The role of microglia in regulating AIS integrity in health and disease

I. Introduction

Multiple Sclerosis (MS) is a disease of the central nervous system that disrupts communication within the brain (Clark et al. 2016). The loss of myelin, an electrically insulating substance that surrounds nerve cells within the central nervous system (CNS), is accepted as a primary causative event resulting in motor, sensory and cognitive disability encountered by patients suffering from MS (Lassmann, 1999). In addition to myelin loss, inflammation also contributes to the CNS dysfunction experienced by victims of this disease.

Although the precise pathological mechanism is not known, it is believed that the loss of myelin results in axonal pathology (Trapp et al. 1998). Several studies have reported that following myelin loss, specific domains, known as nodes of Ranvier (Figure 1), lose their structural integrity resulting in loss of nerve neuronal function.

In order for the axon to function appropriately, electrical signals must travel down the axon. These signals are propagated by the generation of action potentials at each nodal domain. If the action potential cannot be regenerated at downstream nodes, then signal transmission and vital communication fails. Prolonged domain disruption and failure of signal transmission results in axonal degeneration, neuron cell death and failed brain function.

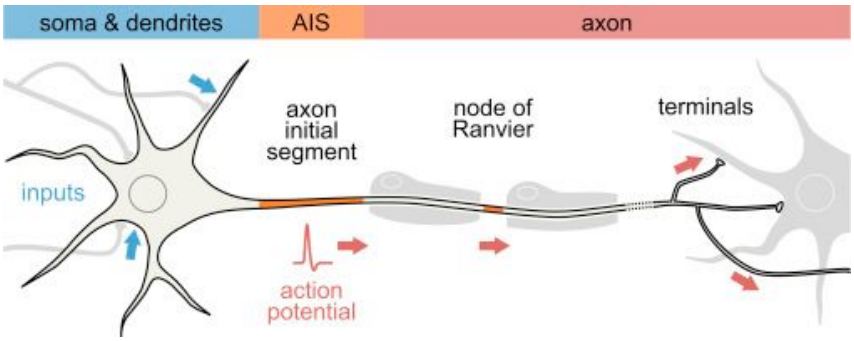

Fig. 1) The Axon Initial Segment (AIS)
Adapted from Fig. 1, Leterrier C. 2016

Although more thoroughly studied, the node of Ranvier is not the only axonal domain critical for communication within the brain. Immediately distal to the neuron soma is the axon initial segment (AIS), (Figure 1). The AIS is responsible for generating and modulating action potentials—the same signal that is regenerated at each node of Ranvier ensuring CNS communication. Briefly, the AIs is the initial 20 microns of the axon immediately distal to the soma and this region maintains a dense cluster of sodium channel ions, which are required for action potential generation. If the AIS of a neuron is compromised either by the loss of sodium ion channel clustering or in the length of the AIS, the AIS does not function appropriately. In cases of MS, it is generally assumed that damage to the AIS is the product of demyelination (Papadopoulos D et al. 2005; Clark et al. 2016; Lassmann, 1999). However recent research has suggested that AIS maintenance functions independently of myelination and that demyelination does not directly damage AIS stability (Hamada and Kole 2015; Clark et al. 2016).

Recently the Dupree lab published a paper using two mouse models that mimic different aspects of MS. The first model is known as the toxicity model of cuprizone (Morell and Matsushima, 1999). In this model the toxin cuprizone is fed to the mice resulting in massive demyelination; however, following demyelination, the AIS exhibited no change in structure. In parallel to the cuprizone model, the Dupree lab also used a mouse model that mimics the inflammatory environment of MS. This model is known experimental autoimmune encephalopathy (EAE). In the EAE-induced mice, the AISs were disrupted. The AISs lengths were shortened, followed by a complete loss of sodium ion channel clustering.

Although demyelination was not observed, the loss of the AISs coincided with the activation of microglia, the resident immune defense cells of the CNS. It is the job of the microglia to survey the brain and react to injury or disease. Interestingly, it has recently been observed that a population of microglia contact the AIS in the healthy brain (Figure 2), (Baalman

et al., 2015). These microglia are surveying microglia and perhaps play a role in stabilizing the AIS; however, such a role for microglia has not been studied and remains to be clarified. In contrast to a possible role of surveying microglia in stabilizing the AIS, the Dupree lab reported that reactive microglia also contact the AIS, in the EAE model, but AIS stability is lost (Figure 2). This presents the question as to whether surveying microglia stabilize the AIS while reactive microglia attack the AIS.

