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14. ABSTRACT

The premise of this proposal which utilized a mouse model of MS known as experimental allergic encephalomyelitis was two-fold: first- that as animals age they become more susceptible to demyelinating disease and secondly that a molecule known to mediate the connection of myelin to the axonal membrane known as neurofascin was a key factor in determining the susceptibility of animals to demyelination. The research substantiated both of these premises: although older animals came down with disease at a somewhat later time point than younger cohorts, they became significantly sicker at this later time point; in addition when neurofascin was greatly decreased in a transgenic mouse the consequence was that these mice became sicker much quicker than a cohort of mice with normal levels of neurofascin. Therefore we have identified a key molecule related to susceptibility to demyelination (neurofascin) and showed that increasing age renders animals to a more profound disease state even though it occurs at a later time point.

15. SUBJECT TERMS susceptibility to EAE, effect of age on EAE, role of neurofascin in demyelinating disease

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Introduction

The severity of MS increases with the age of incidence for reasons that are not understood. This proposal sought to provide evidence for the basis of this observation. In particular previous studies reported that with aging the connections which anchor the myelin to the axon are compromised. Therefore when challenged with the as yet unknown factors which trigger MS, the compromised myelin integrity would leads to a more severe form of the disease. Additionally if the molecule known to anchor the myelin to the axon were lost, it would lead to a more rapid onset and more severe form of disease. Evidence to support or refute these ideas was obtained in the research supported by this proposal. Using a mouse model for MS known as experimental allergic encephalomyelitis (EAE), we initiated EAE in mice of different ages to observe the onset and severity of disease. In addition we challenged transgenic mice that had greatly diminished amounts of the molecule which links myelin to the axon (neurofascin) to also observe the onset and severity of disease in these animals. The results are documented in the "Accomplishments" section below.

Keywords-

EAE, aging, neurofascin, transgenics, demyelinating disease, multiple sclerosis.

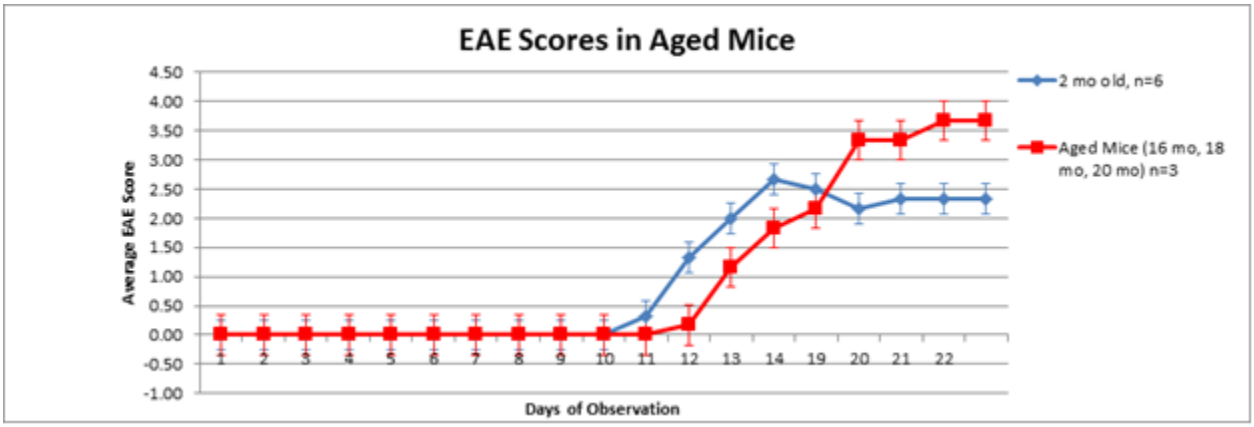
Accomplishments- The accomplishments on this project will be organized according to the tasks and subtasks submitted with the original proposal except for task 2 which was revised in our request for a one year extension submitted on 09/o5/2014.

Task 1- Evaluate the relationship of age to the onset, time course and severity of EAE (months 1-6)

1a. File the appropriate IACUC forms for approval of animal use (for both Task 1 and Task 2) –This has been done.

1b. Induce EAE in aged C57Bl/6 mice (12 in each age group) as follows: 3 months,8 months, 22 months; evaluate clinical severity with time in each age group.

