**Investigating the Integrity of the Axon Initial Segment**

**In the event of Microglia Attenuation**

By: Kevin Limlengco

**Introduction**

Multiple Sclerosis (MS) is a highly degenerative, auto-immune disease of the central nervous system (CNS) (Yadav et al, 2015). MS is identifiable by the degradation of the myelin sheath (Vassall et al, 2015). The myelin sheath, which insulates portions of the axon, facilitates energy-efficient and rapid travel of electrical nerve signals along the axon mediating communication between neurons or between the neuron and the target organ(Simons et al, 2013). Importantly, the myelin sheath is not a continuous structure but is segmented. Between two adjacent myelin segments is an axon domain known as the node of Ranvier (NOR).Within the node are clustered ion channels that allow the influx of sodium ions resulting in the propagation of action potentials along the axon (Arcancibia et al,2014). The continued propagation of these action potentials is essential for the conduction of the electrical nerve signals. Following demyelination, the ion channel clusters are disrupted and action potential propagation along the axon fails resulting in the loss of function of the CNS (Lubetzki & Stankoff, 2014).



In addition to demyelination, another prominent pathology associated with MS is inflammation, which presents as entry of peripheral immune cells such as T cells and macrophages into the CNS and by the activation of resident CNS immune cells known as microglia (Patejdl et al, 2016). In healthy conditions, entry of T cells and macrophages into the CNS is limited but in certain disease cases, including MS, barriers that regulate cell entry are disrupted. The entry of these cells into the CNS also results in the activation of the microglia (Bogie et al, 2014).

Microglia have several functions including surveying the CNS for indicators of injury or invasion (Wake et al, 2014). When microglia detect a potential insult, they become activated and respond in a number of ways including the release of pro-inflammatory molecules including reactive oxygen species. The release of these molecules is believed to be designed to target injured cells and invading cells for death (Wirendfeldt et al, 2011). After killing the cell, the microglia become phagocytic and “eat” the injured or invading cell. However, in MS, there is the possibility that this targeted attack no longer excludes healthy, CNS cells but also attacks the myelin sheath resulting in an auto-immune aspect of the disease. These attacks result in demyelination and consequential disruption of the NOR (Thompson et al, 2017).

Graphical representation of the Neuron and its segments: (Adapted from Maarten et al, 2016)

In addition to targeting the myelin sheath, the Dupree lab has recently published an article that implicates activated microglia in the disruption of another axonal domain known as the axon initial segment (Clark et al, 2016).

 Action potential is necessary to the processing of electrical signals along the axon, it is an adaptation that allows vertebrates to rapidly transmit electrical impulses across large distance (Hamada et al, 2016). To ensure proper propagation along the axon, both NOR and the myelin insulation needs to be maintained. Coincidentally both NOR and myelin sheath serves as potential target for MS, this is accomplished by the recruitment of reactive oxygen species (ROS) resulting for the degradation of both segments.

Along with the NOR and myelin sheath is the Axon initial segment (AIS), a highly reactive region against ROS. AIS is situated relative to the soma, and much like the NOR, ion channels are resided in this area. AIS aids with the proper initiation of the action potential, a feat mediated by its cytoskeletal structure and bundles of protein (Rodriguez, 2014). Maintenance of the AIS is critical for the process of proper propagation of the action potential, this is why several experimental design have been proposed to better understand its entirety. One finding that has been proven several times is that the microglia is among the prime suspects for the degradation of myelin and axonal membrane. AIS pathology isn’t solely subjugated under microglia’s jurisdiction, AIS plasticity has also been proven to advance the pathology of MS (Ilin et al, 2013), but for the sake of this experiment our focus would be on the microglia.

Microglia is among the primary cells that is concentrated in the CNS, it is a highly volatile cell that helps with the maintenance and recovery of the CNS should an injury occur. Microglia ensures proper scarring of the CNS when it’s subjected to harm (Yang et al, 2015). Microglia also helps get rid of intruding cells, trough it’s highly phagocytic behavior (Greenhalgh & David, 2014). However, despite its multiple role in maintaining a well-functioning CNS, microglia ultimately is the reason for multiple neurodegenerative pathologies. Microglia recruits a highly reactive reagent known as the ROS, and as previously stated ROS is responsible for the degradation of myelin sheath, protein and ion-channels residing in the AIS.