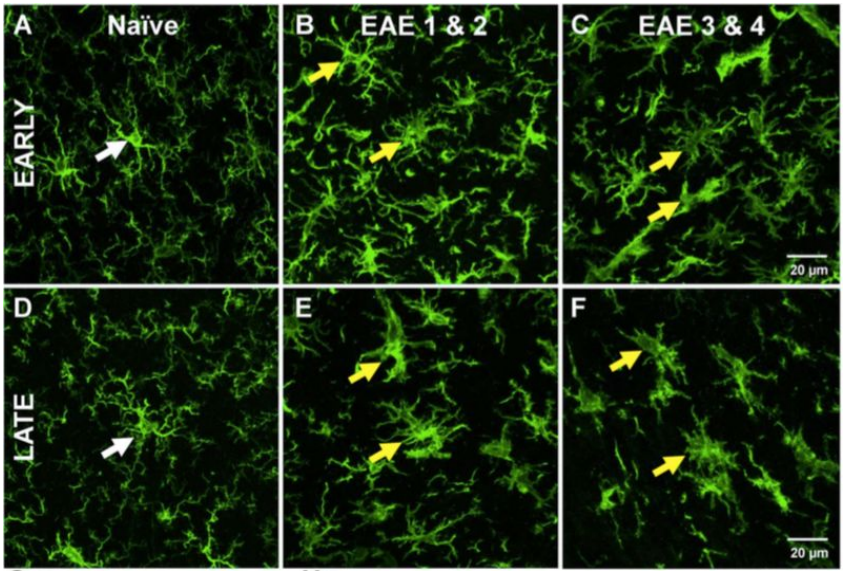

Fig. 2) Non-reactive and reactive microglia in healthy and EAE models. Adapted from Fig. 5, Clark et al. 2016

In an effort to address this question, I will exploit a mild head trauma model based on the work of the Povlishock lab (Greer et al., 2012). In this previous work, Greer et al. reported that the majority of axonal injury following mild head injury was localized to the AIS; however, the microglial activation was not assessed. Although not assessed by Greer et al. other studies have reported microglial activation following head trauma. Therefore to more directly investigate the role that microglia play in AIS integrity, I propose to administer a mild head injury, as induced by Greer et al (2013), but in the absence of microglia. The feasibility of the removal of microglia from the CNS has recently been shown by the lab of Dr. Kim Green (Elmore et al., 2014). Elmore et al. (2014) used an inhibitor against colony stimulating factor 1 receptor (CSF1R). The administration of this inhibitor resulted in effectively destroying ~99% of CNS microglia. After removing the resident microglia from the mice, a range of behavioral tests was conducted to assess the health of the subject. These tests did not suggest any significant

impairment to the subjects' learning, memory, or motor functions (Elmore et al. 2014). The results are surprising in that they suggest that microglial populations do not play a critical role in CNS health and function. On the contrary, I propose that based on the findings of the Dupree lab (Clark et al., 2016) and the Povlishock lab (2013) that reactive microglia have a devastating effect on AIS integrity and that the removal of these reactive cells will attenuate AIS pathology that results from mild traumatic brain injury.

II. Experiment

Mice

To deplete the mice of microglia, I will exploit the (Cre)/LoxP ablation methodology. Based on the work of the Green lab, I will ablate the gene for colony stimulating factor 1 receptor (CSF1R). I will obtain the floxed (flanking loxP) CSF1R mouse from Dr. JW Pollard (Li Chen and Pollard, 2006), which has been shown to effectively deplete CSF1R following cre activation. The floxed mouse refers to a mouse that has two loxP sites flanking regions of the CSF1R gene. In order to specifically ablate CSF1R in microglia, I will mate the floxed CSF1R with the Cx3cr1-cre mice. The site specific Cre recombinase enzyme will be utilized to delete the CSF1R gene; however, the Cre protein will be fused to a mutant estrogen binding domain (ER). The fusion of Cre to the estrogen binding domain will restrict Cre's entry into the nucleus of the microglia until tamoxifen is present. This will allow the mouse to develop into a normal and healthy mouse, whereas the immediate deletion of CSF1R would profoundly harm the mouse's development. Tamoxifen will be injected at a dose of 1mg/kg body weight for 5 consecutive days. Immunostaining for the IBA-1 protein, a microglia specific calcium-binding protein, will be utilized to confirm the successful knockout of CNS microglia (Clark et al. 2016).

