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TITLE: Investigating the role of creatine in oligodendrocyte regeneration during CNS remyelination

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14. ABSTRACT: Chronic oligodendrocyte and myelin loss contributes of axonal dysfunction and neurodegeneration in multiple sclerosis (MS). Although oligodendrocyte precursor cells (OPCs) are abundant in the CNS, and are able to regenerate myelin in the early stages of MS, it remains unknown why remyelination fails in the chronic stage of MS. One possibility, which remains to be investigated, is that regenerated oligodendrocytes, despite differentiating from OPCs, fail to survive in MS lesions. It is known that oligodendrocytes appear abnormal and die in MS lesions. Therefore strategies to enhance survival of newly regenerated oligodendrocytes in MS would improve their ability to remyelinate axons. We have found that creatine, a compound involved in cell survival and energetic metabolism, promotes oligodendrocyte survival in culture. When experimental demyelination was performed on mice lacking the expression of Gamt, the enzyme responsible for creatine synthesis, we found that most of the newly regenerated oligodendrocytes died instead of restoring myelin. Remarkably, when creatine was injected directly into the demyelinated tissue, we found that the number of regenerated oligodendrocytes and the extent of myelin labeling in lesions increased significantly. These unexpected and intriguing observations suggest that brain-synthesized creatine plays a crucial role in stimulating remyelination by enhancing regenerated oligodendrocyte survival. Therefore, the goal of this project is to investigate the protective and proregenerative effect of creatine on regenerated oligodendrocyte survival and remyelination in mice. To achieve our goals, we will examine a genetically modified mouse mutant that does not express Gamt in oligodendrocytes, and assess its ability to maintain survival of regenerated oligodendrocytes and myelin after demyelination. We will also determine if altered creatine levels and reduced regenerated oligodendrocyte survival occurs in aged mice, since advanced age has been suggested as a contributor to remyelination failure and disease progression in MS.

15. SUBJECT TERMS
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Principal Investigator: Jeffrey K. Huang
CDMRP 2nd year report

1. INTRODUCTION

The goal of this study is to test the hypothesis that rOL protection through creatine improves CNS remyelination. To this end, we will determine i) if creatine synthesis in rOLs is required for rOL survival and remyelination, ii) if diminished creatine levels and rOL integrity contribute to remyelination impairment associated with aging, and iii) if the systemic administration of creatine and cyclocreatine (a blood brain barrier permeable creatine analog) can improve CNS remyelination. We will also determine iv) what transcripts and pathways in rOLs are affected with creatine deficiency.

2. KEYWORDS: Creatine, guanidinoacetate methyltransferase (GAMT), remyelination, oligodendrocytes, transgenic mice, experimental demyelination, aging.

3. ACCOMPLISHMENTS

The major goals of this reporting period was to analyze Plp1-CreERT;Gamt-fl/fl mice (Aim 1, Major Task 1); to complete analysis of mice under specialized creatine diets (Aim 1, Major Task 2); begin analysis of aged mice with and without creatine treatment (Aim 2, Major Tasks 1 and 2). Major activities described below:

<u>Aim 1: Examine the role of creatine in rOL survival and remyelination</u>.

Major Task 1: Analyze rOL viability and remyelination in OL-specific Gamt knockout mouse.

1. **Characterization of the new Gamt-fl/fl transgenic mouse line.** In the prior year, we had generated preliminary results suggesting GFP was detected in oligodendrocytes in the CMV-Cre;Gamt-fl/fl line. We have since performed additional characterization for other cell types in the brain with this line, and confirmed that GAMT is only expressed in oligodendrocytes in the brain (**Fig. 1**). Our results confirm that Gamt is only expressed in oligodendrocyte lineage cells in the postnatal brain.

