared in cells oriented at different angles with respect to the section plane.

Images were collected from the lumbar region of the spinal cord. Since EAE is characterized by ascending motor paralysis, the lumbar region is the first area of the spinal cord to be affected in the mouse.

Three sections of the lumbar spinal cord were used per slide. For each section, an image was taken of the dorsal funiculus, ventral funiculus, and four lateral funiculi. This was routinely done using a 40x objective with water magnification.

In each image, the Caveolin-1, marking blood vessels, appeared green. The DAPI, representing cell nuclei, appeared blue. Finally, the expression of either *Zic3*, *Foxq1, Foxf2* (BBB-inducing transcription factors) or *Egfl7* (neoangiogenesis marker) appeared red. A series of optical sections through each image were taken. These could be viewed individually or projected together to reconstruct the image of the blood vessel. The reconstruction of each image was processed using the ImageJ program (which is freely available from NIH), and then analyzed for quantification of expression.

Data Analysis (Performed by the Student)

The ImageJ program was used to quantify the endothelial cells positive for staining of either the angiogenic marker (*Egfl7*) or the transcription factors (*Zic3, Foxq1, Foxf2*). However, multiple quantification methods were performed in order to identify the most accurate method.

Fiji, an open source image processing package based on ImageJ, was used to quantify the images. The first method of quantification was pixel intensity spatial correlation analysis. In each image, the red (*Zic3, Foxq1, Foxf2* or *Egfl7* expression) and green (Caveolin-1) channels were identified to be analyzed with each other, then split into separate images. To express the intensity correlation of the colocalizing objects in each component of a dual-color image, Pearson's correlation coefficient was calculated. However, the colocalization of the red and green channels included all cells that were positive with expression. In order to observe the expression of the transcription factors and neoangiogenesis in blood vessels, the only type of cells necessary to include were endothelial cells. The inclusion of other cells inaccurately increased the number of positive cells.

An additional method of quantification was conducted in order to quantify positive endothelial cells exclusively. This method included manually calculating the percentage of positive cells for the total number of endothelial cells. In each image, the total number of endothelial cells was recorded in an Excel spreadsheet. The number of endothelial cells that exhibited expression was also recorded. The percentage was calculated by dividing total endothelial cells by positive endothelial cells. This method did not account for the intensity of

the signaling in positive endothelial cells. In order to understand the expression of the transcription factors and neoangiogenesis, it is not only essential to calculate the cells positive for expression, but also the levels of expression.

The mean fluorescent intensity was the final method of quantification using Fiji. In each image, the red (*Zic3, Foxq1, Foxf2,* or *Egfl7* expression) and green (Caveolin-1) channels were identified to be analyzed with each other, then split into separate images. In the green channel image, the area of the green signaling was selected to include the blood vessel in the image. The mean area was calculated in Fiji and recorded. Then, the location and shape of the green channel area was placed onto the red channel image. In this area, Fiji was used to calculate the mean fluorescent intensity of the red channel. The mean intensity was also recorded. To obtain the mean fluorescent intensity in the blood vessel, the mean fluorescent intensity of the red channel was divided by the mean area of the green channel. This method of quantification accounted for only positive endothelial cells and the intensity of the expression. Using this quantification method, paired Student's t-tests were conducted to compare the levels of expression between the four groups of mice: P3, healthy adult, acute EAE, and chronic EAE.

Results

Presence of BBB-Inducting Transcription Factors During EAE

The lumbar region of the spinal cord cross sections of acute EAE, chronic EAE, and healthy adult mice, along with the brain cross section of P3 mice were stained using FISH for the expression of three BBB-inducing transcription factors: *Zic3*, *Foxq1,* and *Foxf2*.

Zic3 is expressed by many developing blood vessels at P3 but is low or absent in blood vessels of healthy adult spinal cord (Fig. 1). Unlike the other two transcription factors, *Zic3* is low or absent in inflamed blood vessels in lesions at peak EAE (Fig. 1). During chronic EAE, however, *Zic3* is induced at very low levels (Fig. 1). The quantification table (Fig. 2) shows this trend, with the notable low expression of *Zic3* during acute EAE. Compared to the expression of *Zic3* during P3, the expression of *Zic3* is expressed at significantly lower levels in spinal cord samples of healthy adult, acute EAE, and chronic EAE mice ($p < .05$). The results from the immunofluorescence were consistent with this trend. Expression of *Zic3* was high in P3 brain, but expression was low and even absent in inflamed vessels during acute EAE (Fig. 3).

Foxq1 is highly expressed in P3 developing brain blood vessels (Fig. 4). It is then induced in inflamed blood vessels at peak EAE (Fig. 4). This trend of expression is seen in the quantification table (Fig. 5). Expression of *Foxf2* was exhibited in P3 brain, healthy, acute EAE, and chronic EAE spinal cord (Fig. 6, 7).

Neoangiogenesis in CNS Blood Vessels During EAE

Cross sections of the lumbar region of the spinal cord of acute EAE, chronic EAE, and healthy adult mice, along with the brain cross section of P3 mice were stained using FISH for the expression of *Egfl7*, a marker of angiogenesis.

