# CUPRIZONE MODEL OF DEMYELINATION IN THE PRESENCE OF MTOR INHIBITOR RAPAMYCIN

By

Joshua Horeczko

A capstone project submitted for
Graduation with University Honors

February 2, 2018

University Honors
University of California, Riverside

APPROVED

_____
Dr. Seema Tiwari-Woodruff
Department of Biomedical Sciences

_____
Dr. Richard Cardullo, Howard H Hays Chair and Faculty Director, University Honors
Interim Vice Provost, Undergraduate Education

## Abstract

Multiple sclerosis (MS) is an autoimmune, demyelinating neurodegenerative disease with deleterious symptoms causing patients to have vastly diminished quality of life. A common mouse model used to study MS is the cuprizone (CPZ) diet model. The neurotoxin cuprizone, a copper chelator, induces demyelination in specific regions of the brain when included with the diet. Along with demyelination, brain areas also undergo spontaneous remyelination due to oligodendrocyte progenitor cells (OPCs). Unfortunately, this model has some complications as mice can at times experience spontaneous remyelination due to oligodendrocyte progenitor cells (OPCs) differentiating into mature oligodendrocytes (OL). Simultaneous demyelination and remyelination complicates studies that require pure demyelination. Inhibiting siganaling pathways such as the mammalian target of rapamycin (mTOR) that may cause uniform demyelination is vital for OPC differentiation (Wahl et al., 2014). The proposed study investigates whether the addition of Rapamycin, an inhibitor of mTOR, to the cuprizone diet will inhibit oligodendrocyte maturation in vivo and reduce spontaneous remyelination.

**Acknowledgments**

I would like to thank my faculty mentor, Dr. Tiwari-Woodruff, for her guidance and mentorship throughout my time at the University of California, Riverside. My thanks also go largely to all members of the Tiwari-Woodruff Lab, specifically Hana Yamate-Morgan who helped me immensely and who I have had the pleasure to call my colleague – it has been an honor to be a part of such a vibrant and welcoming team of gifted scientists.

## Table of Contents

## Introduction

Multiple sclerosis (MS) afflicts about 2.5 million people worldwide, causing serious neurological and physical disability in young adults (Browne et al., 2014). Myelin, the insulating cover of most neuronal axons in the brain and spinal cord is significantly decreased in MS due to ongoing demyelination and inflammation. This decrease in axon myelination disrupts communication between neurons within the central nervous system (CNS), resulting in a range of symptoms including visual, motor, and cognitive deficits (Staff et al., 2009).

Oligodendrocytes (OLs) are responsible for producing myelin in the (CNS). Only mature OLs are capable of producing myelin and they are derived from an OL progenitor cell (OPC) that underwent a complex process of proliferation, migration, and differentiation (Kuhlman et al., 2009). The cuprizone model of MS consists of cuprizone diet that is toxic to and kills mature OLs causing a reduction in axon myelination in specific regions of the brain (Sachs et al., 2014). This demyelination is not consistent as, after a few weeks on cuprizone, OPCs begin to differentiate into mature OLs and begin repairing the lost myelin in a phenomenon called spontaneous remyelination (Kipp et al., 2009). This imposes a confounding variable to the cuprizone model because it may be difficult to differentiate if a treatment being tested is causing positive results or if the results are merely a product of spontaneous remyelination. Recently in the Macklin lab rapamycin (a known mTOR inhibitor vital for OPC differentiation) was administered at 10 mg/kg 5 for days a week along with 0.3% cuprizone diet over 6 weeks. Treatment with rapamycin plus cuprizone showed significant decrease in myelin protein compared to vehicle with cuprizone after 6 weeks. In conclusion, the addition of rapamycin to the cuprizone diet established a more uniform demyelinating model (Sachs et al. 2014). We

hypothesize that **the addition of rapamycin with cuprizone for 4.5 weeks will diminish the number of OPCs differentiating into mature OLs inducing consistent demyelination but increased axon damage.**

## Methods

**Animal/Tissue groups:** Five groups of C57BL/6 female 14 week old mice were prepared. Group one was fed a diet of normal chow which serves as our control. Group two was given 0.2% cuprizone diet for 4.5 weeks. Group three was given 4.5 weeks cuprizone diet supplemented with intraperitoneal injection of rapamycin five days per week at 10mg/kg. Group four was given 4.5 weeks on cuprizone diet and additional 3 weeks on normal diet to allow remyelination. Group five was given 4.5 weeks on cuprizone diet with addition of rapamycin and then an additional 3 weeks on normal diet alone.

