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14. ABSTRACT

This research study explores ways to enhance myelin repair. The premise upon which the research is founded is that the signaling molecule, Csk, acts as an endogenous suppressor of myelin repair. We have been exploring the possibility that the AMPK pathway is key component of the Csk-induced brake to myelin repair. We have activated AMPK signaling following myelin damage and preliminary indications are that AMPK activation promotes myelin health and/or repair. We also found that AMPK activation enhances oligodendrocyte differentiation from oligodendrocyte progenitor cells using rodent cultures. We are currently exploring other aspects of the oligodendrocyte and myelin phenotypes in both gain- and loss-of-function modes of AMPK signaling, as well as optimizing our strategies for Csk loss in vivo and in vitro.

15. SUBJECT TERMS
Remyelination, AMPK, cuprizone

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1. Introduction

This research study explores ways to enhance myelin repair. The premise of the research isthat the signaling molecule, Csk, acts as an endogenous suppressor of myelin repair. The purpose of the proposed study is to therefore to examine the molecular pathways that are altered by Csk loss in oligodendrocytes, and to explore the possibility that the AMPK pathway is key component of the Csk-induced brake to myelin repair. We plan to determine whether AMPK signaling promotes myelin repair following cuprizone-mediated demyelination using both loss- and gain-of-function approaches. We also plan to identify novel molecular effectors that contribute to the ability of oligodendrocytes to undergo enhanced myelin repair in the absence of Csk. Here, isolated oligodendrocytes will be subjected to several unbiased screens to reveal novel genes or signaling pathways that may be promising candidates to underlie the enhanced myelin repair.

2. Keywords

Myelin repair, signal transduction, AMPK, Csk, oligodendrocyte, cuprizone.

3. Accomplishments

3a. What were the major goals of the project?

The major goals of the project were divided into the two following specific aims, which were each further subdivided into two major tasks, as follows:

- Specific Aim 1: Determine whether AMPK signaling promotes myelin repair
  - Major Task 1: Determine the effect of AMPK loss-of-function on oligodendrocyte dynamics and myelin repair following cuprizone-mediated demyelination (~7.5% complete)
  - Major Task 2: Determine the effect of AMPK activation on oligodendrocyte dynamics and myelin repair following cuprizone-mediated demyelination (~60% complete)
- Specific Aim 2: Uncover novel molecular effectors that contribute to the ability of Csk-KO oligodendrocytes to undergo enhanced myelin repair.
  - Major Task 3: Perform screen to detect molecular changes in acutely isolated Csk-KO oligodendrocytes from brains undergoing myelin repair. (~7.5% complete)
  - Major Task 4: Perform screen to detect molecular changes in cultured oligodendrocytes following acute knockdown of Csk using siRNA. (~20% complete)

3b. What was accomplished under these goals?

During the first several months of the grant period (July 1 – Oct 15), we were in the process of obtaining regulatory approval for mouse work, which essentially comprises all the experiments in the project, so we could not begin the experiments. However, during this time we were still able to test out several key antibody reagents on other tissue samples that we had in the lab, as well as research and optimize experimental strategies, all in preparation for the approved work.

Once regulatory approval was obtained, we began to do project experiments. Thus far we have mostly focused on Major Task 2, which is to determine the effect of AMPK activation on oligodendrocyte dynamics and myelin repair following cuprizone-mediated demyelination. We chose to start with these experiments because these “gain-of-function” experiments are the ones that have the most promise to lead to beneficial outcomes following treatment. In other words, our preliminary data suggested that AMPK activation could jumpstart oligodendrocyte differentiation, which would have a positive outcome if applied to a demyelinating condition such as Multiple Sclerosis.

After several rounds of small pilots to assess dosing and timing (performed in the winter), in the spring we began to assess remyelination in the corpus callosum following cuprizone-mediated demyelination, in the presence or absence of the AMPK activator, metformin. Tissue samples from these studies are still being evaluated, however our initial examination of myelin content of the corpus callosum indicates that metformin treatment may enhance myelin levels. Given the timing of metformin administration (4 weeks into the cuprizone treatment) it is likely that the increase in myelin is due to an increase in myelin repair engaged in by newborn oligodendrocytes. However we have not yet fully assessed the cellular phenotypes in these tissues to make that determination.