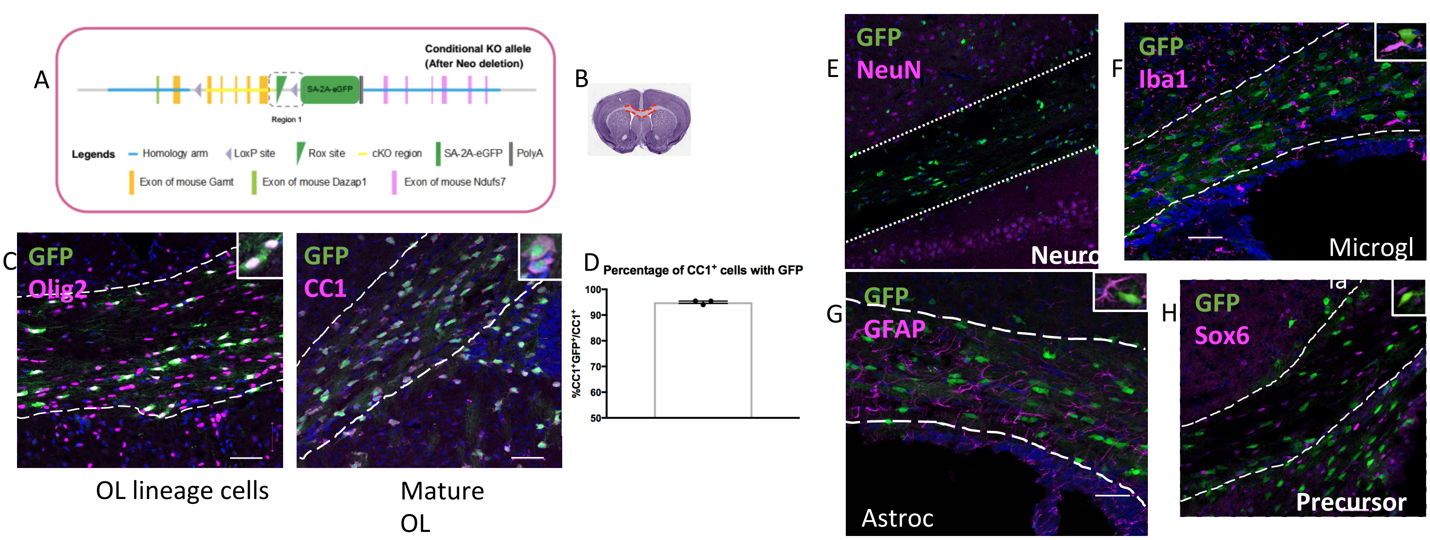


Fig 1. Guandinoacetate methyltransferase (GAMT) is only expressed in mature oligodendrocytes (OL). A) Diagram of new transgenic mouse model where loxP sites are located before exon 2 and exon 6 of mouse *Gamt*. Within excision region lies a stop codon where excision of *Gamt* exons leads to expression of green fluorescent protein (GFP). B) Region of corpus callosum in mouse coronal sections used for images (C) and quantification of OL (D). C) Visualization of colabeling of GFP and Olig2, marker for OL lineage cells, and CC1, marker for mature OL within corpus callosum. D) Quantification of percentage of mature OL ($CC1^+$) with GFP showing 94% colocalization. E) GFP is not colabeled with NeuN, marker of neurons. F) GFP is not colabeled with Iba1, marker of microglia. G) GFP not colabeled with GFAP, marker of astrocytes. H) GFP not colabeled with Sox6, marker for neuronal/ glial precursor cells. Scale bar is 50 microns; quantification n=3.