**Immunohistochemistry**: Immunohistochemistry (IHC) will be performed to examine specific markers for OLs and demyelination within the corpus callosum. Briefly, mice will be euthanized by inhalation of isoflurane, perfused, and their brains dissected. The brains will then be post fixed, cryoprotected, embedded in gelatin, and sliced into 40 μm sections. Various antibodies will be used to assess myelin, oligodendrocytes, astrocytes, and immune cells. Images will be taken using the confocal microscope. The images will be exported into ImageJ and analyzed using software where the intensity of the stain is quantified for each group. An intensity analysis will be used to image myelin, axon health, astrocytes, and inflammation while OLs and OPCs will be counted.

**Table 1.**

| Antibody Name | Target | Vendor | Catalog |
|---|---|---|---|
| MBP | Myelin basic protein | Millipore | AB9348 |
| SMI-32 | Unphosphorylated neurofilament H | Millipore | NE1023 |
| GFAP | Glial fibrillary acidic protein; Astrocytes | Zymed | 18-0063 |
| CD45 | Cluster of differentiation 45; Common leukocyte antigen | BD Pharminogen | BD550539 |
| Olig2 | Oligodendrocytes | Millipore | AB9610 |
| CC-1/APC | Mature myelinating oligodendrocytes | Genetex | GTx16794 |
| Ki67 | Cell cycle related nuclear protein | Millipore | AB9260 |
| Caspase 3 | Cysteine-aspartic acid protease activated in apoptotic cells | Millipore | AM65 |

Table 1. Antibodies such as SMI-32 are used to fluorescently label specific structures within the CNS. In the case of SMI-32 staining in the corpus callosum, axons are labeled which allows visualization of demyelination in neurons.