Graphical representation of resting and Active Microglia. (Cerda et al, 2015)

Thankfully multiple experimental models of MS have been brought up to better understand this highly destructive disease, one of which is Experimental autoimmune encephalomyelitis (EAE). Multiple pathogenesis symptoms of MS have been identified through the use of EAE due to its similarity in process of advancing the pathology (Constantinescu et al, 2011).

One experiment of great importance to us, is one by Clark et al (2016). In their experiment they investigated the AIS integrity when it is attacked by reactive microglia during EAE. EAE as stated above is an experimental model for Multiple sclerosis in an inflammatory environment, it involves the active phagocytic microglia. They started off by injecting a mouse with a predetermined solution which contains myelin oligodendrocyte glycoprotein peptide. Mice showed peak clinical symptoms at 15 days’ post injection. Severity of the mice’s symptoms upon the application of EAE were rated 0 – 4. Upon closer investigation they noticed that microglia’s morphology radically changed into the reactive form (thick and shorter branches). Using VelocityTM they were able to conclude that increase contact between the microglia and AIS greatly contributes to the advancement of the pathology. Knowing this we could hypothesize that elimination of the microglia would greatly improve the condition of EAE induce mice.

One method that shows great promise in regards of microglia attenuation is through the use of genetic engineering, specifically Cre-Lox. Cre-Lox recombination is a site specific recombination developed by Dr. Sauer, however, it wasn’t until 1998 when it was finally recognized as a reliable method to initiate recombination. Cre-Lox consist of two main parts, first off is the Cre recombinase it is a site specific recombinases that catalyzes the recombination of sequences. The other an equally important part of the process is lox-p. Lox-p acts as the marker or recognition sites. It tells the recombinase from which part of the sequence to cut from and what two ends are to be attached (Nagy, 2000).

Graphical representation of Lox points (Kuhn & Torres)

**Experiment**

The purpose of this experiment is to investigate the reactivity of the AIS against microglia in an EAE environment. If our hypothesis is correct attenuation of the microglia through the use of Cre-Lox Recombination should improve the subject’s condition or at least slow down the pathology.

**Colony Stimulating 1 Factor**

As stated before proliferation and activation of microglia is among the leading attributes of several neurodegenerative diseases. So eradication of the microglia should improve the pathology of those affected. One prospective target for this is the microglia’s colony-stimulating factor 1 receptor (CSF1R). CSF1R is responsible for the regulation of the mechanism concerning microglia’s proliferation (Olmos et al, 2016). It was shown buy Olmos et al, that pharmacological targeting of CSF1R greatly improved the condition of the mice, showing better memory and improved behavioral tasks.

Graphical Representation of the Inducible Tamoxifen mediated Recombination (Feil S et al, 2009)

**Cre-Lox**

Much like Clark et al, we are going to attenuate the microglia cells to investigate the integrity of the Axon Initial Segment in the absence of the cells. However, unlike Clark et al we are going to incorporate the Cre- Lox recombination. To accomplish this, we would need to knockout the colony stimulating factor 1 receptor (CSF1R). We would obtain CSF1R flox mice (marked with Lox-P) from Dr. Pollard (Li et al, 2006). However, since we need full control regarding the moment when recombination should take place, CSF1R mice would be mated with Cx3cr1-CreEr mice which we will buy from Jacks Labs. The Cx3cr1-CreER mice allows for conditional knock out of the gene in desire, this is accomplished through the use of nuclear localization capabilities of the estrogen. In this scenario the Cre recombinase is fused with the mutant ligand binding domain, this prohibits it from binding to estrogen. The offspring that would be produce from the following parental combination shouldn’t only allow site specific knock-out of the CSF1R gene but also give us complete control of the moment the desired function should take place (Feil et al, 2009).