Egfl7 is highly expressed in many vessels of the P3 brain and spinal cord, but its expression decreases in adult CNS blood vessels (Fig. 8), with the exception of a few regions where vessels lack a BBB, such as the choroid plexus. *Egfl7* expression was also examined in the CNS blood vessels in spinal cords of mice with active EAE, at scores 2.0 and 2.5. *Egfl7* is expressed in white matter blood vessels of EAE spinal cords at peak disease (score 2.5) (Fig. 8). These vessels are located within active lesions surrounded by immune cell infiltrates indicated by cell nuclei stained blue with DAPI. The quantification table (Fig. 9) shows that *Egfl7* expression is significantly lower in healthy adult mice and chronic EAE mice compared to P3 mice ($p < .05$).

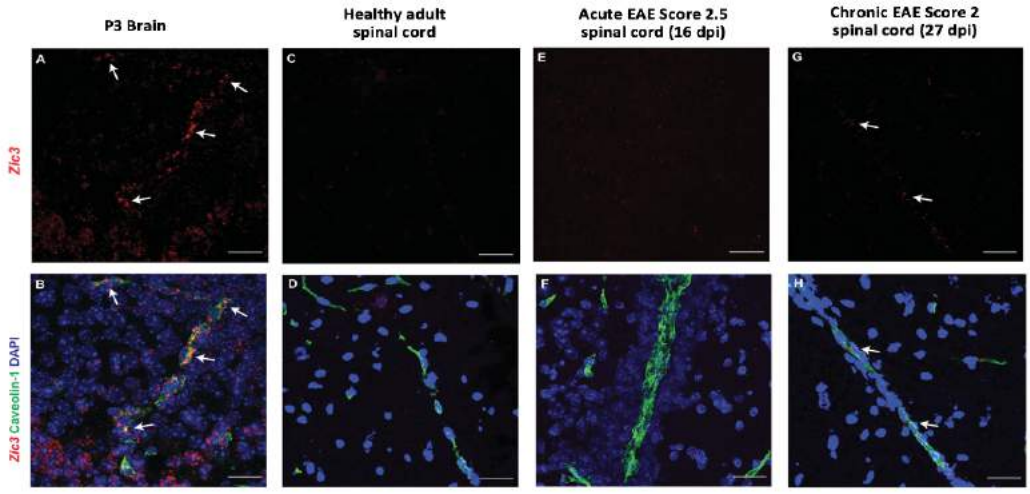

Figure 1. Expression of *Zic3* in P3 brain, healthy adult spinal cord, and EAE spinal cord
Fluorescent RNA in situ hybridization for *Zic3* combined with immunofluorescence for Caveolin-1 and DAPI in P3 and adult healthy mouse spinal cord and MOG35-55 EAE (score 2.5 and score 2). Single channel images for *Zic3* (A, C, E, G) and merged images (B, D, F, H) are both shown.

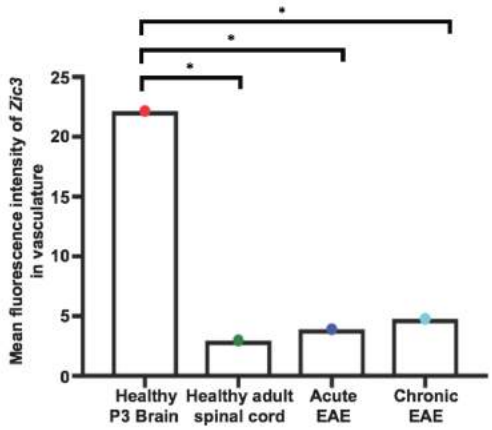

Figure 2. Quantification of *Zic3* expression
The average mean fluorescent intensity of *Zic3* expression in P3 and adult healthy mouse spinal cord and acute and chronic EAE. This trend shows significant decreases in *Zic3* expression levels from the developing healthy P3 to expression levels in healthy adult, acute EAE, and chronic EAE (*p < .05). Since *Zic3* is essential for BBB formation, this trend may indicate the lack of *Zic3* is partially responsible for the failure of Wnt to repair the BBB during EAE.

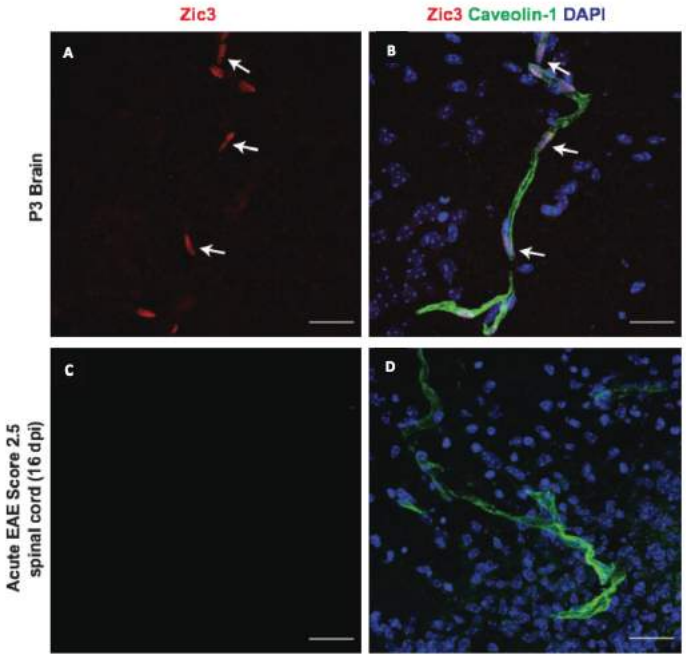

Figure 3. Expression of *Zic3* in P3 brain and EAE spinal cord
Immunofluorescence for *Zic3* combined with immunofluorescence for Caveolin-1 and DAPI in P3 and acute EAE (score 2.5) spinal cord. Single channel images for *Zic3* (A, B) and merged images (C, D) are both shown. Expression of *Zic3* (indicated by white arrows) is seen in P3 brain while expression is low or absent during acute EAE.