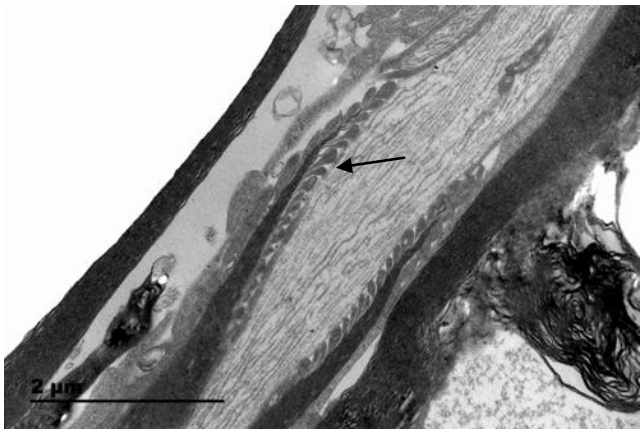
Using standard protocols established in our lab, we induced chronic EAE in cohorts of animals that were either 2 months old, or an aged group which consisted of animals that were 16,18 and 20 months old. The animals were then observed for 24 days after the initiation of EAE and scored for their clinical severity where 1=limp tail, 2=failure to right when placed on back, 3= loss of forelimb function and 4= loss of hindlimb function. The result is shown below.



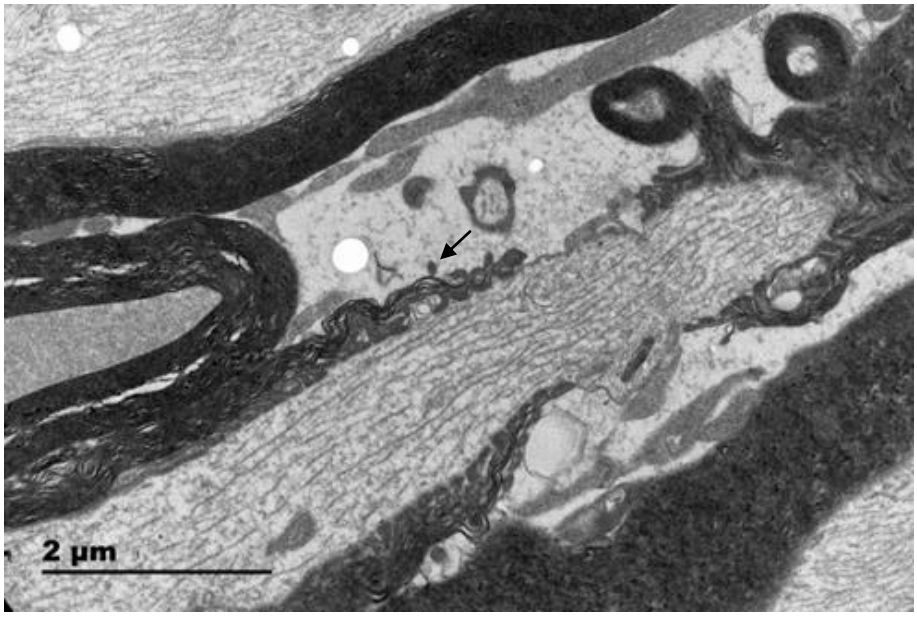
To our surprise, instead of the older animals becoming “sicker quicker” they became “sicker later”. Note that the onset of disease in the older animals was 2 days later than with the younger animals but whereas the younger animals only progressed to an average of stage 2 the older animals progressed until reaching a plateau of approximately four. Also it is interesting to note that the rate of progression is approximately the same.

1c. Perfuse 3 animals from each group and analyze by EM for status of paranode and transverse bands (months 3-4).

Twenty- month old animals at stage 4 of EAE were perfused and examined by electron microscopy. As observed in the electron micrograph below, note that the row of paranodal loops between the arrow is separate and distinct from each other. In normal conditions these loops would be right on top of each other with no space between them. This morphology is indicative of severe paranodal disorganization which would make these axons more susceptible to disruption and loss of function and result in more severe EAE.