## Results

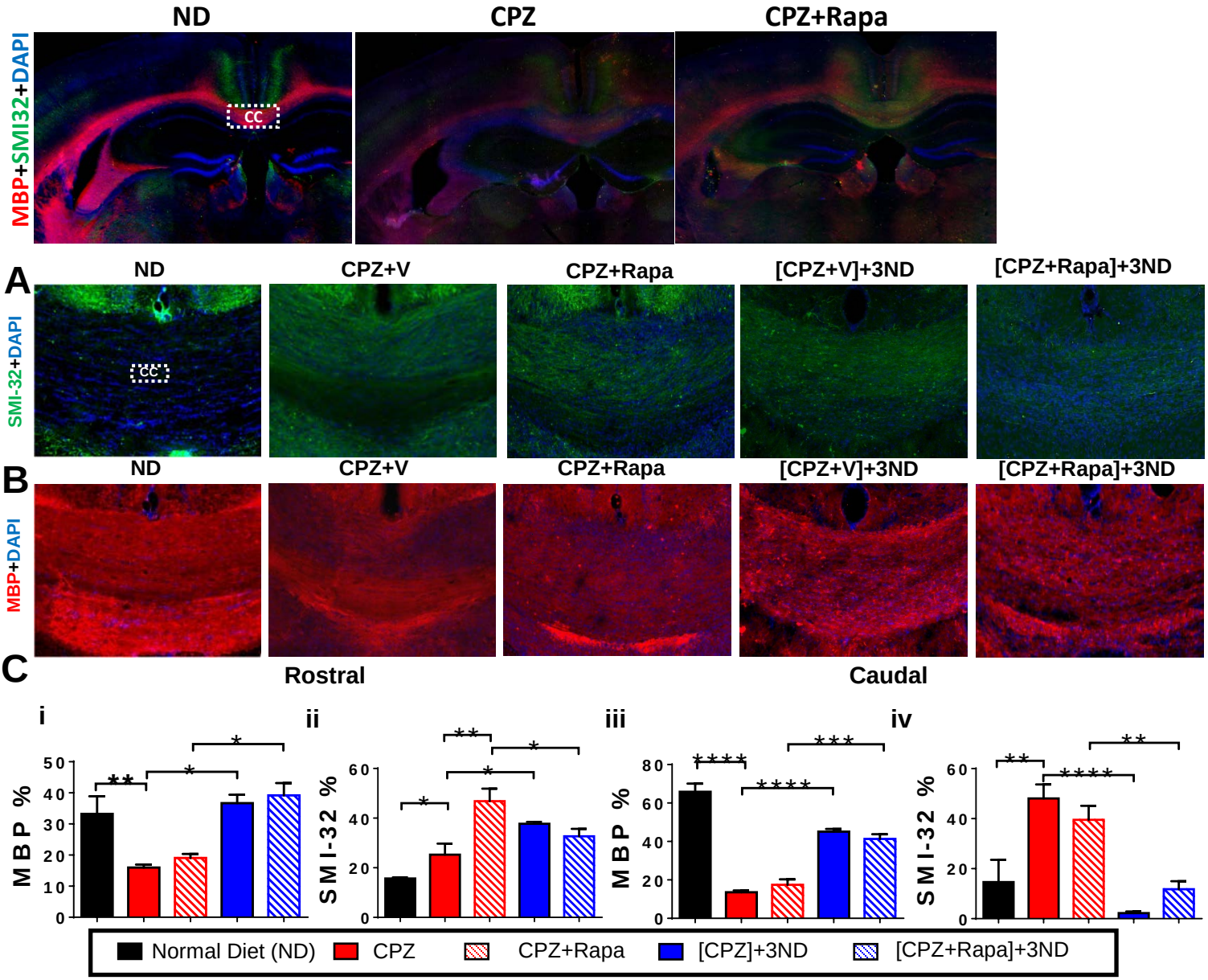


**Figure 1. Rapamycin increases SMI-32+ axon damage without affecting overall myelination levels in the corpus callosum.** Representative 10X images of the corpus callosum (CC, delineated by white dashed box) for normal diet (ND), 4.5 week cuprizone demyelination (CPZ), 4.5 week cuprizone with rapamycin (CPZ+Rapa), 4.5 week cuprizone plus 3 week remyelination ([CPZ]+3ND), and 4.5 week cuprizone with rapamycin plus 3 week remyelination ([CPZ+Rapa]+3ND). (A) Nonphosphorylated neurofilament SMI-32 marker of axon damage (green), and nuclear stain DAPI (blue) Levels of fluorescent SMI-32 intensity were increased during demyelination compared to normal, and were significantly higher in the rostral CPZ+Rapa group compared to CPZ alone. Levels of SMI-32 were decreased during remyelination compared to demyelination, both with and without rapamycin (except for rostral remyelination compared to CPZ, which increased). No difference was observed during remyelination following the addition of rapamycin during demyelination, compared to remyelination following demyelination alone. (B) Myelin basic protein (MBP; red) and DAPI (blue). Levels of fluorescent MBP intensity decreased during demyelination compared to normal, but were recovered during remyelination. No difference was observed as a result of the addition of rapamycin. (C) Quantification of relative fluorescence intensity for ND (black), CPZ (red), CPZ+Rapa (red striped), [CPZ]+3ND (blue), and [CPZ+Rapa]+3ND (blue striped). Data are presented as mean +/- SEM. n=5 mice/group; *p<0.05,**p<0.01,***p<0.001. ANOVA. Holm-Sidak multiple comparisons with posthoc tests.