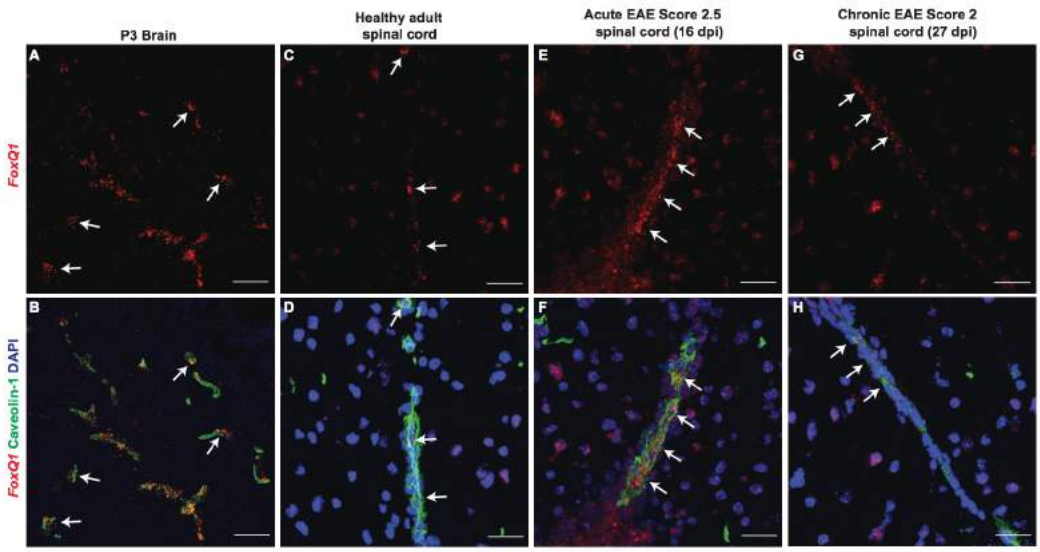

Figure 4. Expression of *Foxq1* in P3 brain, healthy adult spinal cord, and EAE spinal cord
Fluorescent RNA in situ hybridization for *Foxq1* combined with immunofluorescence for Caveolin-1 and DAPI in P3 and adult healthy mouse spinal cord and MOG35-55 EAE (score 2.5 and score 2). Single channel images for *Foxq1* (A, C, E, G) and merged images (B, D, F, H) are both shown.

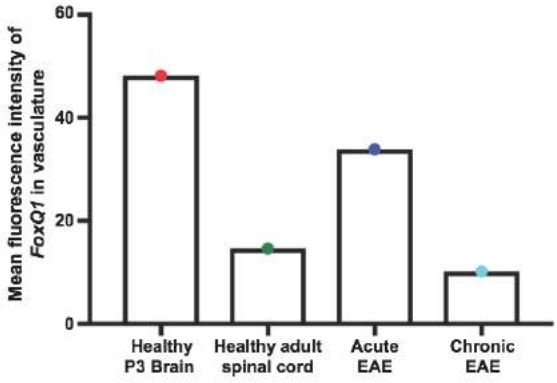

Figure 5. Quantification of *Foxq1* expression
The average mean fluorescent intensity of *Foxq1* expression in P3 and adult healthy mouse spinal cord and acute and chronic EAE. The expression levels of *Foxq1* are non-significant. The presence of *Foxq1* during EAE may play a role of Wnt partial repairing the BBB during EAE.

