OF MANITOBA | Rady Faculty of Health Sciences

Advanced Degrees
Education in Medicine Max
Rady College
of Medicine
Rady Faculty of
Health Sciences
University of Manitoba

# Bachelor of Science in Medicine Degree Program
# End of Term Final Report

**Student Name:** Graham A McLeod **Date:** 08/05/18

**Project Title:** Development of new therapies for promoting myelin repair in multiple sclerosis

**Primary Supervisor Name:** Dr Soheila Karimi
**Department:** Physiology and Pathophysiology

**Co-Supervisor Name:** Dr Eftekhar Eftekharpour
**Department:** Physiology and Pathophysiology

**Summary (250 words max single spaced):**

Multiple Sclerosis (MS) is a progressive autoimmune disease of the central nervous system (CNS) characterized by immune destruction of myelin sheath (demyelination) and resultant functional decline. Spontaneous remyelination afford periods of remission during early stages of MS; however, recurrent autoimmune demyelination causes death of oligodendrocytes (CNS myelin-producing cells) and permanent degeneration of axons (nerve fibres), resulting in irreparable damage to the CNS circuitry. Current therapies for MS are mostly immunomodulatory in nature, attenuating relapses and decreasing the rate of functional decline; but these therapies do not address the problems of progressive demyelination and cell loss. Thus there is an urgent need for therapies that promote endogenous repair and remyelination.

The healthy CNS is able to endogenously renew oligodendrocytes (oligodendrogenesis) from resident populations of precursor cells and thus is able to maintain myelin, but this capacity is diminished in MS. The Karimi lab has made the original observation that downregulation of Factor X signaling in demyelinating lesions seems to be an underlying cause of the limited oligodendrogenesis and remyelination. Factor X is a neuronally-derived growth factor that contributes to differentiation, survival, maturation, and myelinating function of oligodendrocytes. We sought to investigate the impact of Factor X therapy in a clinically relevant mouse model of MS, experimental autoimmune encephalomyelitis (EAE). Here, we demonstrate that bioavailability of Factor X in EAE lesions promotes remyelination and axonal integrity. Our findings suggest the potential of Factor X as a regenerative therapy in MS.

Student Signature
Graham McLeod Digitally signed by Graham McLeod
Date: 2018.08.05 17:00:33 -05'00'

Primary Supervisor Signature
Soheila Karimi Digitally signed by Soheila Karimi
DN: cn=Soheila Karimi, o=University of Manitoba, ou, email=Soheila.Karimi@umanitoba.ca, c=CA
Date: 2018.08.05 17:57:04 -05'00'

**Acknowledgments**: I gratefully acknowledge the sole or partial funding support from the following sponsors;

H.T. Thorlakson Foundation
Dean, College of Medicine
Research Manitoba

Manitoba Medical Service Foundation (MMSF)
Vice-Dean, Research Rady FHS
Health Sciences Centre Research Foundation
Heart and Stroke Foundation

Sponsorship if different than above;

MD/PHD MD/MSc. BSc. (MED) MED II Summer Research Program
Joe Doupe Annual Event Undergraduate Medical Student Research Symposium

**Introduction & Background:** Multiple sclerosis (**MS**) is a progressive autoimmune disease of the central nervous system (**CNS**) characterized by immune destruction of myelin sheath (**demyelination**) and resultant functional decline[1]. MS afflicts an estimated 100,000 Canadians and is the most common non-traumatic cause of neurologic disability in young adults[2,3]. Spontaneous remyelination affords periods of remission during early stages of MS; however, recurrent autoimmune demyelination causes death of **oligodendrocytes** (CNS myelin-producing cells) and permanent degeneration of **axons** (nerve fibres), resulting in irreparable damage to the CNS circuitry. Current therapies for MS are mostly immunomodulatory in nature, attenuating relapses and decreasing the rate of functional decline; but these therapies do not address the problems of progressive demyelination and cell loss. Therapies that promote endogenous repair and remyelination are urgently needed.

The healthy CNS is able to endogenously renew oligodendrocytes (**oligodendrogenesis**) from resident populations of precursor cells and thus is able to maintain myelin[4–6]. However, this capacity is diminished in MS, as recruitment of precursor cells to the lesion is insufficient and the survival and maturation of newly-formed oligodendrocytes into myelinating cells is restricted particularly in chronic lesions[5,7–10]. It is noteworthy that replenishing the lost oligodendrocyte population is crucial in order to regenerate myelin sheaths and restore oligodendrocyte-axon support systems[4].

The Karimi lab has made the original observation that downregulation of **Factor X (which we have been advised not to disclose due to patenting potential)** in demyelinating lesions seems to be an underlying cause of the limited oligodendrogenesis and suboptimal remyelination in demyelinating conditions of the spinal cord including spinal cord injury (SCI) and focal demyelinating conditions. Factor X is a neuronally-derived growth factor vital to CNS development, where it contributes to the differentiation, survival, maturation, and myelinating function of oligodendrocytes[11]. Factor X is expressed primarily by axons, neurons, and oligodendrocytes; a disruption in Factor X signaling results in the loss of oligodendrocytes in the spinal cord during the development[11]. Work in the Karimi lab using a focal demyelinating model of the spinal cord has shown that Factor X replacement promotes the generation and maturation of new oligodendrocytes and accelerates and enhances endogenous remyelination[12]. Building on these new findings, we are investigating the impact of Factor X therapy in a clinically relevant mouse model of MS, experimental autoimmune encephalomyelitis (**EAE**).

In the EAE mouse model, T-cell-mediated autoimmunity is induced to myelin immunogens, resulting in demyelinating lesions in the CNS that are pathologically similar to MS[13]. Importantly, work prior to my arrival in the lab confirmed that Factor X is dysregulated in active MS lesions in humans. My BSc Med project is part of a larger effort, funded by the MS Society of Canada, to uncover the role of Factor X in oligodendrogenesis and remyelination in the EAE model mimicking MS pathology. We hypothesize that Factor X will help foster a pro-regenerative environment which will promote oligodendrogenesis, enhance remyelination, and improve axonal preservation in our EAE model of MS pathology.

**Methods:**

**EAE mouse model and Factor X delivery:** EAE was induced in adult female C57BL/6 mice 8-10 weeks of age. We used female mice because the incidence of MS is known to be two to three times higher in females.[14] At the peak of the disease (day 14-16) mice were randomly assigned to receive daily systemic subcutaneous (SQ) injections of either Factor X or vehicle (saline plus 0.1% of bovine serum albumin) for four weeks. Systemic delivery was used as it is clinically feasible and because Factor X is shown to readily cross the blood-brain barrier.[15] To assess

clinical progression, mice were examined each day and scored on a 15-point EAE clinical scale. **Note: I did not participate in these procedures.**

**Tissue processing**: At 4 weeks following treatment, animals were euthanized through transcardial perfusion with ice-cold phosphate buffer (0.1M) followed by 2% paraformaldehyde (PFA) and 2% glutaraldehyde in phosphate buffer for tissue fixation. Then spinal cord blocks were dehydrated in a graded series of alcohol and embedded in Epon Resin (Electron Microscopy Sciences). In addition to EAE/vehicle and EAE Factor X treated groups, semi-thin section analyses (described below) also included an age-matched *naïve non-EAE* group.