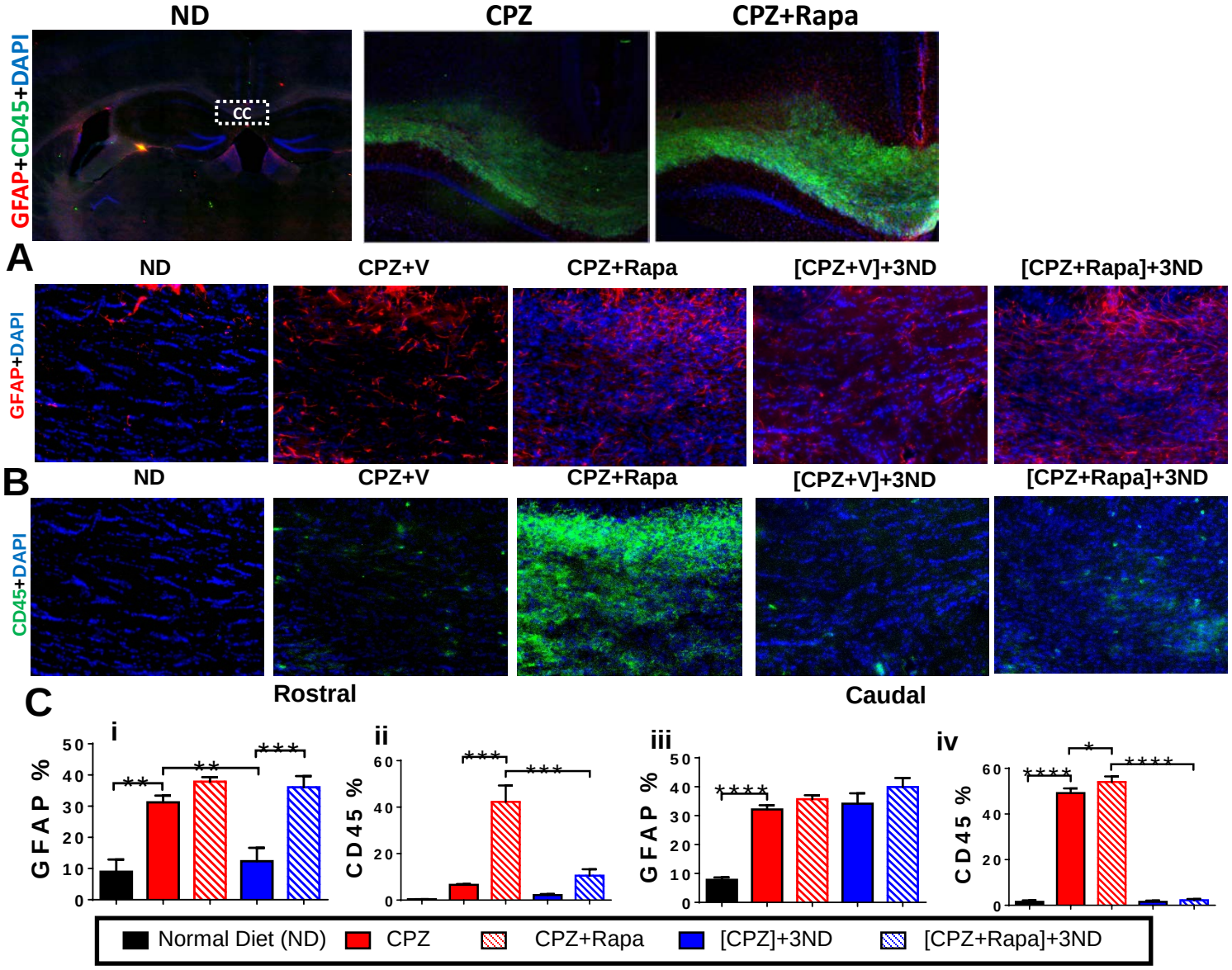


**Figure 2. Rapamycin administration increases levels of leukocyte common antigen CD45 marker of neuroinflammation during demyelination and levels of glial fibrillary acidic protein (GFAP) reactivity during remyelination.** Representative 20X images of the CC (delineated by dashed white box in Fig3A) for normal diet (ND), 4.5 week cuprizone demyelination (CPZ), 4.5 week cuprizone with rapamycin (CPZ+Rapa), 4.5 week cuprizone plus 3 week remyelination ([CPZ]+3ND), and 4.5 week cuprizone with rapamycin plus 3 week remyelination ([CPZ+Rapa]+3ND). (A) Astrocyte glial fibrillary acidic protein marker (GFAP, red) and DAPI (blue). . No difference was observed in GFAP during demyelination as a result of the addition of rapamycin. Although levels of GFAP intensity were increased during demyelination compared to normal, no difference was observed in GFAP that could be linked to the addition of rapamycin. Rapamycin did, however, result in significantly higher levels for [CPZ+Rapa]+3ND compared to [CPZ]+3ND, where remyelination groups overall exhibited sustained high levels of GFAP compared to normals. (B) Common leukocyte antigen cluster of differentiation (CD) microglia/macrophage marker (CD45, green) and DAPI (blue). Levels of CD45 intensity were increased during demyelination compared to normal in caudal sections, and significantly higher levels were observed for CPZ+Rapa compared to CPZ in both rostral and caudal regions. Levels of CD45 were decreased to near-normal levels during remyelination compared to demyelination, both with and without rapamycin. (C) Quantification of relative fluorescence intensity for ND (black), CPZ (red), CPZ+Rapa (red striped), [CPZ]+3ND (blue), and [CPZ+Rapa]+3ND (blue striped). Data are presented as mean +/- SEM. n=5 mice/group; *p<0.05,**p<0.01,***p<0.001. ANOVA. Holm-Sidak multiple comparisons with posthoc tests.