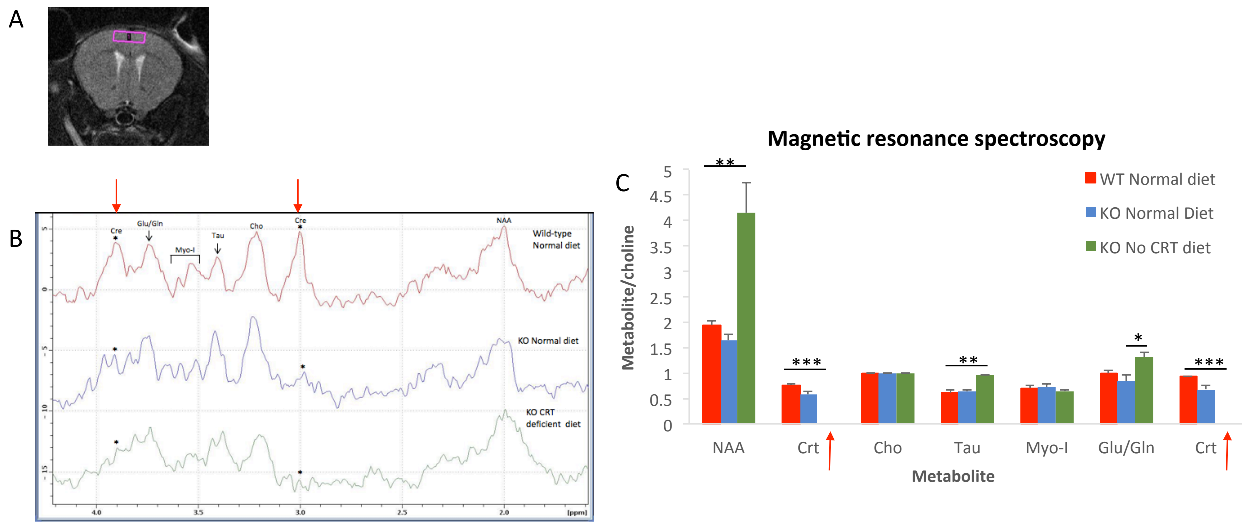


Fig 2. Guandinoacetate methyltransferase (GAMT) global knockout (KO) mice on creatine deficient diet show unmeasurable creatine and metabolite dysregulation in the prefrontal cortex with magnetic resonance spectroscopy. A) Location of voxel in prefrontal cortex of mouse brain for magnetic resonance spectroscopy. B) Image of metabolite peaks between WT on normal diet, KO on normal diet and KO on a creatine deficient diet. C) Quantification of metabolite levels in each animal. KO on creatine deficient diet show unmeasurable levels of creatine and increases in N-acetyl aspartate (NAA), taurine (Tau), and Glutamate/glutamine (Glu/Gln) levels. N=3 in each group, normalized to choline.

2. **Importance of creatine deficient diet in our study.** In our previous publication, we did not observe obvious reduction in oligodendrocytes in the postnatal brain, and only detected increased oligodendrocyte cell death in lesions during remyelination. We have since performed magnetic resonance imaging on our mice and found, remarkably, that creatine obtained from the regular rodent diet provides sufficient creatine in the mouse brain, even in the GAMT-/- mice (**Fig. 2**). This might explain why there was a lack of obvious defect in oligodendrocyte development or brain function in postnatal mice. We have since put all of our transgenic mice on creatine deficient diet, and now found that oligodendrocyte density in the corpus callosum is reduced in GAMT-/- mice fed with creatine deficient diet (**Fig 3**). This important finding indicates that the absence of creatine in the mouse brain (through feeding creatine deficient diet, and deletion of GAMT to remove endogenous creatine synthesis) severely affected oligodendrocyte development. We are now further characterizing these mice for defects in oligodendrocyte lineage cell progression.

3. **Generation of a Pdgfra-CreERT;Gamt-fl/fl mouse line.** To determine if *Gamt* expression in rOL is required for rOL survival and remyelination, we have crossed *Gamt$^{flox/flox}$* with *CMV-Cre* (Jackson Laboratories) to generate a *CMV-Cre*;*Gamt$^{flox/flox}$* mouse line. Moreover, instead of crossing the *Gamt$^{flox/flox}$* mice with a Plp-CreERT driver line, we crossed the mice with a Pdgfra-CreERT line, to conditionally delete GAMT in oligodendrocyte lineage cells (Pdgfra-CreERT), rather than only in mature