In our first time point that was assessed (shown in **Figure 1**), our early indication is that metformin improves either the speed or degree of myelin repair. Of course examination of additional time points will be needed to fully understand the degree of effect as well as the cellular mechanism by which metformin may be influencing post-cuprizone myelin levels, and these are ongoing.

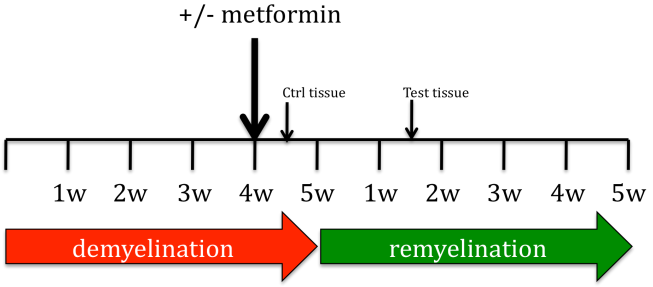

Figure 1. Schematic of a metformin-cuprizone trial.

We have also been making headway on assessing whether metformin can directly influence oligodendrocyte biology, and if so, at what stage and by what mechanism. Previously, we had assessed metformin single hit dosing strategies of OPCs, followed by assessment of lineage stage gene expression. Here we have learned that over the short term, metformin can stimulate increases in OPC differentiation, at the level of changes in mRNA levels of myelin-related genes as well as in the percentages of cells that transition to being CNP- and MBP-positive. We normalized these data relative to Olig2 mRNA levels (used as a readout for oligodendrocyte lineage cells), to guard against the possibility that metformin could shift the percentages of contaminating cell types relative to the oligodendrocyte lineage population (although we did not find substantial differences in normalizing to Olig2 versus normalizing to a housekeeping gene, GAPDH). As seen in earlier assays, we have continued to see that metformin treatment results in increased levels of MBP and CNP mRNA at 24 hours (**Figure 2**) however NG2 and PDGFRa mRNA levels do not significantly change (or slightly trend downwards). We have noted that at day 2 and beyond these changes in gene expression are less pronounced and eventually are no longer statistically significant. We reasoned that the loss of effect over time could be due to metformin stability long term at 37 degrees Celsius. To counter this we designed a new set of experiments in which we changed half the media, adding fresh media, on a daily basis. The analysis of this experiment is currently underway however the first two n reveal that the effects of metformin in the long term (up to 5 days) may be quite robust (not shown).

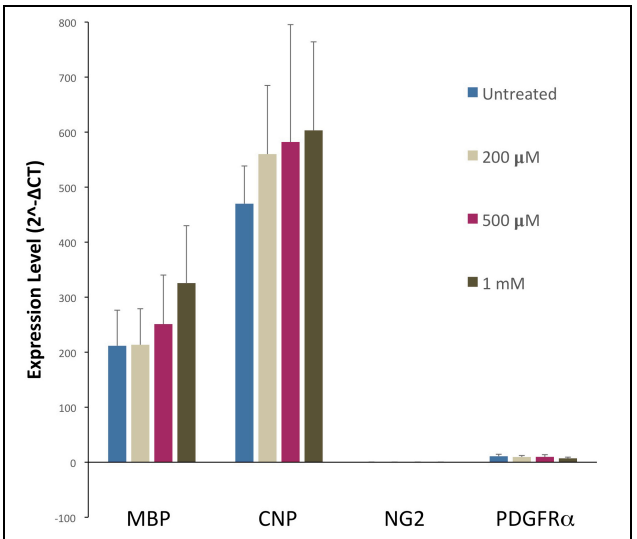

Figure 2. mRNA levels change in response to metformin treatment.