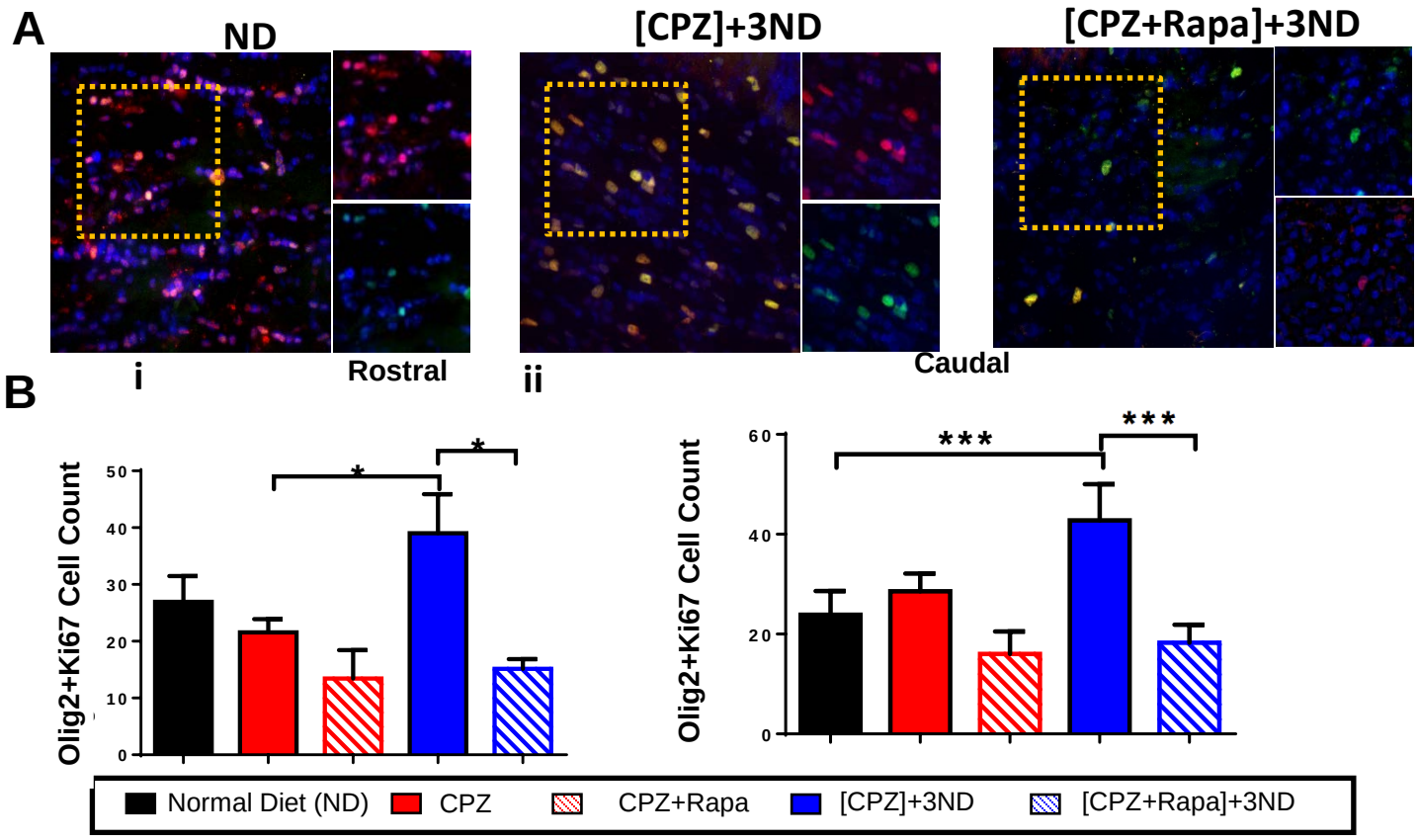


**Figure 3. Rapamycin administration prevents the recovery of colabeled Olig2+Ki67+ proliferating OPCs during remyelination in the CC.** Representative 40X images of the CC for normal diet (ND), 4.5 week cuprizone demyelination (CPZ), 4.5 week cuprizone with rapamycin (CPZ+Rapa), 4.5 week cuprizone plus 3 week remyelination ([CPZ]+3ND), and 4.5 week cuprizone with rapamycin plus 3 week remyelination ([CPZ+Rapa]+3ND). (A) OL transcription factor Olig2 (red), nuclear proliferation marker Ki67 (green) and DAPI (blue). Numbers of proliferating OPCs, identified by colabeling with Olig2 and Ki67, were unchanged between normal and demyelination groups. During remyelination, this OPC population was significantly increased, except in remyelination groups previously given rapamycin during demyelination, which remained at demyelination levels. (B) OPCs stained with Olig2+Ki67+ and overlapping with DAPI nuclei were counted in each of two 40X images of the center CC and are depicted as the average density of cells/mm^2 for ND (black), CPZ (red), CPZ+Rapa (red striped), [CPZ]+3ND (blue), and [CPZ+Rapa]+3ND (blue striped). Data arepresented as mean +/- SEM. n=5 mice/group; *p<0.05,**p<0.01,***p<0.001. ANOVA. Holm-Sidak multiple comparisons with posthoc tests.

Our results show significant increase in SMI-32 signaling (a marker of axonal damage) in the corpus callosum (CC) of rostral brain regions for the groups treated with rapamycin (Figure 1ii). We also see an increase in CD45 (which is a marker for macrophages that are involved in antigen presentation and immune response) in rostral regions of the rapamycin treated groups, suggesting a more severe immune response and more inflammation (Figure 2ii). GFAP (Glial Fibrillary Acidic Protein) is a marker for astrocyte activation. Astrocytes are cells responsible for

neural maintenance but can also be overactive and damaging to the myelin. Astrocyte activation remained higher in the rostral regions of the brain during remyelination of groups treated with rapamycin suggesting that these groups were more active in the demyelination process (Figure 2i). For Olig2 ki67 which are markers for oligodendrocytes and proliferation, we see a significant decrease in amount of OL cells and in proliferation, for the remyelination rapamycin treated groups (Figure3ii). This shows that rapamycin is having its inhibitory effect on OPC proliferation during remyelination. However, we saw no significant difference in levels of MBP (Myelin Basic Protein) which is the main component of the myelin sheath, between rapamycin and CPZ groups for neither the demyelination nor remyelination phase.