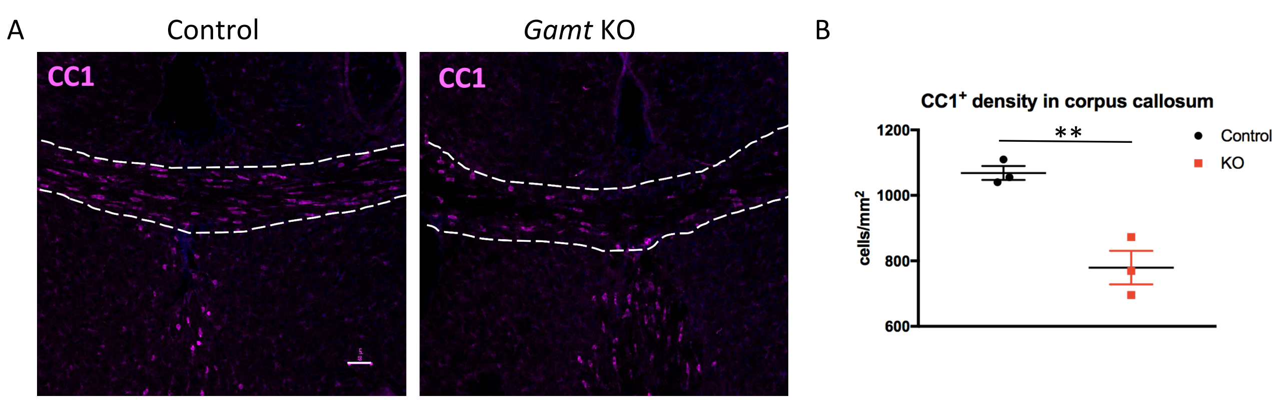


Fig 3. Deletion of *Gamt* leads a reduction in mature OL in the corpus callosum and increase in axonal dystrophy in hippocampus at P14. A) Reduced mature OL (CC1+) in *Gamt* knockout corpus callosum (bottom) compared to control (wild-type) corpus callosum (top). B) Quantification of mature OL (CC1+) in corpus callosum show significant reduction in mature OL in the KO. N=3 unpaired t-test; scale bar is 50 microns.

oligodendrocytes (Plp-CreERT). We have since generated the *Pdgfra-CreERT*;*Gamt*$^{flox/flox}$ mouse line and will use this line in our studies going forward.

4. **Found reduced oligodendrocyte numbers in lesions in Pdgfra-CreERT;Gamt-fl/fl mice.** We have performed focal demyelination on these mice and found a reduction in mature oligodendrocytes in lesions during remyelination thus confirming our hypothesis that GAMT expression in oligodendrocytes is necessary for oligodendrocyte function during remyelination (**Fig. 4**). We plan to further characterize these mice by performing additional immunostaining studies and examine additional timepoints, as well as perform electron microscopy analysis for remyelination. We will also determine if the reduction in oligodendrocytes is due to impaired oligodendrocyte survival.