For the animals destined for immunohistochemical (IHC) analysis, we delivered SQ 5-bromo-2-deoxyuridine (BrdU) during the treatment period to label newly generated cells for detection at a later point. We employed a two-week treatment period for these animals, after which time they were euthanized. Tissue was fixed by transcardial perfusion with cold phosphate-buffered saline (PBS) (0.1M) followed by 3% paraformaldehyde (PFA) in 0.1M PBS and subsequently excised and embedded in optimal cutting temperature (OCT) material to be stored at -80°C. **Note: I did not participate in these procedures; all steps of tissue processing were conducted by other lab members.**

**Myelin g-ratio and axon density in semithin section:** Semi-thin spinal cord cross sections (1 μm thickness) were obtained from EAE, EAE Factor X, and naive animal groups. Following staining with 0.5% toluidine blue, sections were assessed for myelin thickness and axon density. Myelin thickness was assessed by analyzed the **g-ratios**, which is the ratio of inner to outer diameter of the myelin sheath for a circular axon in cross-section. Axon density was assessed by counting the number of axons in images of known size (61 x 61 μm) selected from the lesion and perilesional area.

For these analyses we obtained semi-thin sections from the thoracic spinal cord. Three separate sections that included EAE lesions were analyzed. We collected digital images of the demyelinated lesions using non-overlapping, consecutive microscope fields within and immediately surrounding the lesion area. Lesions typically begin at the spinal cord tissue perimeter, where EAE lesions are formed after the infiltration of peripheral leukocytes , and extend inwards into the tissue. Naïve (non-EAE control) tissue images were obtained from similar regions. A sampling array was used to select random axons for analysis (figure 1D). Using ImageJ software (version 1.52a, https://imagej.nih.gov), 5 equidistant vertical lines were laid atop the image (using the "grid" plugin), and only those axons which crossed the vertical gridlines were measured for g-ratio. For each animal, a minimum 900 axons were analyzed. For axon count, a larger sample was desirable, so we employed 10 equidistant vertical gridlines in the sampling procedure. All the analysis was performed in a blinded manner. Results of g-ratio and axon count were subject to student t-test and one-way ANOVA respectively, followed by *post hoc* pairwise comparison of groups, with results of $p < 0.05$ considered significant. g-ratio frequency distribution was subject to $\chi^2$ test. g-ratio as a function of axon diameter was subject to Mann-Whitney *U* test, with results of $p < 0.05$ considered significant. **Note: I performed these analyses and produced data.**

**Immunohistochemical and imaging procedures:** I also learnt to perform immunohisto-chemistry and imaging on spinal cord tissue section. Serial spinal cord cross sections (14 μm thickness) were cut with a cryotome. EAE lesion sites were located by staining with Luxol Fast Blue (LFB) and hematoxylin and eosin (H&E) stains. Three different sections from the lesion sites (at least 1mm apart) were then subjected to immunohistochemistry (IHC). We employed two sets of staining combinations, one to identify myelination and the other to identify new

oligodendrocytes (figure 3). For myelination immunostaining we probed for Factor X, myelin sheath, and neurofilament-200 (NF-200; a marker of axons). In the oligodendrogenesis assessments we probed for BrdU (identifying proliferating cells), Olig2 (marker of oligodendrocytes), and GFAP (marker of astrocytes). See table 1 for detailed information about the antibodies used.

IHC was performed according to well-established protocols in the Karimi lab. Tissues were briefly blocked for 1 hour at room temperature then incubated overnight at 4°C in the appropriate primary antibody solution (see table 1 for details of the blocking solution and a list of antibodies used in first term). On the next day the tissues were washed thrice with PBS and incubated with the secondary antibody for one hour at room temperature. This protocol was repeated as appropriate for double and triple labelling. Following this, nuclear marker 4',6-diamidino-2-phenylindole (DAPI) was applied for 15 minutes at room temperature, after which the slides were washed thrice with PBS and promptly cover-slipped with Mowiol mounting medium. For BrdU immunolabeling the slides were washed with PBS, incubated in 2 M HCl and 0.5% Triton X-100 for 30 min at 37°C, and washed with 0.1 M sodium borate in PBS for 10 min. Mouse anti-BrdU was applied overnight and followed by incubation with secondary antibody. Immunostained tissues were then imaged with fluorescent microscopy. Some representative images of my IHC work is provided in Figure 3.

**Results:**

**Factor X treatment promotes remyelination and axon preservation in EAE lesions:** At 4 weeks following the peak of EAE, lesions were evident as demyelinated regions with reduced axonal density (figure 2A). Our analysis of EAE lesions confirmed the presence of demyelinated/thinly myelinated axons (higher g-ratios) and axonal loss (assessed by axonal density analysis). Analysis of naïve non-EAE tissue revealed a g-ratio of 0.674, which was increased to 0.699 ± 0.006 in EAE group as a result of demyelination. Factor X treatment decreased g-ratio to 0.664 ± 0.009, indicating an increase in myelin thickness (data represent mean ± SEM, *P = 0.018, student t test, *N* = 4/group, figure 2B). Furthermore, Factor X treatment decreased the proportion of demyelinated/thinly myelinated axons (g-ratio $\geq 0.80$) from 22.3% to 13.7% and increased the proportion of heavily myelinated axons (g ratio < $0.60$) from 21.4% to 29.4%. (figure 2C). It is important to note that the demyelinated axons have a higher g-ratio, and therefore a lower g-ratio represents thicker myelin sheath. Additionally, analysis of individual g-ratios found a significant difference between EAE and EAE Factor X treated groups (Mann-Whitney U test, $p < 0.001$, figure 2D). These data indicate that Factor X treatment promotes remyelination in EAE lesions.

For axon preservation, the number of axons in digital microscope images of a standard size (61 x 61 μm) was quantified. Naive non-EAE tissue was found to have an axonal density of 382 ± 4 axons per $10^4$ $\mu m^2$, which was reduced to 198 ± 12 in EAE group (4-week timepoint) as a result of axonal loss. Factor X treatment significantly increased axonal density in EAE lesions to 277 ± 11 axons per $10^4$ $\mu m^2$, which was 39.9% higher than the number of spared axons in the vehicle treated EAE group (data represent mean ± SEM, *P < 0.001, one-way ANOVA, *N* = 4/group, figure 2E), indicating an important beneficial effect on axonal preservation.

**Oligodendrogenesis and myelination experiments:** The oligodendrogenesis quantification and myelination immunodensity experiments which I began in my first term of BSc Med have been entrusted to new students in the lab, allowing me to concentrate on the myelin thickness and axon preservation analyses in my second term. By performing these experiments in my first term I learned tissue sectioning, histological techniques, immunostaining, and fluorescent imaging.

**Discussion:**

In my BSc Med project, we demonstrate that systemic Factor X treatment facilitates remyelination and axonal integrity in EAE demyelinating lesions. It is known that axonal loss and neurologic disability are correlated in MS[16]; preventing axon loss is therefore highly relevant to improved clinical outcomes. Importantly, parallel neurological assessment by our group has shown the promising effects of Factor X in improving clinical recovery from EAE. Therefore, we propose that these treatment effects are partly due to enhanced remyelination and axonal protection promoted by increased bioavailability of Factor X in EAE lesions. Currently, several concurrent experiments are underway to characterize the effects of Factor X in EAE mice.