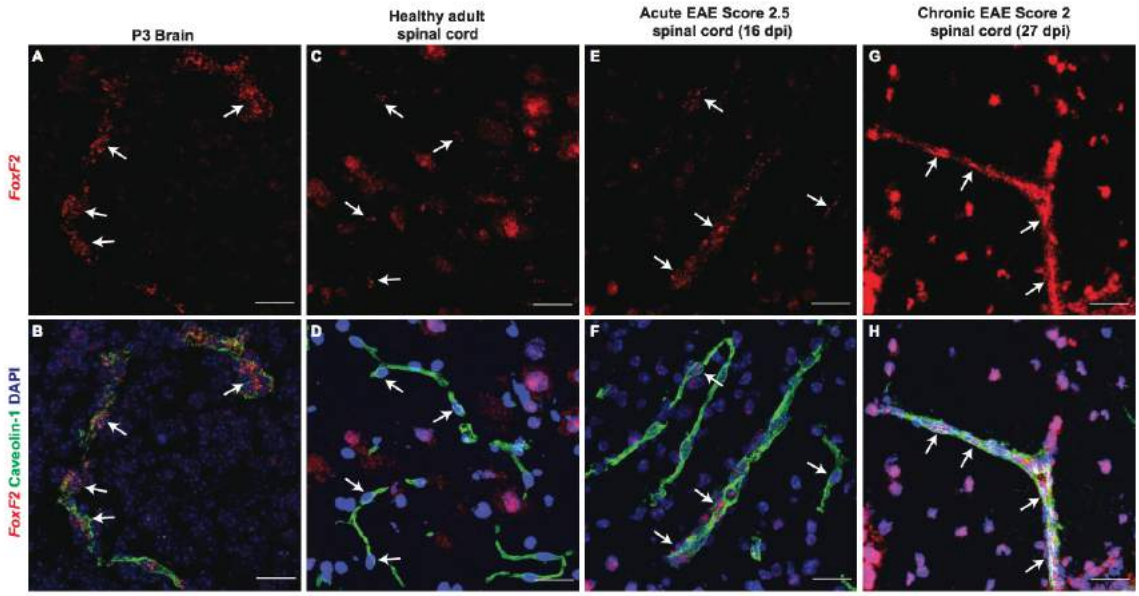

Figure 6. Expression of *Foxf2* in P3 brain, healthy adult spinal cord, and EAE spinal cord
Fluorescent RNA in situ hybridization for *Foxf2* combined with immunofluorescence for Caveolin-1 and DAPI in P3 and adult healthy mouse spinal cord and MOG35-55 EAE (score 2.5 and score 2). Single channel images for *Foxf2* (A, C, E, G) and merged images (B, D, F, H) are both shown.

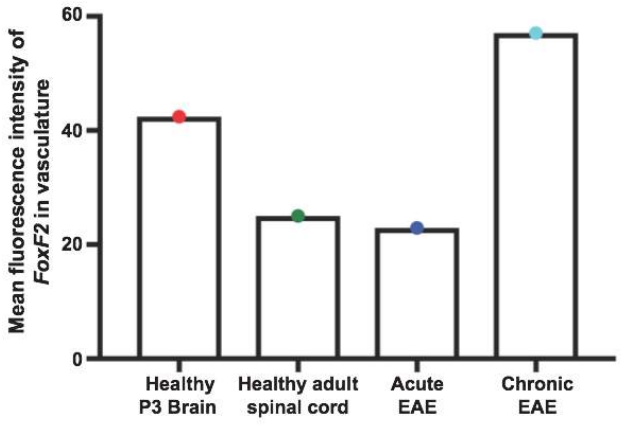

Figure 7. Quantification of *Foxf2* expression
The average mean fluorescent intensity of *Foxf2* expression in P3 and adult healthy mouse spinal cord and acute and chronic EAE. The presence of *Foxf2* during EAE may play a role of Wnt partial repairing the BBB during EAE. The observed increased expression of *Foxf2* during chronic EAE may indicate increased BBB repair explaining the lower clinical scores of chronic EAE (score 2) compared to acute EAE (score 2.5).

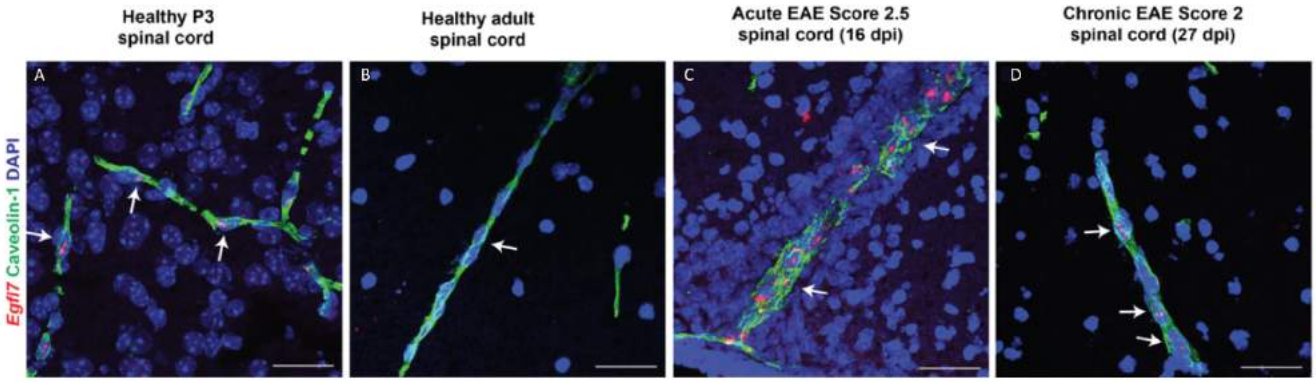

Figure 8. Expression of *Egfl7* in P3 brain, healthy adult spinal cord, and EAE spinal cord
Fluorescent RNA in situ hybridization for *Egfl7* combined with immunofluorescence for Caveolin-1 and DAPI in P3 and adult healthy mouse spinal cord and EAE (score 2.5). **(A)** *Egfl7* is expressed by many developing Caveolin-1 blood vessels at P3. **(B)** *Egfl7* expression is reduced in blood vessels of the adult spinal cord. **(C)** *Egfl7* is induced in inflamed blood vessels of white matter lesions in acute EAE which are surrounded by many cellular DAPI infiltrates. **(D)** During chronic EAE, *Egfl7* levels are reduced again.