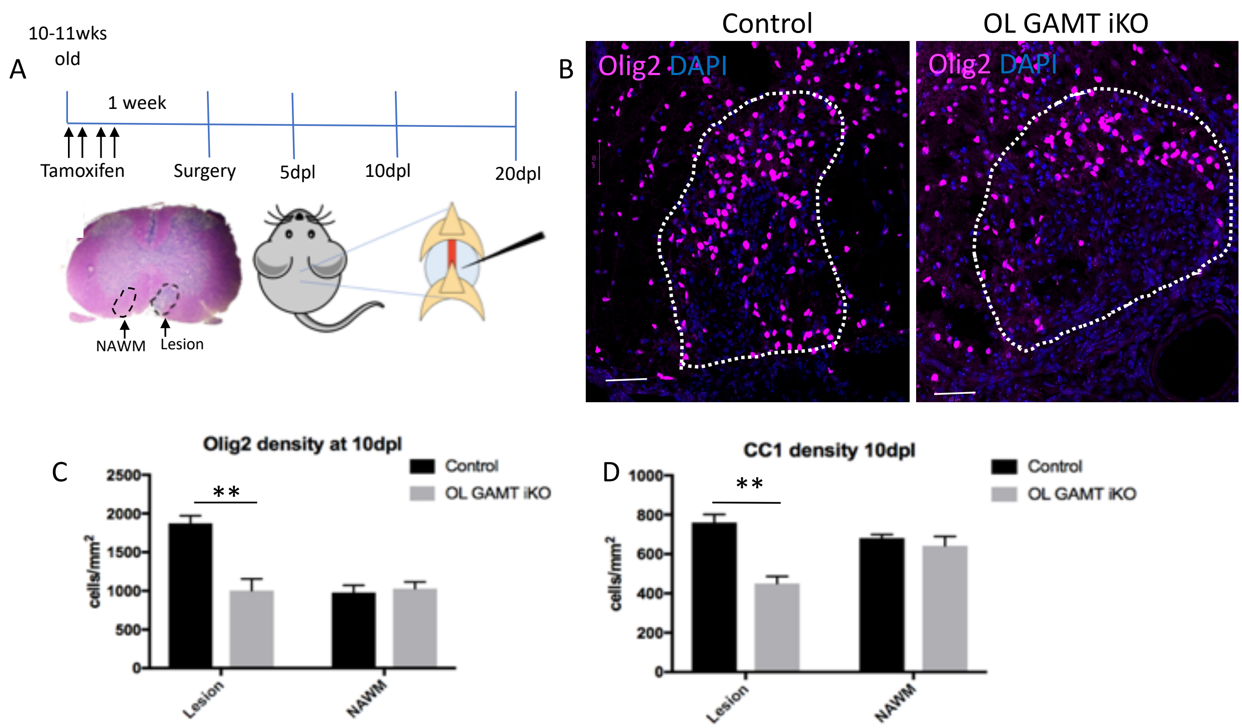
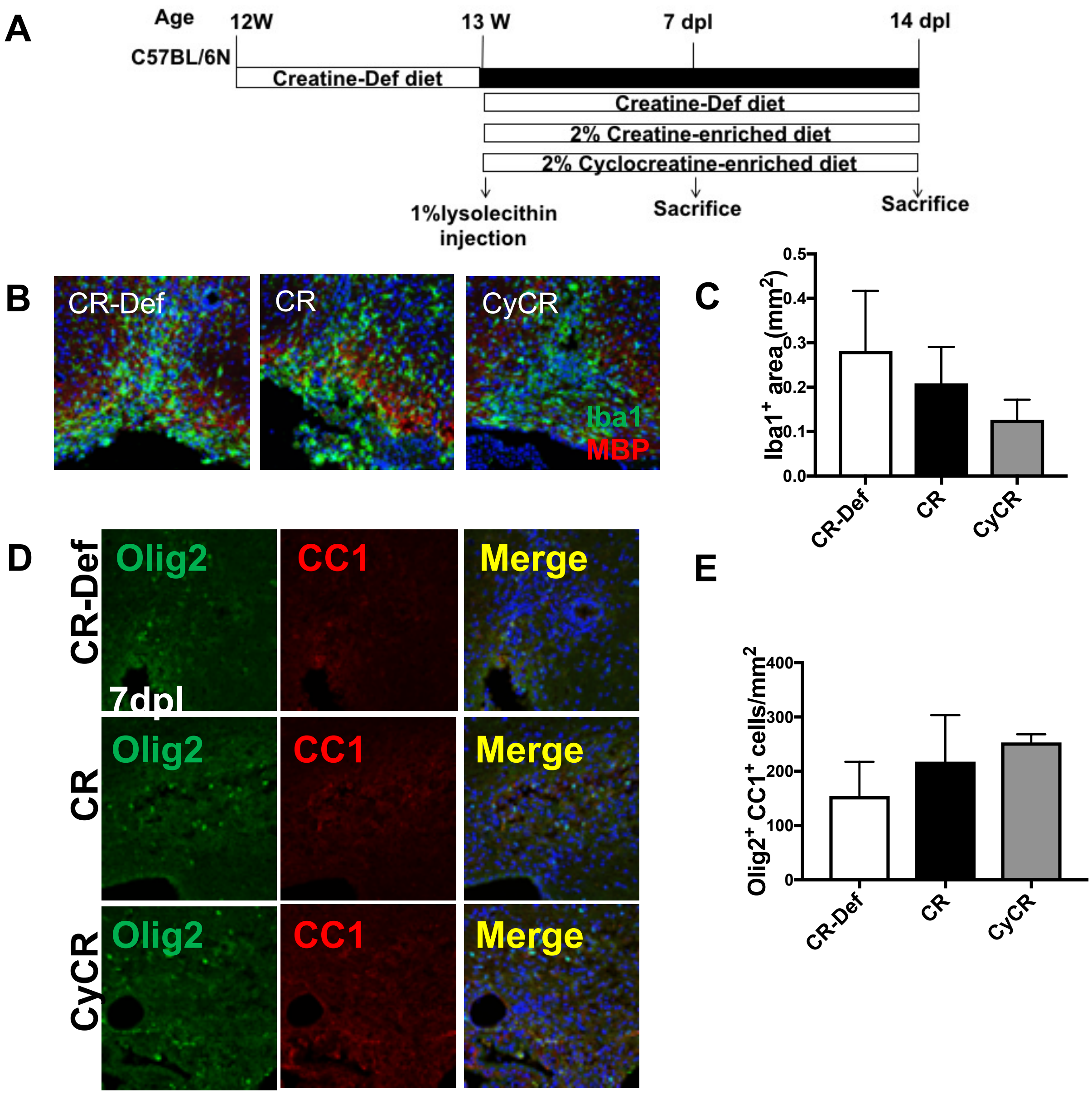


Fig 4. Deletion of *Gamt* from OL lineage cells (Pdgfra-CreER;Gamtfl/fl) leads to a reduction in mature OL after demyelinating injury at 10 days post lesion (dpl). A) Diagram of demyelinating injury protocol following tamoxifen injections for four days to induce removal of *Gamt* in OL lineage cells (OL GAMT iKO). Lysolecithen is injected into dorsal white matter of spinal cord and lesion is compared to normal appearing white matter (NAWM) on opposite side of spinal cord. B) Reduction in total OL in the OL GAMT iKO lesions compared to controls with no difference in NAWM at 10dpl. C) Quantification of total OL reduction in OL GAMT iKO compared to controls at 10dpl. D) Reduction in mature OL in OL GAMT iKO in addition to reduction in total OL at 10dpl. N=3; 2-way ANOVA with Tukey multiple comparisons; scale bar is 50 microns.