In addition to mRNA levels we have continued our work on assessing whether OPCs differentiate to different lineage stages (i.e., CNP-positive, MBP-positive) in response to metformin. Here we can assess individual cells rather than a cumulative level of mRNA in the whole culture, and thus this assay is predicted to be more sensitive. We find that within 24 hours in SATO+T3 (differentiation medium conditions), metformin treatment results in an increase in the percentage of cells that are either CNP-positive or MBP-positive (**Figure 3**). At the same time, metformin treatment results in a trend towards fewer NG2-positive cells (**Figure 4**). Again, we are currently assessing longer term treatment of metformin using our daily dosing strategy described above.

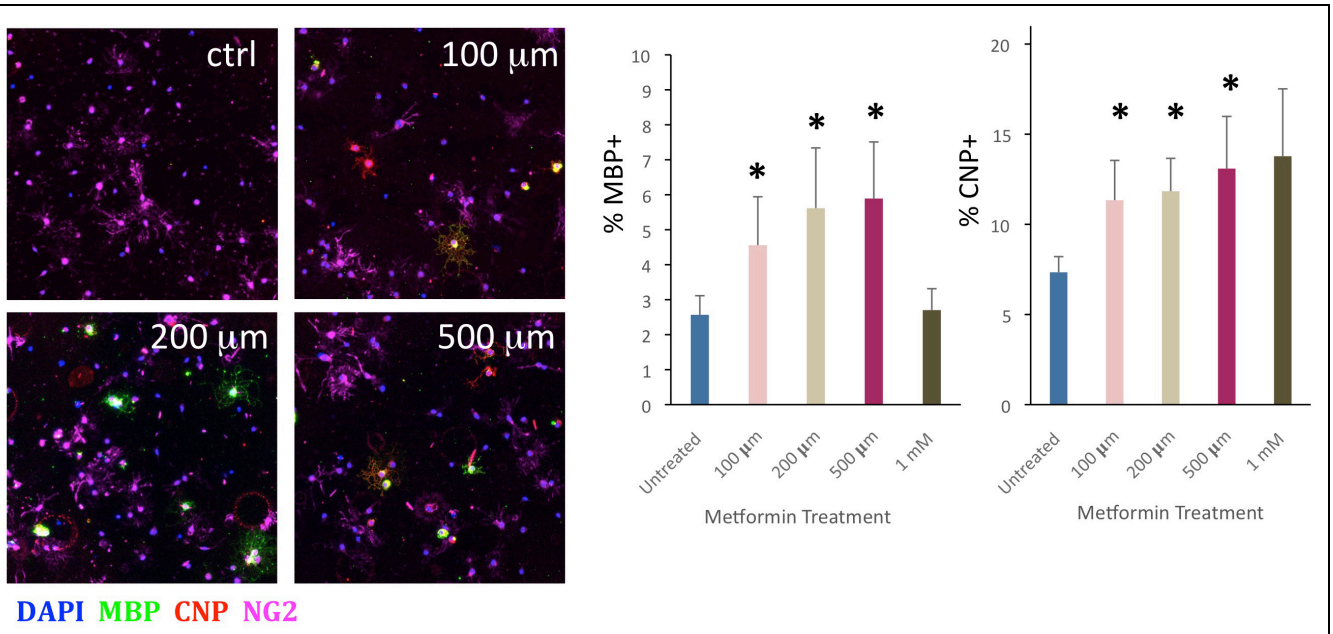

Figure 3. Oligodendrocyte differentiation in response to metformin treatement.