Myelin plays a critical role in CNS function, and its loss in demyelinating conditions such as MS or neurotrauma results in permanent neurological impairment. Therefore, renewal of myelin (i.e. re-myelination) is important for functional recovery. CNS myelin is composed of concentric circles of oligodendrocyte cell membrane wrapped around an axon, with segments of myelin laid end-to-end along the axon's length. Myelin confers a precise molecular organization to the axon, ordering it into discrete, repeating modules, with each module housing a unique population of ion channels that are precisely organized by the myelin[17–28]. This repeating modular arrangement enables **saltatory conduction**, which facilitates fast signal transduction within the brain and spinal cord while also requiring far less energy than traditional signal conduction[29]. Demyelination disrupts the networks which hold the ion channels in place, causing their abnormal distribution along the axonal membrane, which abolishes the possibility of saltatory conduction[30–32]. This downgrades the conduction speed of the axon while simultaneously increasing the metabolic requirements. Additionally, certain channel types are upregulated following demyelination, further increasing the metabolic needs of the axon[30]. Thus, for proper axon function, remyelination is necessary.

Our data show that Factor X treatment facilitates remyelination in EAE lesions over a 4 week treatment period. Like the MS lesions they mimic, EAE lesions suffer from a reduced capacity for replacement of oligodendrocytes and thus fail to adequately replace lost myelin. This diminished endogenous activity to regenerate myelin is owed to a paucity of pro-regenerative factors and an abundance of inhibitory factors. We hypothesized that replenishing Factor X would help promote oligodendrogenesis. Indeed, at the 4-week time point, the EAE group treated with Factor X was significantly remyelinated compared to the EAE group that received vehicle treatment. However, the next step is to add an intermediate 2-week timepoint to our analysis, as it is plausible that Factor X may have accelerated the process of remyelination at earlier stage that has been missed in our assessment at the 4-week timepoint. Since some degree of spontaneous remyelination happens in EAE lesions, an earlier timepoint would allow us to determine whether Factor X can expedite remyelination.

While our data show that remyelination has occurred in EAE group treated with Factor X, it remains to be investigated whether this new myelin is of physiological quality. In the early stages of MS there is a variable degree of spontaneous remyelination, which produces the remission periods that characterize the relapsing-remitting course[33]. However, the remyelinating capacity declines over time in advanced stages of disease due to progressive cell death, and patients often enter a secondary progressive clinical course[34,35]. Although spontaneous remyelination is usually sufficient to improve some function and confer clinical benefit, the new myelin segments are often shorter and thinner, the axonal ion channels may be disorganized, and the axons remain susceptible to metabolic dysfunction[36]. Clinically, inadequately myelinated axons can lead to *Uhtoff's phenomenon*: temporary neurological deficit co-occurring with heightened body temperature, such as when the patient is exercising, bathing, or febrile, or when the ambient temperature is high, etc.[37–39] While the exact pathophysiology of this phenomenon remains to be

characterized, it relates to high temperatures causing stress on neural circuits relying on substandard myelin structures[38,39]. Uhtoff's phenomenon might be mistaken for a relapse; however, symptoms resolve as body temperature lowers, and so the criteria for relapse are not met. (Criteria for relapse are that symptoms must last at least 24 hours and not be coincident with a fever or intercurrent illness[37]).

In light of the inadequacies of spontaneous remyelination, it is worthwhile to assess the precise structure of the newly-developed myelin which is facilitated by our Factor X treatment. These important investigations are already underway in experiments being conducted by other members of the Karimi lab. Notably, IHC analyses will assess the molecular organization of crucial ion channels in Naïve, EAE, and EAE Factor X groups. Given Factor X's important role in developmental myelination, we hypothesize that the newly-generated myelin will be similar to normal, physiologic myelin.

Beyond remyelination, our data further show that Factor X treatment has an important beneficial effect on axonal preservation in EAE, leading to a significantly higher (39.9%) axonal density at the 4-week timepoint. There are likely other mechanisms through which this axonal preservation is mediated. As mentioned above, demyelination increases the metabolic needs of an axon. Perversely, demyelination also diminishes an axon's capacity to meet those needs, and metabolic catastrophe is a major basis of axonal loss in MS[28]. Since myelinated axons are ensheathed by myelin for most of their length and therefore have limited access to the extracellular environment, they depend on glial cells to provide them with nutrients and metabolic substrates[40]. Recent discovery of the **axo-myelinic synapse** reveals that oligodendrocytes have an especially intimate role in this provision[41]. The axo-myelinic synapse refers to the following arrangement: oligodendrocytes express AMPA and NMDA receptors on the inner tongue of the myelin sheath, and in times of high energetic demand, axons release glutamate into the periaxonal space to stimulate these receptors[41]. Stimulation of this receptor causes the oligodendrocyte to upregulate its expression of the GLUT1 transporter, increasing the uptake of glucose[42]. Once inside the oligodendrocyte, this glucose is metabolized into lactate, which is subsequently released to the axon through monocarboxylate transporter 1 (MCT1)[40,43]. The importance of this energy-provision system is evidenced by experiments showing that MCT1 disruption is lethal to axons[43]. Autoimmune demyelination disrupts the axo-myelinic synapse, dysregulating the metabolic coupling of the axon and its oligodendrocyte. Beyond this, progressive oligodendrocyte loss in MS guarantees that the MCT1 system will inevitably be disrupted, worsening the metabolic predicament of the demyelinated axons.

The impairment of axonal metabolism in demyelination goes further still. The distal parts of an axon are supplied with nutrients, macromolecules, and mitochondria via an anterograde transport process which has been shown to rely on oligodendrocytes and myelin[44–46]; demyelinating models disrupt the transport, causing further metabolic impairment[47,48]. Retrograde transport, which functions to clear aggregated cellular materials and transport distant signalling molecules to the soma, is also impaired in demyelination[48]. Interestingly, there is emerging evidence for oligodendrocyte-to-axon transfer of ribosomes[49], and *in vitro* evidence indicates that supportive factors (of unknown identity) are passed from oligodendrocyte to axon via exosomes[50]. Clearly, oligodendrocytes perform many important tasks to support axons, but these tasks are discontinued in demyelinating pathology, contributing to axon loss in MS.

Beyond saltatory conduction, nutrient supply, axonal transport facilitation, and other provisions, there are additional ways in which oligodendrocytes support their myelinated axons that are less well-understood. To this effect, there are many experiments showing that strategic deletion of certain myelin components leads to axonal degeneration. Mice lacking *Cnp1*, an

oligodendrocyte gene which encodes 2',3'-cyclic nucleotide phosphodiesterase (CNP), produce myelin that is structurally normal, yet axons swell and degenerate[51]. Mice lacking *Plp1*, an oligodendrocyte gene which encodes proteolipid protein (PLP/DM20), assemble normal myelin but with abnormal periodicity; over time these axons swell and degenerate[52,53]. Myelin-associated glycoprotein (MAG), which is essential for the initiation of myelination[54], is also known to be essential for axon survival and integrity[55]. Demyelination reneges an axon's access to these essential elements, and this is another way that demyelination can lead to axonal death. Our data show that Factor X treatment promotes axonal preservation in EAE. Further study is needed to characterize the precise mechanisms by which axon survival is facilitated.