Major Task 2: Analyze effect of creatine/cyclocreatine administration on rOL survival and remyelination in Gamt deficient mice.
Progress: Gamt-/- mice produce very few pups (average 3 pups/litter). We have scaled up our breeding so we can obtain enough animals for this experiment.

<u>Specific Aim 2: Analysis of creatine biosynthesis and rOL viability in aged mice.</u>
Major Task 1: Compare creatine levels and rOL survival between aged and young mice.
Progress: We have lesioned both P70 and P360 mice, and have collected tissue sections from these mice. Analysis of enzymes associated with creatine synthesis in these mice is underway.

Major Task 2: Analyze effect of systemic creatine/cyclocreatine administration on rOL survival and remyelination in aged mice.
Progress: We have fed aged mice with creatine and cyclocreatine enriched diet. These mice have been perfused. We have performed immunostaining analysis and generated preliminary data showing that mice with creatine or cyclocreatine treatment exhibited reduced inflammation, and increased



oligodendrocyte lineage cells in lesions (**Fig. 5**). We will analyze more mouse sections for statistical analyses in the coming year. We have also generated samples for electron microscopy analysis.

4. IMPACT: Nothing to Report

5. CHANGES/PROBLEMS: We found that it is important that GAMT knockout mice are fed creatine deficient diet to ensure complete absence of creatine in the brain. Other than this issue, this is no significant changes in the project or direction.

6. PRODUCTS: We published a review article on oligodendrocyte bioenergetics in The Neuroscientist, and an original article in PNAS on the use of vital dye labeling to analyze remyelinating lesions.

Fig 5. Lysolecithin induced demyelination was performed on aged mice (P360). A) Mice were fed with creatine deficient diet for 1 week prior to demyelination. After demyelination, mice were fed with specialized diet for 14 days before sacrifice. B) Immunostaining analysis for Iba1 and MBP show the area of demyelination. C) Analysis of Iba1 intensity reveals a possible reduction in inflammation in the lesion in creatine and cyclocreatine treated mice. D) Immunostaining analysis for oligodendrocytes. E) Analysis of oligodendrocytes in lesions reveal a possible increase in oligodendrocytes in creatine and cyclocreatine treated mice.

1. Tracking the evolution of CNS remyelinating lesion in mice with neutral red dye. Baydyuk M, Cha DS, Hu J, Yamazaki R, Miller EM, Smith VN, Kelly KA, Huang JK. Proc Natl Acad Sci U S A. 2019 Jun 24. pii: 201819343. doi: 10.1073/pnas.1819343116. [Epub ahead of print]. PMID: 31235582
2. Oligodendrocyte bioenergetics in health and disease. Rosko L, Smith VN, Yamazaki R, Huang JK. The Neuroscientist. Review. 2018 Aug 20:1073858418793077. doi: 10.1177/1073858418793077.

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Name:	*Reiji Yamazaki*
Project Role:	*Postdoc*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*9*
Contribution to Project:	*Dr. Yamazaki has performed work on characterizing mouse remyelination in mice treated with creatine and cyclocreatine.*

Name:	*Lauren Rosko*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	12
Contribution to Project:	*Ms. Rosko has performed work on characterizing novel transgenic GAMTfl/fl mice in development and after demyelinating injury, and performed MRI studies.*

GOALS FOR YEAR 3:

1. To complete analysis of Aims 1 and 2.
2. Publication of creatine and cyclocreatine administration of aged mice.
3. Begin RNA-sequencing analysis of brain and demyelinated lesions from Gamt-/- and WT mice (Aim 3). We have developed a novel strategy to label demyelinated lesion using neutral red, a vital dye. This approach allows us to dissect out lesions during remyelination. This work was recently published in PNAS. We plan to use this strategy in Year 3 to perform RNA-sequencing analysis of demyelinated lesions from Gamt-/- and WT mice.