Another avenue that we have been exploring is whether metformin alters the proportion of oligodendrocytes by affecting the small proportion of non-oligodendrocyte cells (astrocytes and microglia) in our primary cultures. In other words, we wanted to determine if changes in oligodendrocyte numbers, or gene expression profiles, could simply be due to gain or loss of other cell types in the culture. With an n of 3 thus far, we do not see significant changes in either astrocytes (determined as the % GFAP+ cells) or microglia (determined as the % Iba1+ cells) in our cultures. We have also begun to assess microglial phenotypes, to see if metformin has influences on pro- or anti-inflammatory phenotypes. Thus far (n = 3) we have determined that there is no change in the percentage of Iba1+ cells that co-express Arg1 (virtually 100%), an anti-inflammatory state readout. However the oligodendrocyte culture conditions contain progesterone, which is a strong driver of the Arg1+ anti-inflammatory microglial state, and may not be the best scenario to assess potential effects of metformin on microglia. We will therefore assess the "big picture" of all relevant cells, including microglia, in as we assess our mice that have undergone cuprizone treatment in the presence or absence of metformin.

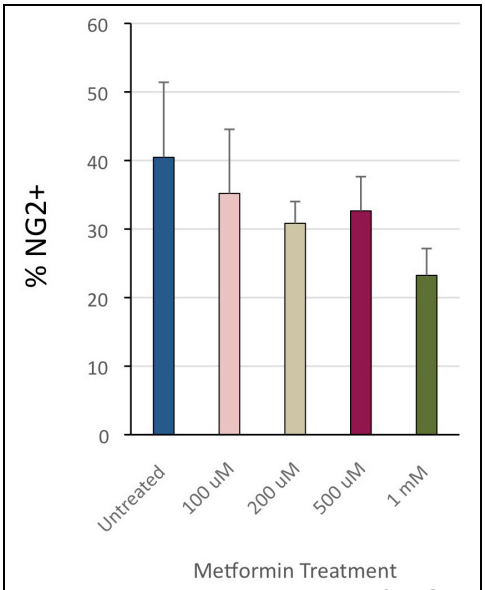

Figure 4. The percentage of NG2+ cells in response to metformin.

3c. What opportunities for training and professional development has the project provided?

There have been extensive training opportunities during the project. The PI has been training a graduate student in oligodendrocyte purifications and cell culture assay techniques, as well as in mouse handling, perfusions, brain tissue dissections, and processing for immunohistochemistry. As a result this graduate student (Mr. Narine) has been making substantial contributions to Aim 1 of the project during the past ~8 months. This graduate student attended an international conference on Glial Cells in Health in Disease in July, and presented our early research findings as a poster presentation (see conference abstract in Appendix). This was a strong professional development opportunity for the trainee.

3d. How were the results disseminated to communities of interest?

As mentioned in 3c, results from the project were presented as a poster at international conference on Glial Cells in Health in Disease in July. In addition, a graduate student working on the project presented his work as a seminar presentation to the Program in Neurosciences here at Stony Brook University.

3e. What do you plan to do during the next reporting period to accomplish the goals?

We hope to expand the AMPK conditional knockout mice in order to make progress on Major Task 1, which thus far has centered on planning and optimizing assays and immunohistological methods. In addition, we will start pilot experiments and optimizing for planned molecular screens of Csk knockout or knockdown oligodendroglia.

4. IMPACT

4a. What was the impact on the development of the principal discipline(s) of the project?
There is considerable interest in the Multiple Sclerosis research community in finding mechanisms that may promote oligodendrocyte health, maturation, and capacity to ensheath multiple neuronal axons with myelin (i.e., myelination capacity). Therefore our early findings that metformin, which activates the AMPK "energy sensor" pathway in cells, enhances oligodendrocyte maturation has the potential to open further inquiry into the role of energy utilization and production in key oligodendrocyte behaviors including myelination.

4b. What was the impact on other disciplines?
Given the potential effect of metformin on oligodendrocyte development we think that metformin will be useful in other disease conditions in which oligodendrocyte pathology or dysfunction plays a role, such as Alzheimer's disease.

4c. What was the impact on technology transfer?
Nothing to report.

4d. What was the impact on society beyond science and technology?
Nothing to report.