We hypothesize that one plausible mechanism by which Factor X promotes remyelination and axonal preservation is the generation of new oligodendrocytes which mature into the myelinating phenotype. In adulthood, new oligodendrocytes can arise from oligodendrocyte progenitor cells (**OPCs**) and neural progenitor cells (**NPCs**). OPCs are found throughout the CNS, self-renewing to maintain the oligodendrocyte lineage[56]. NPCs reside in the subventricular zone of the brain and the central canal of the spinal cord, and tend to be recruited for oligodendrogenesis primarily after injury to the brain and spinal cord[57]. Following white matter injury or demyelination, nearby OPCs proliferate and migrate into the lesion[56]. Upon arriving in the lesion, OPCs restore myelin by first differentiating into oligodendrocytes, then maturing into the myelinating phenotype. Each step in this process is subject to positive and negative regulation by numerous environmental factors (see Alizadeh 2016 for excellent review[58]). In MS, auto-immune mediated damage of oligodendrocytes and myelin is the major basis of this pathology[59]. In MS, oligodendrogenesis is limited due to insufficient levels of pro-regenerative factors and an abundance of inhibitory signals. Factor X is a pro-regenerative factor known to enhance numerous steps in the oligodendrogenesis process, including OPC survival, OPC differentiation into oligodendrocytes, oligodendrocyte survival, oligodendrocyte maturation, and achievement of myelinating phenotype[60–62]. Previous studies in our lab have shown the promise of Factor X in promoting oligodendrocyte replacement in focal demyelination models and spinal cord injury (SCI). Therefore, we hypothesize that Factor X treatment will enhance oligodendrogenesis in EAE lesions.

Our prior work in focal demyelinating models and SCI have also shown a beneficial immunomodulatory role for Factor X in these pathologies[12,63,64]. Notably, our lab has shown that Factor X promotes a pro-regenerative immune response by attenuating pro-inflammatory cytokine expression, enhancing regulatory cytokine expression, and promoting a regulatory immune cell phenotype[12,63,64]. Parallel studies in our lab address the hypothesis that Factor X will promote a pro-regenerative response in EAE pathology as well. If this is indeed the case, this would likely contribute to the axonal preservation effects we have observed, as it is known that in MS the degree of axon loss correlates with the degree of inflammation[65]. Our previous works have also shown that Factor X attenuates upregulation of chondroitin sulfate proteoglycans (CPSGs)[12,63,64], which are well-known to inhibit multiple steps in the oligodendrogenesis process[66–68]. Investigations are in progress to illuminate whether Factor X may have similar beneficial effects in EAE.

## Conclusion

MS is a debilitating progressive autoimmune condition for which disease-reversing therapy is needed. The most promising route to disease reversal is through regenerative therapies which will promote oligodendrogenesis, remyelination, and axonal preservation. We show that Factor X promotes remyelination and axonal preservation and may promote oligodendrogenesis in the animal model of MS, EAE, presenting promising evidence for Factor X as a novel regenerative therapy in MS. Future directions include 1) adding a 2-week timepoint to our remyelination and

axon preservation analyses, 2) assessing the molecular structure and physiological properties of the newly-generated myelin, and 3) determining the immunomodulatory role of Factor X in EAE pathology.

## References


1. Garg N, Smith TW. An update on immunopathogenesis, diagnosis, and treatment of multiple sclerosis. *Brain Behav*. 2015;5(9):n/a-n/a. doi:10.1002/brb3.362.
2. Statistics Canada. Health Statistics Division. *Health Reports*. Statistics Canada; 1996. https://www.statcan.gc.ca/pub/82-003-x/2018001/article/54902-eng.htm. Accessed April 29, 2018.
3. Dutta R, Trapp BD. Mechanisms of neuronal dysfunction and degeneration in multiple sclerosis. *Prog Neurobiol*. 2011;93(1):1-12. doi:10.1016/j.pneurobio.2010.09.005.
4. Plemel JR, Liu W, Yong VW. Remyelination therapies : multiple sclerosis. *Nat Rev Drug Discov*. 2017. doi:10.1038/nrdnrd.2017.115.
5. Franklin RJM. Regenerative Medicines for Remyelination: From Aspiration to Reality. *Cell Stem Cell*. 2015;16(6):576-577. doi:10.1016/j.stem.2015.05.010.
6. Pukos N, Yoseph R, M. McTigue D. To Be or Not to Be: Environmental Factors that Drive Myelin Formation during Development and after CNS Trauma. *Neuroglia*. 2018;1(1):7. doi:10.3390/neuroglia1010007.
7. Ohori Y, Yamamoto S, Nagao M, et al. Growth factor treatment and genetic manipulation stimulate neurogenesis and oligodendrogenesis by endogenous neural progenitors in the injured adult spinal cord. *J Neurosci*. 2006;26(46):11948-11960. doi:10.1523/JNEUROSCI.3127-06.2006.
8. Prineas JW, Kwon EE, Goldenberg PZ, et al. Multiple sclerosis. Oligodendrocyte proliferation and differentiation in fresh lesions. *Lab Invest*. 1989;61(5):489-503. http://www.ncbi.nlm.nih.gov/pubmed/2811298. Accessed April 29, 2018.
9. Karimi-Abdolrezaee S, Billakanti R. Reactive Astrogliosis after Spinal Cord Injury—Beneficial and Detrimental Effects. *Mol Neurobiol*. 2012;46(2):251-264. doi:10.1007/s12035-012-8287-4.
10. Alizadeh A, Dyck SM, Karimi-Abdolrezaee S. Myelin damage and repair in pathologic CNS: challenges and prospects. *Front Mol Neurosci*. 2015;8. doi:10.3389/fnmol.2015.00035.
11. Vartanian T, Fischbach G, Miller R. Failure of spinal cord oligodendrocyte development in mice lacking neuregulin. *Proc Natl Acad Sci U S A*. 1999;96(2):731-735. http://www.ncbi.nlm.nih.gov/pubmed/9892702. Accessed April 29, 2018.
12. Kataria H, Alizadeh A, Shahriary GM, et al. Neuregulin-1 promotes remyelination and fosters a pro-regenerative inflammatory response in focal demyelinating lesions of the spinal cord. *Glia*. 2018;66(3):538-561. doi:10.1002/glia.23264.
13. Stromnes IM, Goverman JM. Active induction of experimental allergic encephalomyelitis. *Nat Protoc*. 2006;1(4):1810-1819. doi:10.1038/nprot.2006.285.
14. Orton S-M, Herrera BM, Yee IM, et al. Sex ratio of multiple sclerosis in Canada: a longitudinal study. *Lancet Neurol*. 2006;5(11):932-936. doi:10.1016/S1474-4422(06)70581-6.
15. Kastin AJ, Akerstrom V, Pan W. Neuregulin-1-beta1 enters brain and spinal cord by receptor-mediated transport. *J Neurochem*. 2004;88(4):965-970. http://www.ncbi.nlm.nih.gov/pubmed/14756818. Accessed April 29, 2018.
16. Bjartmar C, Kidd G, Mörk S, Rudick R, Trapp BD. Neurological disability correlates with spinal cord axonal loss and reduced N-acetyl aspartate in chronic multiple sclerosis patients. *Ann Neurol*. 2000;48(6):893-901.

