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

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13. SUPPLEMENTARY NOTES

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

None Listed

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Grant: W81XWH-17-1-0268
Title: Investigating the Role of Creatine in Oligodendrocyte Regeneration during CNS Remyelination
Principal Investigator: Jeffrey K. Huang

CDMRP 1st year report
STATEMENT OF WORK
PROPOSED START DATE – September 1st, 2017

A PhD student and a postdoc were assigned to this project. Although the project start date was in June 2018, we were unable to start this project until October 2018 when the hiring/contract was finalized for the postdoc to begin in my lab. Below we describe our accomplishments to date with regards to the Major Tasks proposed for Months 0-12:

Aim 1: Examine the role of creatine in rOL survival and remyelination. Prior to this application, we found that creatine addition to OL cultures *in vitro* increased mitochondria density and ATP production, and enhanced OL survival, and that lysolecithin-induced demyelination in *Gamt*$^{-/-}$ mice resulted in a significant reduction in regenerated oligodendrocyte (rOL) density and MBP staining in lesions. However, our previous study relied on mice with global creatine deficiency, therefore **it remains unknown if intracellular creatine synthesis in rOLs is required for rOL survival and remyelination.** To answer these questions, a custom designed conditional *Gamt* deletion mutant (*Gamt*$^{flox/flox}$, generated by Cyagen), containing flanking loxP sites around exons 2 to 6 will be used to enable Cre-mediated *Gamt* knockout (**Fig. 3G**). This mutant also expresses a nuclear GFP (nGFP) upon Cre-mediated excision, thus allows the tracking of rOLs in lesions. To determine if *Gamt* expression in rOL is required for rOL survival and remyelination, we will cross *Gamt*$^{flox/flox}$ with *Plp1-CreERT* (Jackson Laboratories) to generate a *Plp1-CreERT*;*Gamt*$^{flox/flox}$ mouse line.

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

Subtask 2: Perform lysolecithin-mediated demyelination on the following groups of mice:
1. Plp1-CreERT;Gamt-fl/fl mice (n=30; receive tamoxifen)
2. Plp1-CreERT;Gamt-fl/fl mice (n=30; no tamoxifen)
3. Plp1-CreERT (n=30; receive tamoxifen)
4. Gamt–/– mice (n=30)

Timeline: 12-18

Accomplishments:

1. **Breeding in progress.** We have received the Gamt-fl/fl mice and are currently breeding them with the Plp1-CreERT line to accomplish this task. This has taken some time because we needed to generate a breeding line for Gamt-fl/fl (custom made and received from Cyagen), and Plp1-CreERT (ordered from Jackson Labs).

2. **Characterization of the new Gamt-fl/fl transgenic mouse line.** Upon receipt of the Gamt-fl/fl mice, we wanted to first test if this mouse line allowed us to confirm Gamt expression in oligodendrocyte lineage cells in the CNS before testing its deletion in oligodendrocytes by using the Plp1-CreERT mouse line. Therefore we crossed the Gamt-fl/fl mice with a CMV-Cre line to confirm that Gamt deletion could be induced with Cre recombinase in vivo, and to determine if GFP expression could be detected in the CNS. We have generated new data showing that GFP was highly expressed in oligodendrocyte lineage cells in the CMV-Cre;Gamt-fl/fl line in white matter tracts of adult mice (**Fig. 1a**). Moreover, following demyelination, GFP expression was mainly detected in oligodendrocyte lineage cells in lesions of CMV-Cre;Gamt+/- heterozygotic mice and not in control CMV-Cre;Gamt+/+ mice (**Fig. 1b and c**). These results confirm that Gamt is primarily expressed in oligodendrocyte

lineage cells in development and during remyelination, and supports the analysis of GAMT loss of function in oligodendrocyte lineage cells in Major Task 1.

3. **Analysis of creatine on ER stress in oligodendrocytes *in vitro*.** Previous studies have demonstrated that oligodendrocytes require functional ER stress proteins during remyelination, suggesting that increased ER stress may be informative indicator of rOL dystrophy. While the mice are being bred, we tested the effect of creatine application on ER stress in primary oligodendrocytes. We induced ER stress in primary oligodendrocytes by addition of tunicamycin, and evaluated the expression of CHOP, a marker of ER stress, in oligodendrocytes. Preliminary data so far show that creatine application did not reduce ER stress (based on CHOP immno-labeling) in oligodendrocytes (**Fig. 2**). We are currently testing different tunicamycin and creatine concentrations in our *in vitro* study, and will compare our results *in vitro* to *in vivo* study in the coming year.

