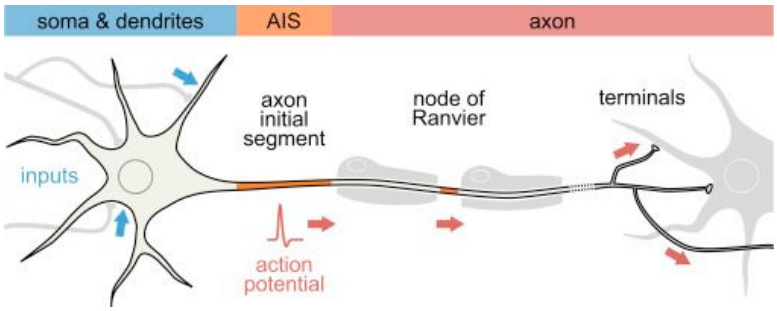
Consequences to the axon initial segment after near total removal of microglia in healthy central nervous system and central nervous system suffering recent injury

I. Introduction

Multiple Sclerosis (MS) is a disease of the central nervous system that disrupts communication within the brain and between the brain and the body (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 chief responsibility for disability encountered in patients suffering from MS (Lassmann, 1999). However, axonal pathology also plays a role in producing disability (Clark et al. 2016). One particular axonal domain, the axon initial segment (AIS), is the focus of this proposal.



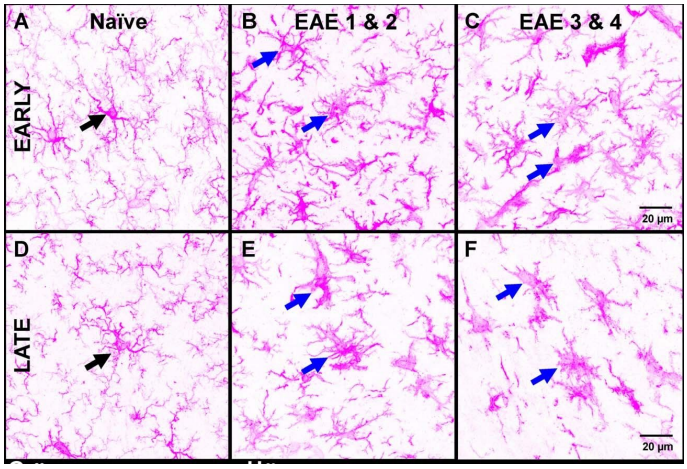
The Axon Initial Segment (AIS)
Adapted from Fig. 1, Leterrier C. 2016

The AIS is responsible for generating and modulating action potentials-- the signals that play a central role in cell to cell communication. 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). This begs the question, what does bring about damage to the AIS?

Studies have exploited a model of experimental autoimmune encephalopathy (EAE) in mice in order to investigate the consequences to axonal domains in the presence of local

inflammation resembling that of MS (Hanisch UK, Kettenmann H. 2007; Buffington SA, Rasband MN. 2011). In one particular instance, AIS stability was investigated in an EAE model; the environment produced through EAE precipitated inflammation in the AIS. While local demyelination did not damage the AIS, inflammation produced through EAE had a significantly destructive effect upon local AISs (Clark et al. 2016).

It has been observed that microglial cells, the resident immune defense cells of the CNS, form small populations that contact the AIS (Baalman et al 2015). The relationship these microglia cells have with AIS stability and function is poorly understood. In models of EAE, these local microglia cells have been observed to exhibit a reactive phenotype, whereas microglia contacting the AIS in healthy models display a non-reactive, surveying phenotype. Considering this observation, when the CNS of an EAE model is treated with an anti-inflammatory drug, reactive microglia populations are nearly completely attenuated, and AIS deterioration is inhibited and is actually reversed in regard to AIS length (Clark et al. 2016). This leads to the conclusion that reactive microglia in contact with the AIS play an active role in exacerbating or producing inflammation and precipitating AIS damage. The experiment suggested in this proposal aims to shed light upon the relationship between microglia populations and the AISs they contact.



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

The relationship between CNS microglia and general organismal function in healthy models has also been investigated. A recent study tested this relationship through inhibition of a

key regulating gene that is crucial to the health of CNS microglia, the colony stimulating factor 1 receptor (CSF1R). This inhibition effectively destroyed ~99% of CNS microglia. After removing resident microglia from murine models, a range of behavioral tests were 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). While this finding may suggest that microglial populations do not play a critical role in CNS health and function, the effect near total removal of microglia holds upon the AIS in particular is of interest. This proposal aims to suggest investigation into this relationship; the effect upon the AIS after near total knockout of CNS microglia.