http://www.ncbi.nlm.nih.gov/pubmed/11117546. Accessed April 30, 2018.
17. Amor V, Feinberg K, Eshed-Eisenbach Y, et al. Long-Term Maintenance of Na+ Channels at Nodes of Ranvier Depends on Glial Contact Mediated by Gliomedin and NrCAM. *J Neurosci*. 2014;34(15):5089-5098. doi:10.1523/JNEUROSCI.4752-13.2014.
18. Chiu SY, Ritchie JM. Potassium channels in nodal and internodal axonal membrane of mammalian myelinated fibres. *Nature*. 1980;284(5752):170-171. http://www.ncbi.nlm.nih.gov/pubmed/6244497. Accessed July 20, 2018.
19. Poliak S, Peles E. The local differentiation of myelinated axons at nodes of Ranvier. *Nat Rev Neurosci*. 2003;4(12):968-980. doi:10.1038/nrn1253.
20. Traka M, Goutebroze L, Denisenko N, et al. Association of TAG-1 with Caspr2 is essential for the molecular organization of juxtaparanodal regions of myelinated fibers. *J Cell Biol*. 2003;162(6):1161-1172. doi:10.1083/jcb.200305078.
21. Rasband MN, Trimmer JS, Schwarz TL, et al. Potassium channel distribution, clustering, and function in remyelinating rat axons. *J Neurosci*. 1998;18(1):36-47. http://www.ncbi.nlm.nih.gov/pubmed/9412484. Accessed July 20, 2018.
22. Zhou D, Lambert S, Malen PL, Carpenter S, Boland LM, Bennett V. AnkyrinG is required for clustering of voltage-gated Na channels at axon initial segments and for normal action potential firing. *J Cell Biol*. 1998;143(5):1295-1304. http://www.ncbi.nlm.nih.gov/pubmed/9832557. Accessed July 20, 2018.
23. Sherman DL, Brophy PJ. Mechanisms of axon ensheathment and myelin growth. *Nat Rev Neurosci*. 2005;6(9):683-690. doi:10.1038/nrn1743.
24. Susuki K, Chang K-J, Zollinger DR, et al. Three Mechanisms Assemble Central Nervous System Nodes of Ranvier. *Neuron*. 2013;78(3):469-482. doi:10.1016/j.neuron.2013.03.005.
25. Coman I, Aigrot MS, Seilhean D, et al. Nodal, paranodal and juxtaparanodal axonal proteins during demyelination and remyelination in multiple sclerosis. *Brain*. 2006;129(12):3186-3195. doi:10.1093/brain/awl144.
26. Einheber S, Zanazzi G, Ching W, et al. The axonal membrane protein Caspr, a homologue of neurexin IV, is a component of the septate-like paranodal junctions that assemble during myelination. *J Cell Biol*. 1997;139(6):1495-1506. http://www.ncbi.nlm.nih.gov/pubmed/9396755. Accessed July 20, 2018.
27. Lyons DA, Talbot WS. Axonal Domains: Role for Paranodal Junction in Node of Ranvier Assembly. *Curr Biol*. 2008;18(18):R876-R879. doi:10.1016/j.cub.2008.07.070.
28. Alizadeh A, Dyck SM, Karimi-Abdolrezaee S. Myelin damage and repair in pathologic CNS: challenges and prospects. *Front Mol Neurosci*. 2015;8:35. doi:10.3389/fnmol.2015.00035.
29. Trapp BD, Stys PK. Virtual hypoxia and chronic necrosis of demyelinated axons in multiple sclerosis. *Lancet Neurol*. 2009;8(3):280-291. doi:10.1016/S1474-4422(09)70043-2.
30. Craner MJ, Newcombe J, Black JA, Hartle C, Cuzner ML, Waxman SG. Molecular changes in neurons in multiple sclerosis: Altered axonal expression of Nav1.2 and Nav1.6 sodium channels and Na+/Ca2+ exchanger. *Proc Natl Acad Sci*. 2004;101(21):8168-8173. doi:10.1073/pnas.0402765101.
31. Karimi-Abdolrezaee S, Eftekharpour E, Fehlings MG. Temporal and spatial patterns of Kv1.1 and Kv1.2 protein and gene expression in spinal cord white matter after acute and chronic spinal cord injury in rats: implications for axonal pathophysiology after neurotrauma. *Eur J Neurosci*. 2004;19(3):577-589. http://www.ncbi.nlm.nih.gov/pubmed/14984408. Accessed July 20, 2018.
32. Eftekharpour E, Karimi-Abdolrezaee S, Wang J, El Beheiry H, Morshead C, Fehlings MG. Myelination of Congenitally Dysmyelinated Spinal Cord Axons by Adult Neural Precursor Cells Results in Formation of Nodes of Ranvier and Improved Axonal Conduction. *J*

*Neurosci*. 2007;27(13):3416-3428. doi:10.1523/JNEUROSCI.0273-07.2007.
33. Bramow S, Frischer JM, Lassmann H, et al. Demyelination versus remyelination in progressive multiple sclerosis. *Brain*. 2010;133(10):2983-2998. doi:10.1093/brain/awq250.
34. Eriksson M, Andersen O, Runmarker B. Long-term follow up of patients with clinically isolated syndromes, relapsing-remitting and secondary progressive multiple sclerosis. *Mult Scler J*. 2003;9(3):260-274. doi:10.1191/1352458503ms914oa.
35. Lublin FD, Reingold SC, Cohen JA, et al. Defining the clinical course of multiple sclerosis: The 2013 revisions. *Neurology*. 2014;83(3):278-286. doi:10.1212/WNL.0000000000000560.
36. Chari DM. Remyelination In Multiple Sclerosis. In: *International Review of Neurobiology*. Vol 79. ; 2007:589-620. doi:10.1016/S0074-7742(07)79026-8.
37. Olek MJ, Narayan RN, Frohman EM, Frohman TC. Manifestations of multiple sclerosis in adults - UpToDate. UpToDate. https://www-uptodate-com.uml.idm.oclc.org/contents/manifestations-of-multiple-sclerosis-in-adults?search=uhthoff phenomenon&source=search_result&selectedTitle=1~5&usage_type=default&display_rank=1. Published 2018. Accessed July 31, 2018.
38. Selhorst JB, Saul RF. Uhthoff and his symptom. *J Neuroophthalmol*. 1995;15(2):63-69. http://www.ncbi.nlm.nih.gov/pubmed/7550931. Accessed July 30, 2018.
39. Opara JA, Brola W, Wylegala AA, Wylegala E. Uhthoff`s phenomenon 125 years later - what do we know today? *J Med Life*. 2016;9(1):101-105. http://www.ncbi.nlm.nih.gov/pubmed/27974923. Accessed July 30, 2018.
40. Saab AS, Tzvetanova ID, Nave K-A. The role of myelin and oligodendrocytes in axonal energy metabolism. *Curr Opin Neurobiol*. 2013;23(6):1065-1072. doi:10.1016/j.conb.2013.09.008.
41. Micu I, Plemel JR, Lachance C, et al. The molecular physiology of the axo-myelinic synapse. *Exp Neurol*. 2016;276:41-50. doi:10.1016/j.expneurol.2015.10.006.
42. Saab AS, Tzvetavona ID, Trevisiol A, et al. Oligodendroglial NMDA Receptors Regulate Glucose Import and Axonal Energy Metabolism. *Neuron*. 2016;91(1):119-132. doi:10.1016/j.neuron.2016.05.016.
43. Lee Y, Morrison BM, Li Y, et al. Oligodendroglia metabolically support axons and contribute to neurodegeneration. *Nature*. 2012;487(7408):443-448. doi:10.1038/nature11314.
44. Millecamps S, Julien J-P. Axonal transport deficits and neurodegenerative diseases. *Nat Rev Neurosci*. 2013;14(3):161-176. doi:10.1038/nrn3380.
45. Kirkpatrick LL, Witt AS, Payne HR, Shine HD, Brady ST. Changes in microtubule stability and density in myelin-deficient shiverer mouse CNS axons. *J Neurosci*. 2001;21(7):2288-2297. http://www.ncbi.nlm.nih.gov/pubmed/11264304. Accessed July 20, 2018.
46. Edgar JM, McLaughlin M, Yool D, et al. Oligodendroglial modulation of fast axonal transport in a mouse model of hereditary spastic paraplegia. *J Cell Biol*. 2004;166(1):121-131. doi:10.1083/jcb.200312012.
47. Lin T-H, Kim JH, Perez-Torres C, et al. Axonal transport rate decreased at the onset of optic neuritis in EAE mice. *Neuroimage*. 2014;100:244-253. doi:10.1016/j.neuroimage.2014.06.009.
48. Sorbara CD, Wagner NE, Ladwig A, et al. Pervasive Axonal Transport Deficits in Multiple Sclerosis Models. *Neuron*. 2014;84(6):1183-1190. doi:10.1016/j.neuron.2014.11.006.
49. Shakhbazau A, Schenk GJ, Hay C, et al. Demyelination induces transport of ribosome-containing vesicles from glia to axons: evidence from animal models and MS patient brains. *Mol Biol Rep*. 2016;43(6):495-507. doi:10.1007/s11033-016-3990-2.
50. Frühbeis C, Fröhlich D, Kuo WP, et al. Neurotransmitter-Triggered Transfer of Exosomes