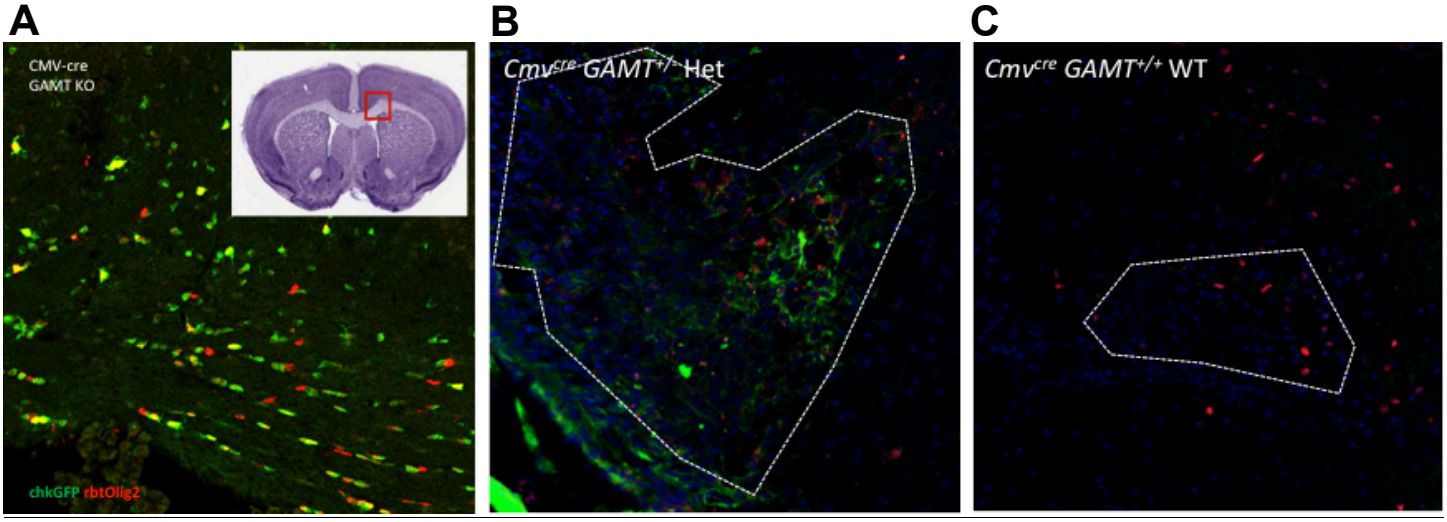

Figure 1. Induction of GFP in oligodendrocyte lineage cells of CMV-Cre;GAMT+/- mice. Mice with heterozygous deletion of Gamt is expected to allow GFP expression. **A**) Immunostaining with anti-GFP and anti-Olig2 (which labels oligodendrocyte lineage cells) in the adult mouse corpus callosum shows strong GFP co-labeling with oligodendrocyte lineage cells. **B**) Following spinal cord demyelination, GFP expression was also co-localized with Olig2 in the lesion (within the dashed lines). **C**) No GFP expression in oligodendrocyte lineage cells was detected in lesion of a control mouse which has both copies of Gamt expressed, thus confirming that GFP expression is only induced upon Gamt deletion.

4. **Analysis of mitochondria density during remyelination**. Since we have previously found that creatine regulates mitochondria density in OLs *in vitro*, we determined if rOLs exhibit altered mitochondrial distribution and density in the cell body and processes. To analyze mitochondria density in rOLs, we examined the extent of mitochondrial density in oligodendrocytes of Gamt-/- mice during remyelination, and the impact of creatine monohydrate administration in lesions on mitochondrial density in oligodendrocytes during remyelination in wildtype and Gamt-/- mice. We have generated new data showing that Gamt-/- mice displayed a significant reduction in Tom20+ mitochondria in oligodendrocytes (**Fig. 3A**), and that creatine administration significantly increased mitochondrial density in oligodendrocytes during remyelination (**Fig. 3B**), thus supporting our previous observation *in vitro*. Once we have the mice bred and lesioned, we will use Tom20 as a marker for mitochondria in oligodendrocytes in lesions.

Major Task 2: Analyze effect of creatine/cyclocreatine administration on rOL survival and remyelination in Gamt deficient mice.

<u>*Subtask 1:*</u> Perform lysolecithin-mediated demyelination on following group of mice:

1. Gamt-/- mice (n=90)

2. WT mice (n=30)

Subtask 2: Feed mice with the following diet from 1 dpl until sacrifice

1. Gamt-/- mice (n=30; receive creatine monohydrate diet)
2. Gamt-/- mice (n=30; receive cyclocreatine diet)
3. Gamt-/- mice (n=30; receive regular diet)
4. WT mice (n=30; receive regular diet)

Subtask 3: Perfuse total n=60 mice at 3 postlesion time points (5 mice per postlesion time point X 4 groups) with paraformaldehyde, and perform cryosectioning of lesioned spinal cords.