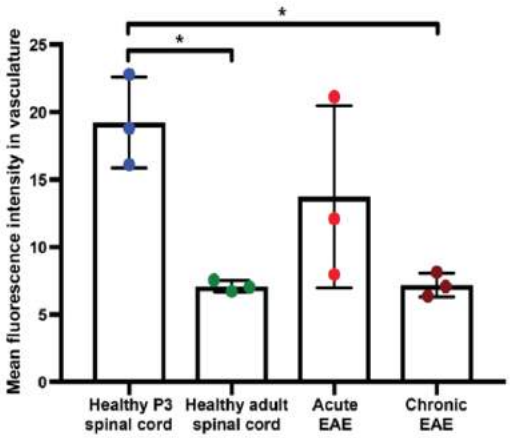

Figure 9. Quantification of *Egfl7* expression
The average mean fluorescent intensity of *Egfl7* expression in P3 and adult healthy mouse spinal cord and acute and chronic EAE. There are significant decreases in *Egfl7* expression in healthy adult mice and chronic EAE compared to P3 healthy mice (*p < .05).

Discussion

Transcription Factors and Future Directions

During healthy development, several key transcription factors (*Zic3, Foxq1, Foxf2*) are expressed in CNS endothelial cells to promote BBB maturation (Hupe et al., 2017). When a healthy BBB forms, tight junction formation enables the BBB to function properly. However,

these preliminary findings show that *Zic3*, an essential transcription factor for BBB formation, is not induced in CNS endothelial cells during EAE despite activated Wnt/β-catenin activity. The lack of *Zic3* may contribute to the failure to maintain BBB integrity.

Zic3 is a downstream target of the Wnt signaling pathway, as well as the Nodal signaling pathway (Purandare et al., 2002; Winata et al., 2013). The Nodal signaling pathway is a signal transduction pathway important in regional and cellular differentiation during embryonic development (Shen, 2007). In both of these pathways, the general role of *Zic3* is as a transcriptional regulator that acts not only alone, but in many instances in conjunction with other transcription factors (Aruga, 2018; Winata et al., 2013). When Wnt signaling is activated, it is anticipated that *Zic3*, in combination with the other two transcription factors, may promote BBB repair in EAE *in vivo*, reduce immune cell infiltration, reduce demyelination, and improve clinical outcomes (Aruga, 2018). These results from this study showed that *Foxq1* and *Foxf2* is expressed during acute EAE. These transcription factors may help in maintaining BBB integrity.. In order to preserve BBB integrity in EAE, all three transcription factors may be necessary.

The exploratory results from this study offer many future directions for this research. Since *Zic3* appears to be absent or expressed at low levels during acute EAE, it is necessary to evaluate this barrier-specific transcription factor when overexpressed prior to disease onset. It is expected that *Zic3* plays a role in forming the BBB in EAE during activated Wnt signaling. To test this, future research should utilize endothelial cell-specific viral delivery (Bourdenx, Dutheil, Bezard, & Dehay, 2014; Palmela et al., 2012). Disease outcomes, demyelination, and BBB structure and function could also be assessed. Additionally, an experiment should be conducted to quantitatively measure barrier integrity. Western blots can assay the tight junction proteins (Poritz, Harris, Kelly, & Koltun, 2011). For the four experimental groups (P3, healthy adult, acute EAE, and chronic EAE), the Wnt reporter mouse should be compared to a healthy mouse. The band densities can be calculated to determine the amount of tight junction proteins present. Then, the amount of proteins present of the Wnt reporter mouse can be compared to the control mouse in order to better understand BBB integrity during upregulated Wnt/β-catenin signaling.

Neoangiogenesis and Future Directions

Neoangiogenesis was evaluated by the expression of *Egfl7* in P3 brain, healthy adult spinal cord, acute EAE spinal cord, and chronic EAE spinal cord (n=3). It was expected that

Egfl7 would be expressed at significantly higher levels during acute EAE as compared to adult healthy spinal cord. In two of the three acute EAE mice, high *Egfl7* expression was observed. However, in one acute EAE mouse, expression was at a similar level to that in healthy adult mice. Although there was no significant increase in expression from adult healthy mice to acute EAE, the expression of *Egfl7* during acute EAE was also not significantly lower than the expression during P3, when *Egfl7* is upregulated. In conversations with the lab PI, it was determined that there is still an interest in continuing the investigation of the original hypothesis that *Egfl7* expression is higher during acute EAE compared to healthy adult mice because of the observed increase of *Egfl7* in two out of the three mice. It cannot be determined if the third mouse was an outlier due to the small sample size. These initial findings suggest that *Egfl7* expression may better our understanding of neoangiogenesis in EAE during activated Wnt/β-catenin signaling.

Although Wnt/β-catenin signaling plays a role in angiogenesis during CNS development, neoangiogenesis may further contribute to leaky blood vessel pathology in the CNS during the neuroinflammatory state in EAE (Poritz et al., 2011). Antiangiogenic drugs have been developed to stop tumors from growing their own blood vessels during cancer to slow the growth of cancer (Jain, 2005). However, there is currently no conclusive findings on the effects of antiangiogenic drugs for EAE or MS (J. Lengfeld et al., 2014). These initial findings of neoangiogenesis and the activation Wnt suggest that the delivery mechanism and target of antiangiogenic drugs may need to be reevaluated to benefit MS patients.