Once our mice reach maturity we would initiate recombination, since CreER recombinases would be binded to a mutant ligand binding domain, recombination won’t take place until we introduce tamoxifen into our mice. Once tamoxifen is injected into our mice, the tamoxifen would bind to the ligand binding protein which should already be binded to the Cre-recombinase. Upon binding of the tamoxifen Cre-recombinase should translocate to the nucleus where it would perform its function knocking out the CSF1R gene. If homologous recombination is a success ablation of the microglial cells should take place.

**EAE**

To evaluate the neuronal integrity of our subject in an inflammatory environment, we will be performing EAE on our mice. We will inject 50 uL of a solution containing 3 mg/mL myelin oligodendrocyte glycoprotein peptide 35-55. Which was emulsified with Freund’s adjuvant containing 2 mg/mL of M. tuberculosis (Invitrogen Life Technologies, Grand Island, NY). It will then be followed up with an injection of intraperitoneal along with 300 ng Pertussis toxin in 200 uL phosphate saline which will be followed by a booster PT injection after 48 hours. If recombination mediated by the tamoxifen was a success, we shouldn’t see any extreme pathological symptoms i.e. malfunctioning hind legs since microglia wouldn’t be able to produce cytokines and recruit more microglia.

**Perfusion**

To avoid degradation of our tissue and cell we’ll perform a series of established method for perfusion. Animals will be deeply anesthetized, the concentration will be dependent on their body weight (0.016 mL/gm). The solution will contain 2.5% of avertin (2,2,2 tribromethanol; Sigma-Aldrich; St. Louis, MO) in 0.9% saline, the subject would then be perfused with 4% paraformaldehyde. Following this, brains will be surgically removed and immerse in PBS solution of 30% sucrose for 48 h to ensure preservation. Brains will be frozen in OCT compound, sectioned into 40 um thick coronal sections and stored at -80C.

**Immunohistochemistry**

To properly visualize the morphological changes in microglia and our AIS we would label each segment with antibodies. Mouse monoclonal antibodies directed against ankryn-G (AnkG; NeuroMab, Davis, CA; N106/36, 1:200) will be used to analyze the AIS, since ankryn-G is required for the establishment and maintenance of AIS (Clark et al, 2016). Aside from ankryn-G, Nav+ channel isoform 1.6 would also be targeted to differentiate between the NOR and action initial segment. IBA-1 would then be used to target the ionized calcium binding adaptor molecule-1 to assess the effect of Cre-Lox against the microglia.

Example of what an AIS image would look like (Adapted from Clark et al, 2016)

**Visualization**

All images will be collected through the use of Zeiss LSM 710 confocal laser scanning microscope. Confocal z-stacks with an optical distance of 25um will be collected from neocortical layer V for each of the six sections per mouse. AIS will be thoroughly inspected for any structural changes or damage, most specifically the length and ion channels. Microglia would also be quantified to determine whether attenuation was a success. Mouse’s severity of pathology would be compared to the amount of microglia present and the damage received by the AIS.

**Discussion**

If all goes well in the cross-breeding of cx3cr1 and creER we should have cx3cr1-creER mouse. However, in rare cases where we might face complication is when parental mouse doesn’t produce cx3cr1-creER offspring. If that’s the case, rebreeding of the mouse could be reinitiated, however, such events would set us back. Another method we can incorporate in our experiment is PCR, this would allow us to ensure that our mice possess the desired gene.

Another concern is that this experiment solely investigates the structural components of the AIS, through the investigation of any morphological changes in the said segment. We also investigate the amount of microglia residing in the AIS hoping to get a better understanding of the relation between the degradation of the AIS and active microglia. Possible improvements in the methodology is to investigate the propagation of the action potential along the axon in the end of the experiment, this could be done through the use of electrophysiology. Electrophysiology is the investigation excitable cells through the use of electrodes attach to the vertebrates of the animal.

Even though Cre-lox recombination is a highly reliable method for site specific recombination it still has its flaws, one possible improvement for this experiment is to use Crispr-Cas9. Crispr-Cas9 uses short palindromic associated proteins obtained from a bacteriophage (Guo et al, 2015).

There are numerous improvements possible to get a better understanding of the AIS and the microglial cells that resides in it however, this experiment’s main goal is to investigate whether a successful attenuation of the microglial cells could improve the condition of mice under EAE.

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