Subtask 4: Perfuse total n=60 mice at 3 postlesion time points (5 mice per postlesion time point X 4 groups) with glutaraldehyde, and tissues processed for embedding, thin sectioning, and electron microscopy.

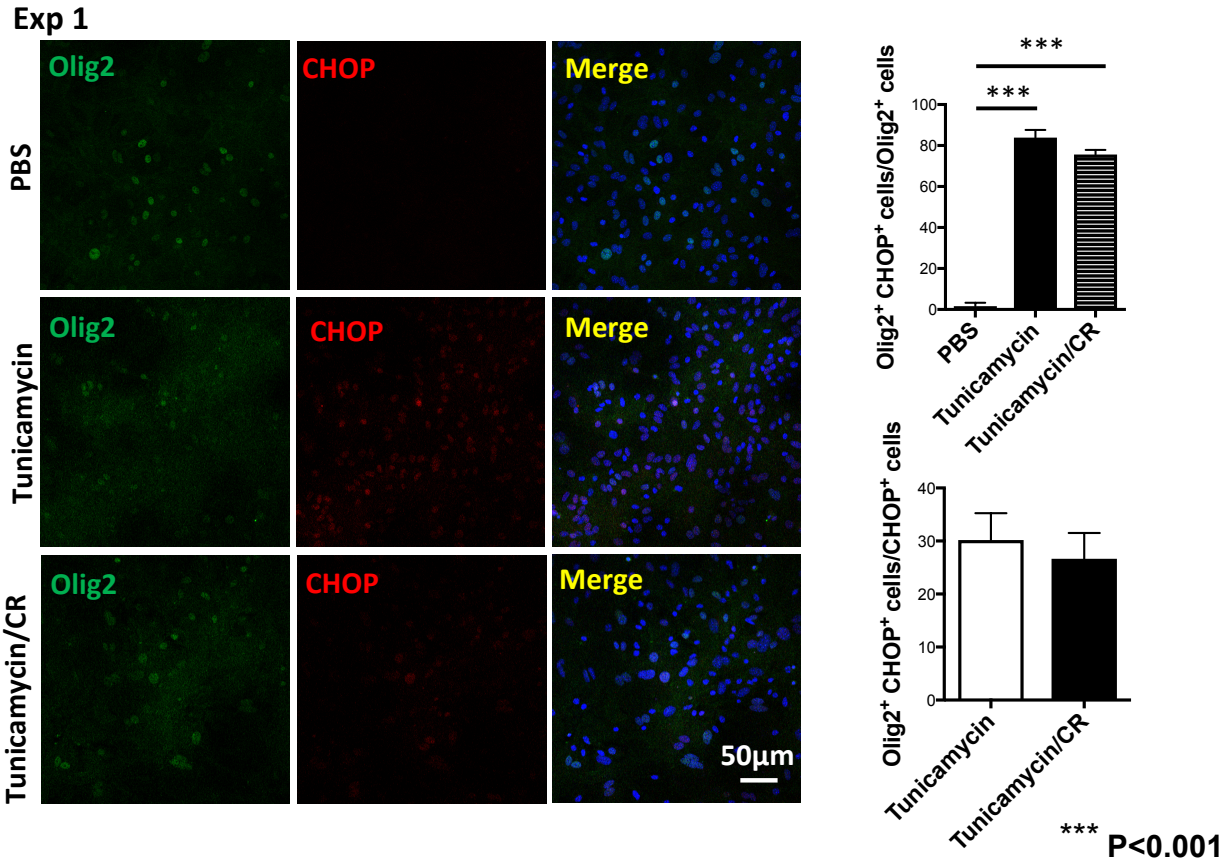

Figure 2. Effect of creatine on ER stress in oligodendrocytes. ER stress was induced on primary rat oligodendrocyte cultures with tunicamycin (10µg/ml), followed by addition of creatine monohydrate (100µM). The ER stress marker CHOP increased significantly in oligodendrocytes after tunicamycin treatment. However, creatine addition (CR) did not appear to significantly reduce CHOP expression.

Accomplishments:

1. We have previously found that Gamt-/- did not exhibit developmental abnormality, but displayed impaired oligodendrocyte survival following demyelination. This observation led us to hypothesize that creatine-derived from mouse diet might have prevented oligodendrocyte abnormality during development. We have since put pregnant mice on creatine deficient diet for at least one month before giving birth, and have now found that pups born from mothers fed on creatine deficient diet appeared smaller than those on regular diet (**Fig. 4**). We are currently characterizing the extent of oligodendrocyte lineage cell development in these mice. Because of this result, we have since decided to put all of our mice on creatine deficient diet before performing the proposed Major Task 2 to

ensure that no endogenous creatine confounds our analysis of oligodendrocyte survival during remyelination.