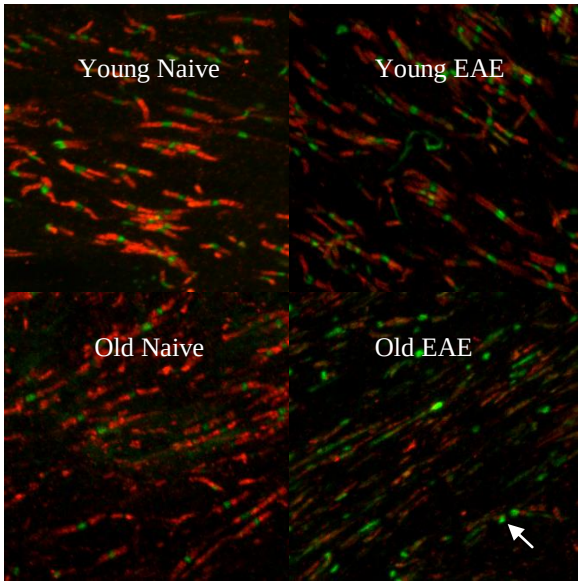
Severe disruption of the paranode can also be seen in the panel below as the arrow indicates one of the paranodal loops of myelin is actually inverted.



Both of these election micrographs support the view that disorganization of the paranodal region makes the aged animals more susceptible to the harmful effects of EAE. These experiments are being repeated to confirm these results. It should be noted that in younger animals these types of paranodal disruptions are never seen.

1d. Perfuse 3 animals from each group and analyze by light microscopy for myelin and axonal stability

Four cohorts of mice (young naïve, old naïve, young EAE stage 4 and old EAE stage 4) were analyzed by light microscopy for stability of a specialized region of the axon known as the node of Ranvier. The light microscopy utilized two markers: a red label (Caspr) for the regions immediately adjacent to the nodes of Ranvier known as the paranodal regions and a green label (Ankyrin G) for the node itself. The results are shown below:



. There is very little difference in the labelling and disposition of the nodes and paranodes among the Young Naïve, Young EAE or Old Naïve. However, there is a huge difference with Old EAE. Note in the old EAE panel, that although the nodes are very evident in most of the field (White arrows) , the paranodes (indicated by the red labelling) are virtually non-existent. In addition the green puncta are much elongated. These observations are consistent with the view that in the case of Old EAE the paranodes are dissipated so that the nodes (indicated by the green labelling) are somewhat elongated since as the paranodes break down the nodes will spread out. Once again this data indicates that in the aged animals the nodes and paranodes are more severely damaged and lost as a consequence of the induction and progression of disease.

Task 2- Using transgenic mice evaluate the relationship of paranodal stability to the onset, time course and severity of EAE.

Revised Subtasks for Task 2-

Task2a-- Rederive the transgenic mouse at VCU to a cleanliness standard suitable for McGuire Animal facilities- (completed)

Task 2b- Receive the male transgenic mice from the investigator at the University of Texas at San Antonio (completed)

Task 2c- Using germinal cells from the transgenic mice fertilize female mice which are suitable for the McGuire Animal facilities (completed)

Task 2d- Breed the offspring and ascertain that the transgenic trait is present – (completed)

Task 3e-Ship the rederived mice to McGuire (completed)

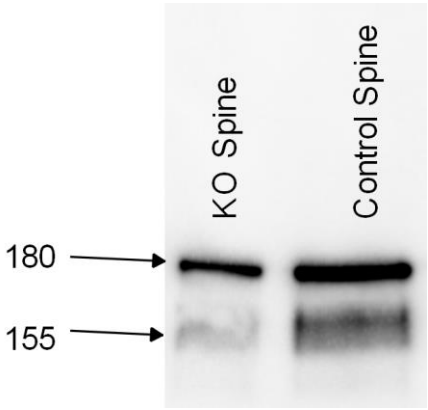
Task 3f- Breed the rederived to obtain sufficient numbers of mice to carry out EAE studies (completed)

Task 3g- Activate the cre-lox construct by injecting tamoxifen in transgenic mice (completed)
There was one slight detour in accomplishing this task. We learned of a new technique to give the tamoxifen in the animal feed which was supposed to be more efficient and reliable than giving the tamoxifen by injection. To our dismay we learned after 40 days spent on this experiment that in fact this method did not work as well as the tradition method of injecting the tamoxifen. Therefore we reverted back to the original technique and had some limited success with deleting the gene by this means.