Administration mild TBI

All mice will receive a surgical procedure to remove a small portion of the cranium. Injured mice will then undergo a procedure known as a central fluid percussion injury. This will be administered by releasing a pendulum onto a fluid-filled piston to induce a brief fluid pressure pulse upon the intact dura mater (Figure 3). The injuries will be classified as mild severity with an impact of 1.7± 0.04 atmospheres. Mice not receiving the injury will be used as controls for the injury and noted as “Sham injured mice.”

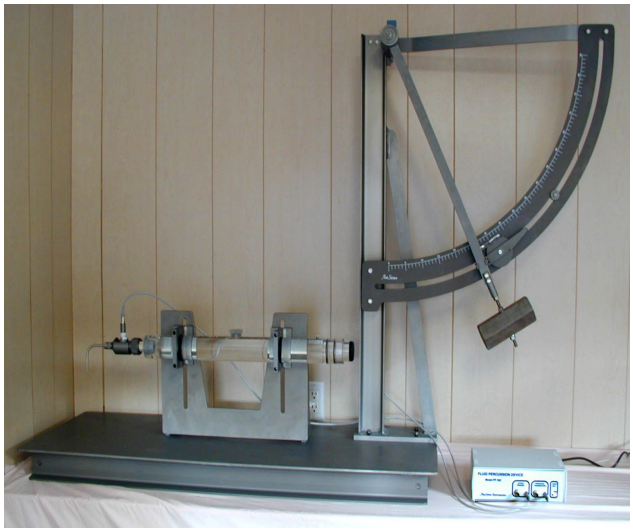

Fig. 3) Fluid Percussion Device. Image provided by Amscien Intsruments

Injury Groups

A total of eight groups will be studied, these groups are as follows: (3 days and 7 days pertains to number days after microglia is knocked out in the selected groups)

1) microglia knockout and TBI-- 3 days

2) microglia knockout and TBI-- 7 days

3) microglia knockout and no TBI-- 3 days

4) microglia knockout and no TBI-- 7 days

5) no microglia knockout and TBI-- 3 days

6) no microglia knockout and TBI-- 7 days

7) (control) no microglia knockout and no TBI-- 3 days

8) (control) no microglia knockout and no TBI-- 7 days

Analyze AIS length and number

To quantify AIS length and number of AISs, brains will be prepared for immunocytochemical labeling. Briefly, mice will be perfused with 4% paraformaldehyde. Brains will be removed, frozen and sectioned. The sections will then be immunolabeled for markers specific for the AIS including sodium ion channels 1.6 and ankyrinG double labeled with the neuronal marker NeuN, as previously described by the Dupree lab (Clark et al., 2017). Using the confocal images, the percent of neurons (NeuN+ cells) that extend as positively labeled AISs will be determined for each mouse. The length of the subjects' AISs will be determined through confocal microscopy and utilization of image analysis software (Baalman et al. 2015, Clark et al. 2016). The groups of mice that retained CNS microglia and did not receive a TBI will be used as a control group.