Future research should focus on the role of Wnt in neoangiogenesis during EAE. The results in this study revealed that *Egfl7* (a marker of neoangiogenesis) was increased during acute EAE. However, the error bar was wide for expression during acute EAE. In order to explore the accuracy of the results, additional experiments should be conducted with an increased number of mice. To understand the function of neoangiogenesis in EAE, it is essential to also assess: 1) neurological deficits, including the day of onset, score at peak disease, and neurological scoring during EAE progression; 2) immune cell infiltration; 3) demyelination; and 4) paracellular and transcellular BBB function (Girolamo et al., 2014; J. Lengfeld et al., 2014).

Limitations

This exploratory study was conducted to better understand the role of Wnt signaling in BBB repair in EAE. However, the results are inconclusive until further research is conducted.

The trends of expression of transcription factors and neoangiogenesis which were observed in this study should be further investigated. The sample size of 3 should be increased to conduct more comprehensive statistical analyses. The estimated sample sizes for animal numbers were calculated with STATA software using the following criteria: 1) type I error probability below 5%; 2) power of analysis above 80%; and 3) an effect size of 1.5. A group size of n=9 is required to conduct unpaired Student's t-tests and a sample size of n=12 is required to conduct a one-way ANOVA test.

Conclusion

Since there is no known mechanism to repair the BBB in EAE, the Wnt/β-catenin pathway was targeted in this study for its role in BBB formation and angiogenesis. The first hypothesis was that several key transcription factors for BBB formation would be absent in acute EAE mouse spinal cord. *Foxq1* expression was induced in acute EAE, and *Foxf1* expression in acute EAE was similar to expression in the healthy adult mouse. However, *Zic3* expression was low or absent in acute EAE. Although *Foxq1* and *Foxf2* expression were present during EAE, the absence of *Zic3* in blood vessels of endothelial cells during acute EAE supports the hypothesis and provides interest to further explore in future research. The second hypothesis was that neoangiogenesis would be increased in the acute EAE mouse spinal cord compared to the healthy adult mouse spinal cord. The preliminary results suggest the *Egl7* expression is increased in acute EAE mice compared to healthy adult mice. Future research with a larger sample size is required in order to further investigate these initial findings.

By assessing the role of Wnt/β-catenin signaling for both BBB integrity and neoangiogenesis, it is possible to understand how future research can utilize this information to create effective therapies to address the leaky BBB in MS patients. Ultimately, in EAE, the Wnt/β-catenin pathway may fail to maintain BBB integrity due to the lack of *Zic3* and may promote inflammation by upregulating neoangiogenesis. However, future research should focus on understanding the Wnt pathway as a mechanism to repair the BBB and prevent inflammation in MS patients. The exploratory results from this study may eventually lead to the development of therapeutics designed to reduce neoangiogenesis and repair BBB function, thereby improving long-term neurological deficits in human MS.

Bibliography

Aruga, J. (2018). Zic family proteins in emerging biomedical studies. In *Advances in Experimental Medicine and Biology*. https://doi.org/10.1007/978-981-10-7311-3_12

Begley, D. J. (2006). Structure and function of the blood-brain barrier. In *Enhancement in Drug Delivery*. https://doi.org/10.1016/j.nbd.2009.07.030

Bourdenx, M., Dutheil, N., Bezard, E., & Dehay, B. (2014). Systemic gene delivery to the central nervous system using Adeno-associated virus. *Frontiers in Molecular Neuroscience*. https://doi.org/10.3389/fnmol.2014.00050

Constantinescu, C. S., Farooqi, N., O'Brien, K., & Gran, B. (2011a). Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *British Journal of Pharmacology*. https://doi.org/10.1111/j.1476-5381.2011.01302.x

Cramer, S. P., Simonsen, H., Frederiksen, J. L., Rostrup, E., & Larsson, H. B. W. (2014). Abnormal blood-brain barrier permeability in normal appearing white matter in multiple sclerosis investigated by MRI. *NeuroImage: Clinical*. https://doi.org/10.1016/j.nicl.2013.12.001

Daneman, R., Agalliu, D., Zhou, L., Kuhnert, F., Kuo, C. J., & Barres, B. A. (2009). Wnt/β-catenin signaling is required for CNS, but not non-CNS, angiogenesis. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.0805165106

Dejana, E. (2010). The role of wnt signaling in physiological and pathological angiogenesis. *Circulation Research*, *107*(8), 943–952. https://doi.org/10.1161/CIRCRESAHA.110.223750

Dendrou, C. A., Fugger, L., & Friese, M. A. (2015). Immunopathology of MS. *Nature Reviews Immunology*. https://doi.org/10.1038/nri3871

Dobbing, J. (1961). The Blood-Brain Barrier. *Developmental Medicine & Child Neurology*. https://doi.org/10.1111/j.1469-8749.1961.tb15323.x

Fitch, M. J., Campagnolo, L., Kuhnert, F., & Stuhlmann, H. (2004). Egfl7, a novel epidermal growth factor-domain gene expressed in endothelial cells. *Developmental Dynamics*. https://doi.org/10.1002/dvdy.20063