5. **CHANGES/PROBLEMS:**

5a. Changes in approach and reasons for change
We are planning to add a panel of metabolic analysis to our study of oligodendrocytes that have alterations in AMPK activity. Given the central role of AMPK in cellular energetics we have taken the opportunity of interacting with a neighboring lab who uses the Seahorse assay to assess changes in energy production, consumption, mitochondrial respiration, and other parameters of cellular energetics. We have done some pilot experiments to add the Seahorse to our "oligodendrocyte culture assay workflow" and we have some of the assays working nicely. Because the Seahorse assays use a small well format with very few cells, we can easily add this in to our cellular analysis plans using the same number of rodent preps of primary oligodendrocytes. This addition will give us further insight into how AMPK changes may regulate oligodendrocyte health and metabolic abilities, which if found, should have a profound impact on remyelination capacity.

5b. Actual or anticipated problems or delays and actions or plans to resolve them.
We have a delay in obtaining the AMPK conditional knockout line to be used in Aim 1A. Currently the needed mice are being expanded at Jackson laboratories and are anticipated to be available in approximately 2 months. In the meantime we have doubled down on Aim 1B, which uses existing mouse strain in combination with the pharmacological activation of AMPK pathways.

5c. Changes that had a significant impact on expenditures.
Due to the delay in being able to use the grant funds while awaiting ACURO approval, we were not able to use funds to support the graduate student working on the project. Instead he was appointed on a T32 Training Grant. Once ACURO was approved we were unable to switch his stipend onto the DOD since the T32 appointment had to go for one year. Once his appointment on the T32 ends in August we will move him to the DOD. Effort did not change.

5d. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.
Nothing to report.

5e. Significant changes in use or care of human subjects.
Not applicable.

5f. Significant changes in use or care of vertebrate animals.
Nothing to report.

5g. Significant changes in use of biohazards and/or select agents.
Not applicable.

6. **PRODUCTS:**

Conference abstract from the European Meeting on Glia in Health in Disease (see appendix).

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

What individuals have worked on the project?

Name:	*Mohaniall Narine*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*9*
Contribution to Project:	*Mr. Narine has assessed the effect of AMPK activation in oligodendrocyte cultures and in mic, using multiple techniques including immunohistochemistry, western blotting, and qRT-PCR. In addition Mr. Narine has been working on assay development, including testing dosing, testing primer conditions, and optimizing antibodies.*
Funding Support:	*NIH T32 Training Grant.*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report.

What other organizations were involved as partners?
Nothing to report.

8. **SPECIAL REPORTING REQUIREMENTS**

Not applicable.

9. **APPENDICES**

See next page.

Abstract #986

An Investigation into the SFK-AMPK Signaling Axis and its Role in CNS Myelination

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Content

Src family kinases (SFK) are a family of non-receptor tyrosine kinases that integrate and transduce information about the extracellular environment to regulate downstream intracellular processes. The SFK member Fyn is needed for normal myelin production, with a global knockout of Fyn or loss of Fyn kinase activity resulting in severe hypomyelination. Conversely, we found that constitutive activation of Fyn in oligodendrocytes (via loss of C-terminal Src Kinase (Csk), a negative SFK regulator) leads to increased myelin wrapping (hypermyelination) in the brain and spinal cord. Hypermyelination in Csk knockouts was, surprisingly, not accompanied by detectable changes in MAPK or mTOR pathway activity, two pathways known to control the extent of myelin wrapping. Instead, Csk loss was accompanied by a 7-fold increase in AMP-kinase (AMPK) transcripts. AMPK acts as the metabolic sensor in the cell and is activated during energy intensive processes (e.g., myelination) to regulate the activity or expression of proteins involved in ATP synthesis. Exogenous activation of AMPK can be achieved with metformin, a drug used to treat type 2 diabetes. We found that metformin treatment of oligodendrocyte progenitor cells increases the expression of myelin proteins (MBP, CNP) and accelerates the generation of mature oligodendrocytes. Studies to evaluate the ability of metformin to influence myelination and/or myelin repair are currently underway. The ability to manipulate the SFK-AMPK signaling axis may prove to be beneficial in the development of remyelination therapies aimed at combating demyelinating diseases such as Multiple Sclerosis.