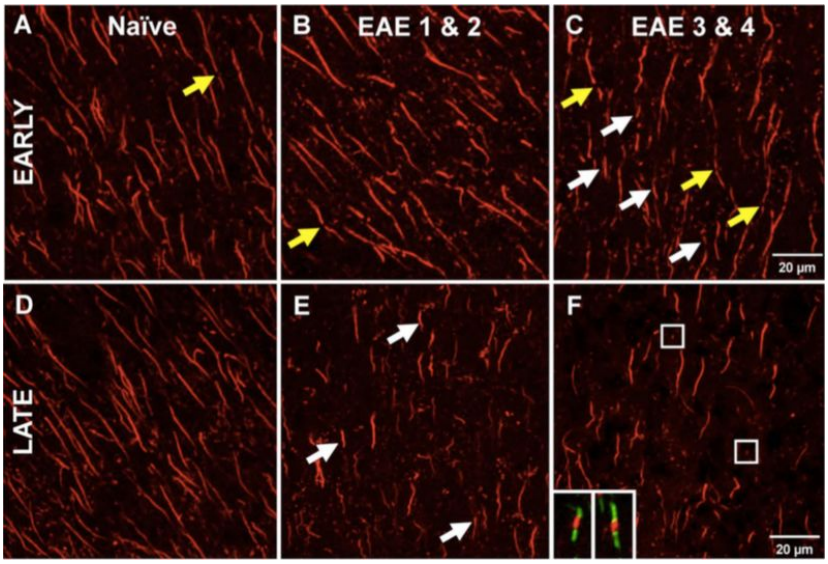

Fig. 4) Numerous healthy AIS in naive mice, diminished population of shorter AIS in EAE mice. Adapted from Fig. 3 Clark et al. 2016

Analyze AIS function

Although change in structure, as assessed by confocal imaging implies altered function, directly testing function is a preferable approach. Any changes in AIS function will be determined through electrophysiology. When analyzing subjects, the brain will be sectioned and sliced, held in a bath of artificial cerebrospinal fluid, and electrodes will be utilized to artificially generate action potentials (Zonta et al. 2011). The size (amplitude), speed (rate of rise), and the current required to drive action potentials within a desired frequency (52-58 Hz) will be analyzed.

III. Discussion

Baalman et al. (2013) reported a mild alteration in AIS length following a blast head injury. Although significant, the reduction in length was less than 2% of the normal length. In a subsequent paper, Baalman et al. (2015) reported that surveying microglia contact the AIS in the healthy CNS suggesting that microglia may play a role in stabilizing the AIS. In our study, we will first deplete the brain of microglia and then administer a head injury. I predict that the absence of the microglia will result in a more profound disruption of the AIS. This disruption may be manifest by shorter AISs or the overall reduction of AISs. Since the microglia will be in a surveying state at the time of depletion, this study is limited to the effect that the depletion of surveying microglia has on the AIS. Although beyond the scope of the current study, it will be interesting to induce reactive microglia and then administer the same injury paradigm following prolonged exposure of the AIS to these reactive microglia. Additionally, it would be interesting to therapeutically inhibit activation of microglia and then administer the injury.

It is difficult to predict the outcome for our study as AISs may be preserved or they may reveal greater disruption. The hypothesis from the Dupree lab with regard to the impact that reactive microglia have on the AIS is that these reactive cells are disruptive to the AIS. Based

on previous studies of head injury, the microglia will become reactive following injury. Therefore, the removal of these cells (no reactive microglia will be present) could result in preservation of the AIS as the reactive microglia could be harmful to AIS integrity. On the other hand, the depletion of the surveying microglia may result in an exacerbation of AIS pathology as the stabilizing effect of these cells would be lost. Since Baalman et al (2013) reported that only a small percentage of the AISs are contacted by microglia, it is possible that the loss of the microglial contact will be minimal and that the depletion of the microglia prior to becoming reactive will have a greater effect. Therefore, I predict that the AISs will be preserved following microglia depletion following head trauma as the consequence of eliminating the effect of reactive microglia outweighs the consequence of losing microglial-induced stability for a small population of AISs.

A potential pitfall arises from the inability of the (Cre)/LoxP knockout to eliminate a large enough quantity of CNS microglia. In this instance, a CSF1R inhibitor like PLX3397 could be substituted and integrated into the diet of healthy mice in order to eliminate CNS microglia (Elmore et al. 2014). Any findings in this experiment may not offer definitive evidence that suggests a clear mechanism through which the AIS is supported by microglial contact. But this experiment hopes to push the understanding of this relationship forward with the hopes that the scientific community might be one small step closer to understanding how these microglial populations relate to the AIS.

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