Recently a relationship between traumatic brain injury (TBI) and reactive microglia populations contacting AISs has been established. This study found that reactive microglia lost contact with the AIS after a TBI (Baalman et al. 2015, Baalman et al. 2013). This relationship is poorly understood. It may be conjectured that investigation into this phenomenon could shed light upon the relationship between local microglial populations and AIS stability and function.

There are a number of studies that provide insights and observations allowing one to draw inferences into how microglial contact affects the AIS (Baalman et al, 2015; Clark et al. 2016; Elmore et al. 2014). However these studies have yet to confidently determine a viable function for the microglial populations that contact the AIS. The experiment outlined in this proposal does not aspire to offer definitive evidence to a primary role played by microglia contacting AISs. However, through eliminating CNS microglia and examining the effect upon the length and function of AISs, another step toward understanding this relationship may be achieved.

II. Experiment

As briefly mentioned above, the goal of this experiment is to examine the change in length and the change in function to the AIS after virtually all of the CNS's microglia have been removed. The relationship between AIS microglia populations and traumatic brain injuries (TBI) will also be investigated.

Microglia will be removed through knocking out the CSF1R gene, some subjects will receive a mild TBI immediately after CSF1R knockout through administering a controlled cortical impact, the AIS length will be determined through confocal microscopy, and AIS function will be tested through electrophysiology. 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

<u>(Cre)/LoxP microglia knockout</u>

(Cre)/LoxP recombination will be utilized in order to knockout CNS microglia through deleting the colony stimulating factor 1 receptor (CSF1R) gene, of which leads to the production of CNS

microglia (Elmore et al. 2014, Goldmann et al. 2013). 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 activation until the estrogen antagonist Tamoxifen is introduced. 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. Cre-ER will be introduced into the mouse through replacing the Cx3cr1 gene, a receptor of which microglia exhibit a high expression, with a sequence encoding for Cre-ER to produce $Cx3cr1^{CreER}$ (Goldmann et al. 2013).

A mouse engineered to express $Cx3cr1^{CreER}$ will be bred with a "floxed" mouse engineered to have two LoxP binding sites flanking the CSF1R gene. The offspring of these mice will possess each gene and the deletion of CSF1R will occur upon the introduction of Tamoxifen (Goldmann et al. 2013).

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; Elmore et al. 2014).

Administration mild TBI

Half of the subjects will receive a mild TBI. This will be executed through opening the cranium and conducting a procedure known as a controlled cortical impact, in which a machine will impact the brain at a specified velocity and will produce a brain injury in a controlled manner (Robertson et al. 2013). The effect upon the AIS in these subjects will be cross examined with the length and function of the AISs in the microglia knockout group and the healthy group in order to arrive at a potential relationship between brain injury and microglial populations on the AIS.

Analyze AIS length

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.

Analyze AIS function

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

Greer and Povlishock 2012 suggest that CNS microglia may play a role in stabilizing the AIS (Greer and Povlishock, 2012). Considering this, it is reasonable to predict that either AIS length will decrease or AIS function will become impaired (or both) after microglia are removed from contacting the AIS. To further substantiate this prediction, Greer and Povlishock also found that electrophysiological patterns in injured (mild TBI) CNSs are significantly different from the patterns of healthy CNSs and that these changes in neuronal excitability are likely to precipitate dysfunction (Greer and Povlishock, 2012). However, it is important to note that this finding does not apply to AIS length-- albeit a change in function may prove more consequential when considering the potential for disability.

With this being said, it is interesting to consider the findings of Elmore et al. 2014. This study removed all CNS microglia and observed that the subjects (mice) did not display any sort of cognitive impairment or impairment of motor skills-- in one instance the researchers observed that a group of mice with no CNS microglia completed a cognitive challenge faster than the healthy control group of which retained all CNS microglia (Elmore et al. 2014). Elmore et al. 2014 tested its subjects with behavioral challenges however, and their findings are more difficult to interpret with a high degree of confidence as to the health of the CNS.

In regard to the consequence of TBI to the AIS, Baalman et al. 2013 observed that the AIS is noticeably shortened after the CNS suffers a mild TBI and this observation is substantiated by other studies (Baalman et al. 2013; Buki and Povlishok. 2006; Povlishok et al. 1999). So, of course, it is very reasonable to predict that the AIS will shorten when the CNS encounters injury. However, Baalman et al. 2015 observed that populations of active microglia lose contact with the AIS following mild TBI (Baalman et al. 2015). This relationship between reactive microglia in contact with the AIS and brain injury may produce unexpected results.

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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