Mediates Oligodendrocyte–Neuron Communication. Barres BA, ed. *PLoS Biol*. 2013;11(7):e1001604. doi:10.1371/journal.pbio.1001604.
51. Lappe-Siefke C, Goebbels S, Gravel M, et al. Disruption of Cnp1 uncouples oligodendroglial functions in axonal support and myelination. *Nat Genet*. 2003;33(3):366-374. doi:10.1038/ng1095.
52. Klugmann M, Schwab MH, Pühlhofer A, et al. Assembly of CNS myelin in the absence of proteolipid protein. *Neuron*. 1997;18(1):59-70. http://www.ncbi.nlm.nih.gov/pubmed/9010205. Accessed July 20, 2018.
53. Griffiths I, Klugmann M, Anderson T, et al. Axonal swellings and degeneration in mice lacking the major proteolipid of myelin. *Science (80- )*. 1998;280(5369):1610-1613. doi:10.1126/science.280.5369.1610.
54. Biffiger K, Bartsch S, Montag D, Aguzzi A, Schachner M, Bartsch U. Severe hypomyelination of the murine CNS in the absence of myelin-associated glycoprotein and fyn tyrosine kinase. *J Neurosci*. 2000;20(19):7430-7437. http://www.ncbi.nlm.nih.gov/pubmed/11007902. Accessed July 20, 2018.
55. Nguyen T, Mehta NR, Conant K, et al. Axonal Protective Effects of the Myelin-Associated Glycoprotein. *J Neurosci*. 2009;29(3):630-637. doi:10.1523/JNEUROSCI.5204-08.2009.
56. Hughes EG, Kang SH, Fukaya M, Bergles DE. Oligodendrocyte progenitors balance growth with self-repulsion to achieve homeostasis in the adult brain. *Nat Neurosci*. 2013;16(6):668-676. doi:10.1038/nn.3390.
57. Xing YL, Roth PT, Stratton JAS, et al. Adult Neural Precursor Cells from the Subventricular Zone Contribute Significantly to Oligodendrocyte Regeneration and Remyelination. *J Neurosci*. 2014;34(42):14128-14146. doi:10.1523/JNEUROSCI.3491-13.2014.
58. Alizadeh A, Karimi-Abdolrezaee S. Microenvironmental regulation of oligodendrocyte replacement and remyelination in spinal cord injury. *J Physiol*. 2016;594(13):3539-3552. doi:10.1113/JP270895.
59. Cudrici C, Niculescu T, Niculescu F, Shin ML, Rus H. Oligodendrocyte cell death in pathogenesis of multiple sclerosis: Protection of oligodendrocytes from apoptosis by complement. *J Rehabil Res Dev*. 2006;43(1):123. doi:10.1682/JRRD.2004.08.0111.
60. Gauthier M-K, Kosciuczyk K, Tapley L, Karimi-Abdolrezaee S. Dysregulation of the neuregulin-1-ErbB network modulates endogenous oligodendrocyte differentiation and preservation after spinal cord injury. *Eur J Neurosci*. 2013;38(5):2693-2715. doi:10.1111/ejn.12268.
61. Vartanian T, Goodearl A, Viehöver A, Fischbach G. Axonal neuregulin signals cells of the oligodendrocyte lineage through activation of HER4 and Schwann cells through HER2 and HER3. *J Cell Biol*. 1997;137(1):211-220. http://www.ncbi.nlm.nih.gov/pubmed/9105049. Accessed April 29, 2018.
62. Fernandez P-A, Tang DG, Cheng L, Prochiantz A, Mudge AW, Raff MC. Evidence that Axon-Derived Neuregulin Promotes Oligodendrocyte Survival in the Developing Rat Optic Nerve. *Neuron*. 2000;28(1):81-90. doi:10.1016/S0896-6273(00)00087-8.
63. Alizadeh A, Santhosh KT, Kataria H, Gounni AS, Karimi-Abdolrezaee S. Neuregulin-1 elicits a regulatory immune response following traumatic spinal cord injury. *J Neuroinflammation*. 2018;15(1):53. doi:10.1186/s12974-018-1093-9.
64. Alizadeh A, Dyck SM, Kataria H, et al. Neuregulin-1 positively modulates glial response and improves neurological recovery following traumatic spinal cord injury. *Glia*. 2017;65(7):1152-1175. doi:10.1002/glia.23150.
65. Bjartmar C, Wujek JR, Trapp BD. Axonal loss in the pathology of MS: consequences for understanding the progressive phase of the disease. *J Neurol Sci*. 2003;206(2):165-171. http://www.ncbi.nlm.nih.gov/pubmed/12559505. Accessed August 2, 2018.
66. Lau LW, Keough MB, Haylock-Jacobs S, et al. Chondroitin sulfate proteoglycans in

demyelinated lesions impair remyelination. *Ann Neurol*. 2012;72(3):419-432. doi:10.1002/ana.23599.
67. Keough MB, Rogers JA, Zhang P, et al. An inhibitor of chondroitin sulfate proteoglycan synthesis promotes central nervous system remyelination. *Nat Commun*. 2016;7:11312. doi:10.1038/ncomms11312.
68. Dyck S, Kataria H, Alizadeh A, et al. Perturbing chondroitin sulfate proteoglycan signaling through LAR and PTPσ receptors promotes a beneficial inflammatory response following spinal cord injury. *J Neuroinflammation*. 2018;15(1):90. doi:10.1186/s12974-018-1128-2.