Task 3h- - Allow mice to lose the gene whose loss is activated by tamoxifen (completed)

Task 3i- Check the treated mice by Western blot for the loss of the gene product (completed-see below)
A litter of mice shown to be homologous for the cre-lox construct were injected with either peanut oil (control) or tamoxifen in peanut oil for 10

consecutive days when they were one month old. After 43 days, the spinal cord was obtained from a sentinel mouse and subjected to Western blot analysis using a pan-specific antibody to neurofascin. The results are shown below:

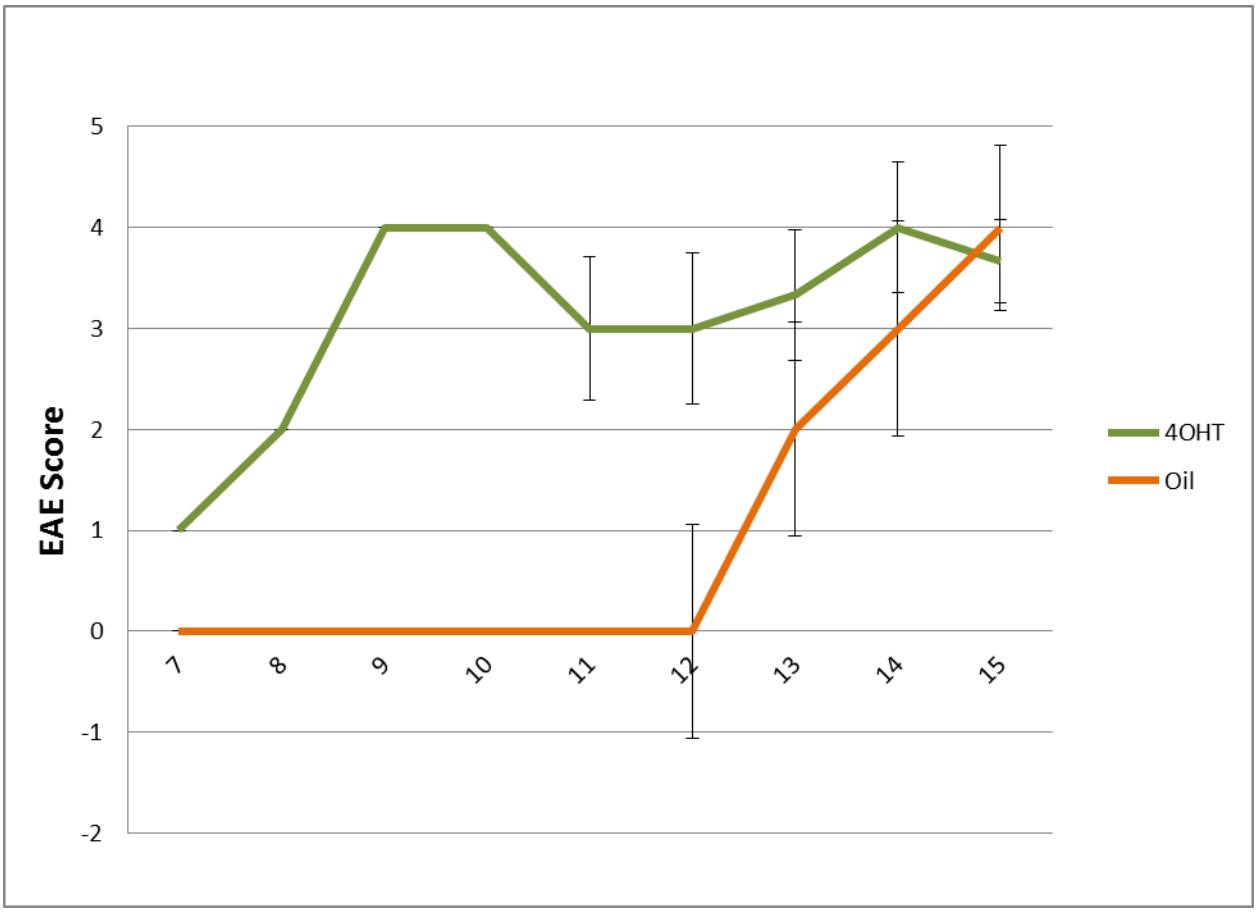


The band at 180 represents neuronal neurofascin which we would not expect to be altered in this transgenic mouse. The 155 band is due to neurofascin on the myelin mem brane at the internodes and is the molecule which we are interested in eliminating. Note that in the knock-out spine the concentration of this band is greatly reduced although not eliminated. These are the mice that we then used for the induction of EAE.