Floris, S., Blezer, E. L. A., Schreibelt, G., Döpp, E., Van Der Pol, S. M. A., Schadee-Eestermans, I. L., … De Vries, H. E. (2004). Blood-brain barrier permeability and monocyte infiltration in experimental allergic encephalomyelitis: A quantitative MRI study. *Brain*, *127*(3), 616–627. https://doi.org/10.1093/brain/awh068

Frischer, J. M., Bramow, S., Dal-Bianco, A., Lucchinetti, C. F., Rauschka, H., Schmidbauer, M., … Lassmann, H. (2009). The relation between inflammation and neurodegeneration in multiple sclerosis brains. *Brain*. https://doi.org/10.1093/brain/awp070

Girolamo, F., Coppola, C., Ribatti, D., & Trojano, M. (2014). Angiogenesis in multiple sclerosis and

experimental autoimmune encephalomyelitis. *Acta Neuropathologica Communications*. https://doi.org/10.1186/s40478-014-0084-z

Glass, C. K., Saijo, K., Winner, B., Marchetto, M. C., & Gage, F. H. (2010). Mechanisms Underlying Inflammation in Neurodegeneration. *Cell*. https://doi.org/10.1016/j.cell.2010.02.016

Hupe, M., Li, M. X., Kneitz, S., Davydova, D., Yokota, C., Kele-Olovsson, J., … Gessler, M. (2017). Gene expression profiles of brain endothelial cells during embryonic development at bulk and single-cell levels. *Science Signaling*, *10*(487), 1–13. https://doi.org/10.1126/scisignal.aag2476

Jain, R. K. (2005). Normalization of tumor vasculature: An emerging concept in antiangiogenic therapy. *Science*. https://doi.org/10.1126/science.1104819

Kermode, A. G., Thompson, A. J., Tofts, P., Macmanus, D. G., Kendall, B. E., Kingsley, D. P. E., … Mcdonald, W. I. (1990). Breakdown of the blood-brain barrier precedes symptoms and other mri signs of new lesions in multiple sclerosis: Pathogenetic and clinical implications. *Brain*, *113*(5), 1477–1489. https://doi.org/10.1093/brain/113.5.1477

Lengfeld, J., Cutforth, T., & Agalliu, D. (2014). The role of angiogenesis in the pathology of multiple sclerosis. *Vascular Cell*. https://doi.org/10.1186/s13221-014-0023-6

Lengfeld, J. E., Lutz, S. E., Smith, J. R., Diaconu, C., Scott, C., Kofman, S. B., … Agalliu, D. (2017). Endothelial Wnt/β-catenin signaling reduces immune cell infiltration in multiple sclerosis. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.1609905114

Liebner, S., Corada, M., Bangsow, T., Babbage, J., Taddei, A., Czupalla, C. J., … Dejana, E. (2008). Wnt/β-catenin signaling controls development of the blood - brain barrier. *Journal of Cell Biology*, *183*(3), 409–417. https://doi.org/10.1083/jcb.200806024

Lubetzki, C., & Stankoff, B. (2014). Demyelination in multiple sclerosis. In *Handbook of Clinical Neurology*. https://doi.org/10.1016/B978-0-444-52001-2.00004-2

Lund, H., Krakauer, M., Skimminge, A., Sellebjerg, F., Garde, E., Siebner, H. R., … Hanson, L. G. (2013). Blood-Brain Barrier Permeability of Normal Appearing White Matter in Relapsing-Remitting Multiple Sclerosis. *PLoS ONE*, *8*(2). https://doi.org/10.1371/journal.pone.0056375

Lutz, S. E., Smith, J. R., Kim, D. H., Olson, C. V. L., Ellefsen, K., Bates, J. M., … Agalliu, D. (2017). Caveolin1 Is Required for Th1 Cell Infiltration, but Not Tight Junction Remodeling, at the Blood-Brain Barrier in Autoimmune Neuroinflammation. *Cell Reports*, *21*(8), 2104–2117. https://doi.org/10.1016/j.celrep.2017.10.094

Mazzoni, J., Smith, J. R., Shahriar, S., Cutforth, T., Ceja, B., & Agalliu, D. (2017). The Wnt Inhibitor Apcdd1 Coordinates Vascular Remodeling and Barrier Maturation of Retinal Blood Vessels. *Neuron*. https://doi.org/10.1016/j.neuron.2017.10.025

Miller, S. D., Karpus, W. J., & Davidson, T. S. (2010). Experimental autoimmune encephalomyelitis in the mouse. *Current Protocols in Immunology*. https://doi.org/10.1002/0471142735.im1501s77

Minagar, A., & Alexander, J. S. (2003). Blood-brain barrier disruption in multiple sclerosis. *Multiple Sclerosis*. https://doi.org/10.1191/1352458503ms965oa

Moore, M. A. S. (2002). Putting the neo into neoangiogenesis. *Journal of Clinical Investigation*. https://doi.org/10.1172/JCI0214940

Nag, S. (2003). Morphology and molecular properties of cellular components of normal cerebral vessels. *Methods in Molecular Medicine*, *89*, 3–36. https://doi.org/10.1385/1-59259-419-0:3

Nichol, D., & Stuhlmann, H. (2012). EGFL7: A unique angiogenic signaling factor in vascular development and disease. *Blood*. https://doi.org/10.1182/blood-2011-10-322446