## Figures and tables

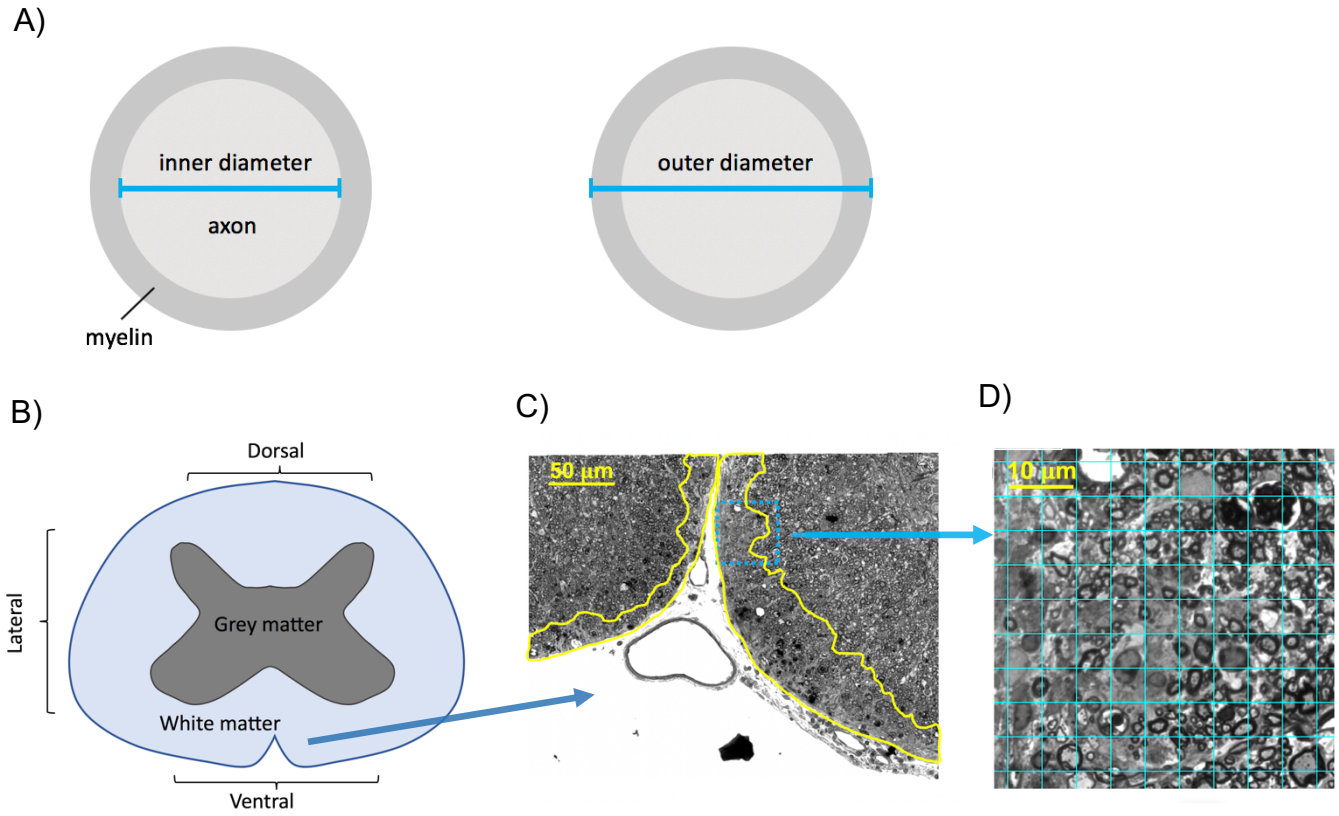


**Figure 1. g-ratio schematic and spinal cord cross section showing EAE lesion.** (A) Technique for measuring g-ratio is depicted. (B) Anatomic depiction of spinal cord in cross section. (C, D) Semi-thin spinal cord cross section showing ventral bilateral EAE lesion (yellow tracing) 28 days after peak of disease. From the lesion and perilesional area, boxes measuring 600 x 600 pixels (61 x 61 µm) were selected for analysis (blue area; enlarged). Number of boxes used was determined by size of lesion. 10 equidistant gridlines were laid atop, and any axon transected by a vertical gridline was analyzed.

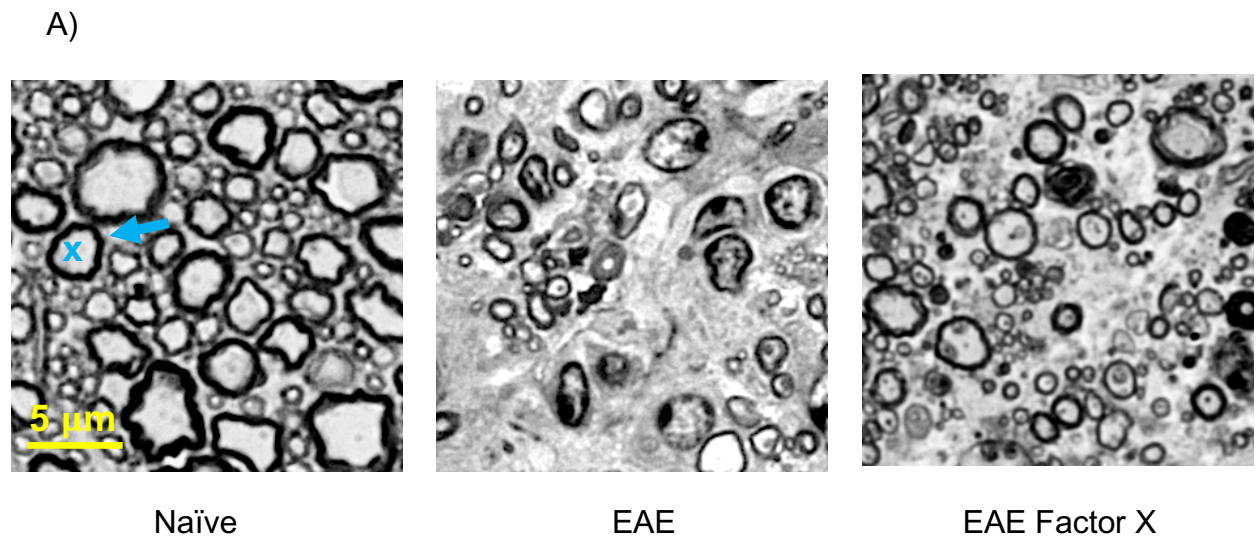
A)
x
5 µm
Naïve
EAE
EAE Factor X
B)
g-ratio
0.75
0.70
0.65
0.60
0.05
0.00
*
EAE
EAE Factor X
C)
% frequency distribution
Naïve
EAE
EAE Factor X
<0.4
0.40-0.45
0.45-0.5
0.5-0.55
0.55-0.6
0.6-0.65
0.65-0.7
0.7-0.75
0.75-0.8
0.8-0.85
0.85-0.9
g-ratio
D)
g-ratio
Mann-Whitney test;
p < 0.001
Axon diameter (µm)
E)
Axons per 10⁴ µm²
Naïve
EAE
EAE Factor X