Task 3j- Induce EAE in the mice with the loss of gene product. (completed)

Task 3k- Score the mice for clinical symptoms from day of induction up to 30 days later (completed –see below)

Cohorts of control (oil) or 4-hydroxytamoxifen (4OHT) injected transgenic mice were induced for chronic EAE using the methods established in our laboratory. The mice were then followed clinically for 15 days. The results are shown below.



Note the profound difference in the onset of disease: the mice with reduced levels of glial neurofascin (green line) begin to get sick at day 8 while the onset of sickness in the controls is at day 13 which is what is expected for normal mice. In addition the animals reach peak disease after only 2 days in contrast to the controls which take 4 days to reach peak disease. Therefore we tentatively conclude that mice which have reduced levels of the glial neurofascin which anchors the myelin to the axon get "sicker-quicker" indicating that the loss of this key molecule (which we have previously shown to occur with aging) leads to increased susceptibility and increased severity of disease. The problem with this study was the size of the animal cohorts that got sick was low, lending uncertainty to the results.

Task 3L- Perfuse affected mice and characterize the CMNS Morphologically by light level and EM studies. (Not completed)

Task 3M- Write up results and submit for publication. (Not completed)

Task 4- Draw overall conclusions from data gathered in task 2 and task 3 and apply for further grant support for this project (Not completed).

4a. Submit preliminary proposal to appropriate administrative review for grant submission (Not completed).

4b. Write up and submit proposals to appropriate funding agencies (Not completed)

Impact

Clearly the only way for the results of this study to have an impact is to present them at national meetings or in publications- neither of which we have been able to do due to complications mentioned below. However we believe that these results will impact our understanding of the consequences of multiple sclerosis in an aging population. In particular we have determined that in the animal model for MS that when older animals are challenged with disease the paranodal regions of the myelinated axons are completely disorganized. This disorganization includes a spreading of the paranodal regions with consequent spreading of the nodal region. This disorganization is consistent with a loss of function of the myelinated axons which are affected. In addition our preliminary results with the transgenic animals indicate that the reduction/loss of glial neurofascin leads to increased susceptibility to disease. Since this molecule appears to be lost with aging, it means that in general aged individuals are more susceptible to MS. Further statistically significant experiments with transgenic animals will be required to solidify these conclusions.

Changes/problems

Frankly obtaining and deriving transgenic animals has been a nightmare. We assumed several pairs of these animals would be available when we started the project. Unfortunately the PI who had these animals transferred to a new institution at the time our grant started. Prior to the move he reduced all his animals to a minimum so that the breeding pairs of animals only became available to us in March of 2014. There was a problem with these animals since they were infected with helicbacter and so when they had to be shipped to our sister institution (VCU) where they were “rederived” to produce mice that were heliobacter free. What this involved is taking a clean female mouse and injecting hormones so that she would

produce eggs removing the eggs and carrying out in vitro fertilization. The eggs are then implanted in a “pseudopregnant” mouse which would then produce “clean” offspring. This whole process took 7 months which meant that the first “clean” pairs of mice were delivered to us from VCU in October of 2014. Meantime we set up the tail clip PCR assays which are required to check the genotype of the mice through subsequent breedings. The next nightmare was breeding mice which were homozygous for the floxed neurofascin gene. Each mating required about 5 weeks from mating to offspring followed by another 5 weeks until the offspring could be screened. Next the CRE would have to be activated with tamoxifen which was a 10 day treatment. As previously mentioned we took a detour at this point since instead of injecting the tamoxifen we gave it to the animals in fed and subsequently found out it did not work since the protein levels never declined. That detour cost us another 3 months. Finally even after the animals had the gene deleted it took at least 40 days for the protein level of neurofascin to drop appreciably. It should be appreciated that with these time requirements we had no problem using our one year extension and even then we did not have the opportunity to induce EAE in the animals. Finally we would up with 3 controls and 4 knock-outs for the EAE experiment. However not all the animals became sick in this experiment although we generated enough data to tentatively make some conclusions about the role of glial neurofascin in susceptibility to EAE.

Our intention was to generate one publication from all this data which would document the role of glial neurofascin in demyelinating disease and aging. Due to the paucity of transgenic animals we now realize this will not be possible. However we do have enough data on the aged animals to publish and we are currently pursuing this course.

Products- none

Other collaborating Organizations-none

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Special Reporting Requirements- none

Appendices- none