Palmela, I., Brites, D., & Brito, M. A. (2012). Blood -brain barrier in health and disease. In *The Blood-Brain Barrier: New Research*. https://doi.org/10.1002/ana.23648

Poritz, L. S., Harris, L. R., Kelly, A. A., & Koltun, W. A. (2011). Increase in the tight junction protein claudin-1 in intestinal inflammation. *Digestive Diseases and Sciences*. https://doi.org/10.1007/s10620-011-1688-9

Purandare, S. M., Ware, S. M., Kwan, K. M., Gebbia, M., Bassi, M. T., Deng, J. M., … Casey, B. (2002). A complex syndrome of left-right axis, central nervous system and axial skeleton defects in Zic3 mutant mice. *Development*.

Schmidt, M. H. H., Bicker, F., Nikolic, I., Meister, J., Babuke, T., Picuric, S., … Dikic, I. (2009). Epidermal growth factor-like domain 7 (EGFL7) modulates Notch signalling and affects neural stem cell renewal. *Nature Cell Biology*. https://doi.org/10.1038/ncb1896

Schmidt, M., Paes, K., De Mazière, A., Smyczek, T., Yang, S., Gray, A., … Ye, W. (2007). EGFL7 regulates the collective migration of endothelial cells by restricting their spatial distribution. *Development*. https://doi.org/10.1242/dev.002576

Shen, M. M. (2007). Nodal signaling: Development roles and regulation. *Development*. https://doi.org/10.1242/dev.000166

Shi, J., Chi, S., Xue, J., Yang, J., Li, F., & Liu, X. (2016). Emerging Role and Therapeutic Implication of Wnt Signaling Pathways in Autoimmune Diseases. *Journal of Immunology Research*. https://doi.org/10.1155/2016/9392132

Shimomura, Y., Agalliu, D., Vonica, A., Luria, V., Wajid, M., Baumer, A., … Christiano, A. M. (2010). APCDD1 is a novel Wnt inhibitor mutated in hereditary hypotrichosis simplex. *Nature*. https://doi.org/10.1038/nature08875

Sospedra, M., & Martin, R. (2016). Immunology of Multiple Sclerosis. *Seminars in Neurology*. https://doi.org/10.1055/s-0036-1579739

Stenman, J. M., Rajagopal, J., Carroll, T. J., Ishibashi, M., McMahon, J., & McMahon, A. P. (2008). Canonical Wnt signaling regulates organ-specific assembly and differentiation of CNS vasculature. *Science*. https://doi.org/10.1126/science.1164594

Suryawanshi, A., Manoharan, I., Hong, Y., Swafford, D., Majumdar, T., Taketo, M. M., … Manicassamy, S. (2015). Canonical Wnt signaling in dendritic cells regulates Th1/Th17 responses and suppresses autoimmune neuroinflammation HHS Public Access canonical wnt signaling in DCs limits effector T cell responses and represents a potential therapeutic approach to contro. *J Immunol. J Immunol*, *194*(7), 706–721. https://doi.org/10.4049/jimmunol.1402691

Vos, C. M. P., Geurts, J. J. G., Montagne, L., Van Haastert, E. S., Bö, L., Van Der Valk, P., … De Vries, H. E. (2005). Blood-brain barrier alterations in both focal and diffuse abnormalities on postmortem MRI in multiple sclerosis. *Neurobiology of Disease*, *20*(3), 953–960. https://doi.org/10.1016/j.nbd.2005.06.012

Weiss, N., Miller, F., Cazaubon, S., & Couraud, P. O. (2009). The blood-brain barrier in brain homeostasis and neurological diseases. *Biochimica et Biophysica Acta - Biomembranes*. https://doi.org/10.1016/j.bbamem.2008.10.022

Weksler, B. B., Subileau, E. A., Perrière, N., Charneau, P., Holloway, K., Leveque, M., … Couraud, P. O. (2005). Blood-brain barrier-specific properties of a human adult brain endothelial cell line. *FASEB Journal*. https://doi.org/10.1096/fj.04-3458fje

Winata, C. L., Kondrychyn, I., Kumar, V., Srinivasan, K. G., Orlov, Y., Ravishankar, A., … Mathavan, S. (2013). Genome Wide Analysis Reveals Zic3 Interaction with Distal Regulatory Elements of Stage Specific Developmental Genes in Zebrafish. *PLoS Genetics*. https://doi.org/10.1371/journal.pgen.1003852

Wu, G. F., & Alvarez, E. (2011). The Immunopathophysiology of Multiple Sclerosis. *Neurologic Clinics*. https://doi.org/10.1016/j.ncl.2010.12.009

Zhao, Z., Nelson, A. R., Betsholtz, C., & Zlokovic, B. V. (2015). Establishment and Dysfunction of the Blood-Brain Barrier. *Cell*. https://doi.org/10.1016/j.cell.2015.10.067

Zhou, Y., Wang, Y., Tischfield, M., Williams, J., Smallwood, P. M., Rattner, A., … Nathans, J. (2014). Canonical WNT signaling components in vascular development and barrier formation. *Journal of Clinical Investigation*, *124*(9), 3825–3846. https://doi.org/10.1172/JCI76431