2. We have now received all the specialized diets. In light of the possibility that creatine in regular diet could affect our analyses, we have ordered extra creatine deficient diet so that mice described in Major Task 2, Subtask 1 will receive creatine deficient diet first before demyelinating lesion is performed on them. After injury, the mice will be put on the specialized diets as described in Subtask 2.

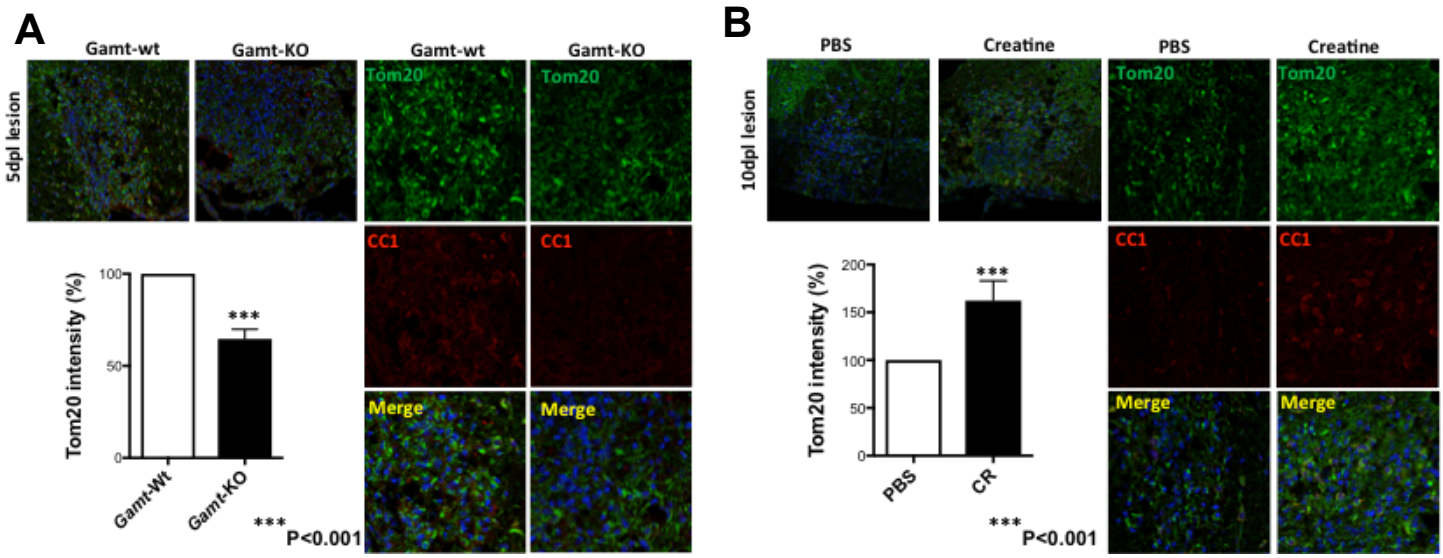

Figure 3. Altered mitochondrial density in oligodendocytes during remyelination in Gamt deficient and creatine treated mice. Immunostaining analysis for the mature oligodendrocyte marker CC1 and the mitochondria marker Tom20 was performed on spinal cord demyelinated lesions at 10dpl. **A**) Compared to control mice, Gamt-/- mice displayed a significant reduction of Tom20 labeling in oligodendrocytes. **B**) Injection of creatine monohydrate into mouse lesions, significantly increased Tom20 expression in oligodendrocytes compared to control mice treated with PBS. These results suggest that creatine in oligodendrocytes regulate mitochondrial density *in vivo*.

Goals for Year 2:

1. To complete analysis of Plp1-CreERT;Gamt-fl/fl mice (Aim 1, Major Task 1).
2. To complete analysis of mice under specialized creatine diets (Aim 1, Major Task 2).
3. Begin analysis of aged mice with and without creatine treatment (Aim 2, Major Tasks 1 and 2).
4. Begin obtaining mice for RNA sequencing analysis of rOLs at the end of Year 2 (Aim 3).

Figure 4. Creatine deficiency affects growth in mice. Mice born from mothers that were fed on creatine deficient diet exhibited significantly reduced size compared to those on regular diet. After weaning, mice continued to be on creatine deficient diet. So far, these mice do not appear to catch up in size/weight. This data so far raises the question whether myelination is impaired in the developing CNS.