**Figure 2. Factor X treatment facilitates remyelination and promotes axonal preservation in EAE lesions.** (A) Representative images from semi-thin spinal cord cross sections show axon density and extent of myelination in non-EAE naïve animal tissue, an EAE animal at 28 days after the peak of disease, and an EAE lesion site from an animal treated with Factor X. An axon (+) and its surrounding myelin (arrow) are labelled as an example. Non-EAE naïve tissue contains a high density of myelinated axons as expected, whereas EAE lesions show evidence of axon loss and demyelination. EAE Factor X group showed considerably more axons in the lesion site, with fewer demyelinated axons, compared to the EAE untreated group. (B) Quantitative analysis of myelin thickness by g-ratio revealed a value of 0.674 in naïve non-EAE tissue (dotted line), which was increased to 0.699 ± 0.006 in EAE group as a result of demyelination. Factor X treatment decreased g-ratio to 0.664 ± 0.009, indicating an increase in myelin thickness (data represent mean ± SEM, *P = 0.018, student t test, *N* = 4/group). (C) Frequency distribution of g-ratio is depicted, ranging from 0.40 to 0.85 with an interval of 0.05. The difference between groups was non-significant by $\chi^2$ test, however, Factor X treatment decreased the proportion of demyelinated/thinly myelinated axons (g-ratio $\geq 0.80$) from 22.3% to 13.7% and increased the proportion of heavily myelinated axons (g ratio < $0.60$) from 21.4% to 29.4%. Importantly, decreased frequency of thinly myelinated axons suggests remyelination, and is likely to produce clinical benefit. (D) Scatter plot showing g-ratios of individual myelinated axons as a function of the respective axon size. Black and red dots represent EAE and EAE Factor X treated groups, respectively. g-ratio was significantly different between EAE and EAE Factor X group (Mann-Whitney U test, $p < 0.001$). The linear regression of the g-ratio measurements for each animal (*N* = 4/group) is shown by the black and red lines representing EAE and EAE Factor X treated groups, respectively. (E) Quantitative analysis of axon density revealed that naïve normal tissue has 382 ± 4 axons per $10^4$ μm$^2$, which was decreased to 198 ± 12 in EAE group. Factor X treatment significantly increased axonal density by 39.9% to 277 ± 11 axons per $10^4$ μm$^2$, indicating an important beneficial effect on axonal preservation (data represent mean ± SEM, *P < 0.001, one-way ANOVA, *N* = 4/group).

**Table 1. Antibody preparations for IHC.**

| Primary | | | Secondary | | |
|---|---|---|---|---|---|
| **Antibody** | **Source** | **Dilution** | **Antibody** | **Source** | **Dilution** |
| Factor X | Santa Cruz (Rb) | 1:100 | anti-Ms Alexa 488-conjugated | Invitrogen (Gt) | 1:400 |
| Fluoromyelin dye | Thermo Scientific | 1:300 | anti-Ch Alexa 488-conjugated | Invitrogen (Gt) | 1:200 |
| NF-200 | Sigma (Ms) | 1:500 | anti-Ms Alexa 568-conjugated | Invitrogen (Gt) | 1:400 |
| GFAP | AVES (Ch) | 1:500 | anti-Rb Alexa 647-conjugated | Invitrogen (Gt) | 1:400 |
| Olig-2 | Chemicon (Rb) | 1:1000 | | | |
| BrdU | BD (Ms) | 1:400 | | | |

Rb = rabbit, Ms = mouse, Ch = chicken, Gt = goat. Antibodies were diluted in blocking solutions of 1% bovine serum albumin (BSA), 5% non-fat milk, and 0.3% Triton X in PBS. Factor X primary antibodies were diluted in a modified blocking solution containing 2% goat serum rather than 1% BSA. Fluoromyelin dye, which stains myelin sheath, was diluted in PBS and did not require a secondary antibody solution.

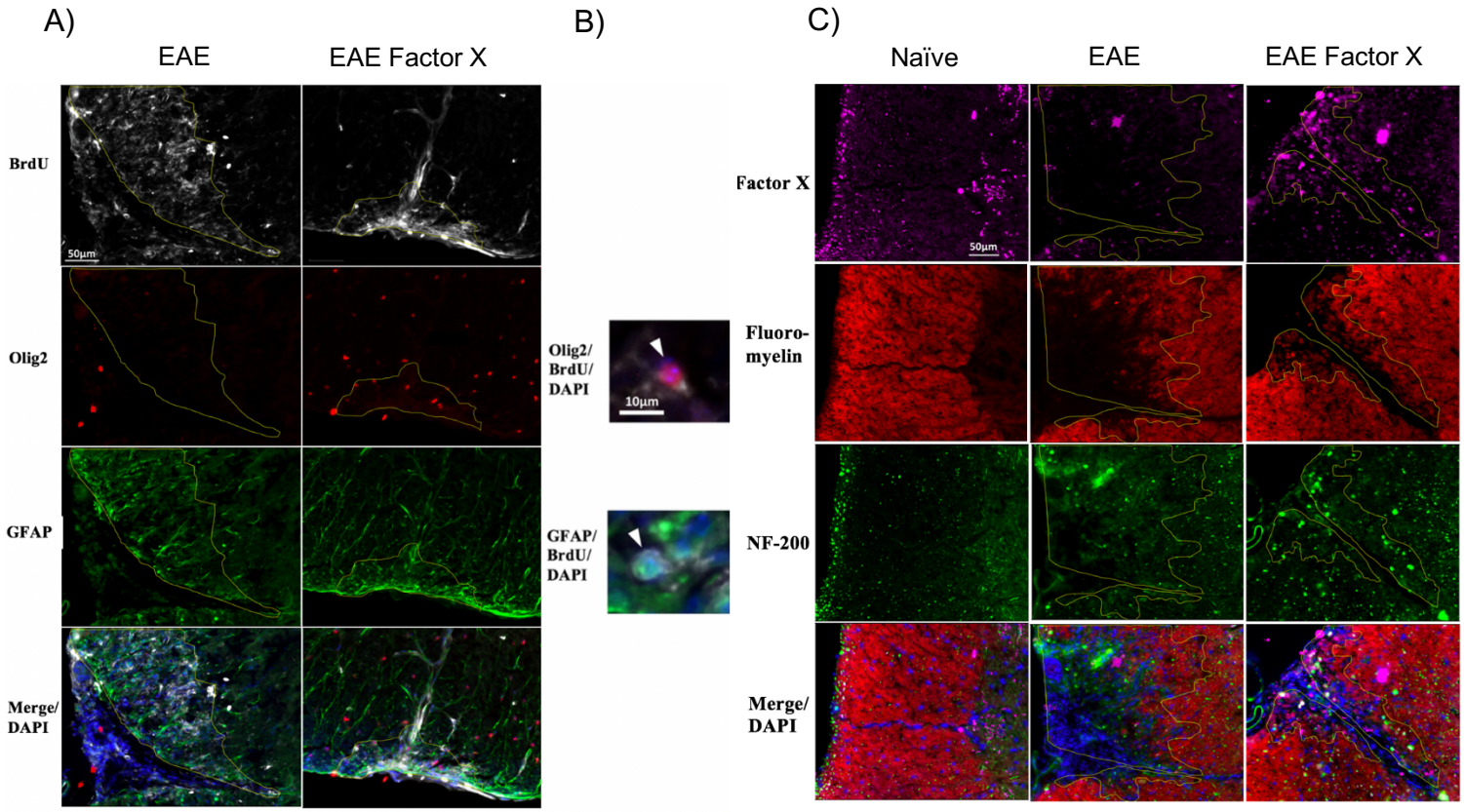


**Figure 3. Immunohistochemical images of EAE lesions for oligodendrocyte and astrocyte proliferation and markers of Factor X, myelin, and axons**. (A) Immunofluorescence images show BrdU (white; newly generated cells), Olig2 (red; marker of oligodendrocytes), and GFAP (green; marker of astrocytes) within the lesion areas in EAE untreated group and EAE Factor X treatment group. (B) Newly generated oligodendrocytes co-labelled with BrdU and Olig2 and newly generated astrocytes co-labelled with BrdU and GFAP; denoted by white arrows. (C) Immunofluorescence images show Factor X (purple), myelin (red; stained with fluoromyelin dye), and NF-200 (green; marker of axons) within the lesion areas in EAE group and EAE Factor X treatment group, compared to healthy controls (naïve). Note the large demyelinating lesion in the EAE untreated group.