## Discussion

We expected to see more extensive demyelination and axon damage in the rapamycin treated groups as inhibition of the mTOR pathway should prevent OPCs from proliferation, survival, migration, and differentiation into mature OLs thus reducing spontaneous demyelination and decreasing trophic support to axons. Our results show a decrease in OPC proliferation for remyelination with rapamycin and a increase in SMI-32 axon damage in the demyelination rapamycin treated groups which supports our hypothesis. Between MBP groups we do not see a significant difference it is possible that because we conducted our experiment for 4.5 weeks we did not see as extensive demyelination as seen when done over 6 weeks. Overall our results show a more intense immune system response when given rapamycin. In remyelination groups there was a significant reduction in mature OLs along with an increase in astrocyte activation. Which implies that our hypothesis is accurate in that rapamycin is having an inhibitory effect on oligodendrocyte proliferation.

**References**


Browne P, Chandraratna D, Angood C, Tremlett H, Baker C, Taylor BV, Thompson AJ (2014) Atlas of Multiple Sclerosis 2013: A growing global problem with widespread inequity. Neurology 83:1022–1024.

Staff, Nathan P., et al. “Multiple Sclerosis With Predominant, Severe Cognitive Impairment.” Archives of Neurology, vol. 66, no. 9, 2009, doi:10.1001/archneurol.2009.190.

Kuhlmann, Tanja, and Wolfgang BrÃ¼ck. “Oligodendroglial Pathology in Multiple Sclerosis.” The Biology of Oligodendrocytes, pp. 171–185., doi:10.1017/cbo9780511782121.010.

Wood, T. L., Bercury, K. K., Cifelli, S. E., Mursch, L. E., Min, J., Dai, J., & Macklin, W. B. (2013). mTOR: A Link from the Extracellular Milieu to Transcriptional Regulation of Oligodendrocyte Development. *ASN Neuro*. http://doi.org/10.1042/AN20120092

Wahl, S. E., McLane, L. E., Bercury, K. K., Macklin, W. B., & Wood, T. L. (2014). Mammalian Target of Rapamycin Promotes Oligodendrocyte Differentiation, Initiation and Extent of CNS Myelination. *Journal of Neuroscience*. http://doi.org/10.1523/JNEUROSCI.4311-13.2014

Sachs, H. H., Bercury, K. K., Popescu, D. C., Narayanan, S. P., & Macklin, W. B. (2014). A new model of Cuprizone-Mediated demyelination/remyelination. *ASN Neuro*. http://doi.org/10.1177/1759091414551955

Narayanan, S. P., Flores, A. I., Wang, F., & Macklin, W. B. (2009). Akt Signals through the Mammalian Target of Rapamycin Pathway to Regulate CNS Myelination. *Journal of Neuroscience*. http://doi.org/10.1523/JNEUROSCI.0232-09.2009

Kumar, S., Patel, R., Moore, S., Crawford, D. K., Suwanna, N., Mangiardi, M., & Tiwari-Woodruff, S. K. (2013). Estrogen receptor β ligand therapy activates PI3K/Akt/mTOR signaling in oligodendrocytes and promotes remyelination in a mouse model of multiple sclerosis. *Neurobiology of Disease*. http://doi.org/10.1016/j.nbd.2013.04.005

Kipp, M., Clarner, T., Dang, J., Copray, S., & Beyer, C. (2009). The cuprizone animal model: New insights into an old story. *Acta Neuropathologica*. http://doi.org/10.1007/s00401-009-0591-3

Guardiola-Diaz, H. M., Ishii, A., & Bansal, R. (2012). Erk1/2 MAPK and mTOR signaling sequentially regulates progression through distinct stages of oligodendrocyte differentiation. *GLIA*. http://doi.org/10.1002/glia.22281

Dai, J., Bercury, K. K., & Macklin, W. B. (2014). Interaction of mTOR and Erk1/2 signaling to regulate oligodendrocyte differentiation. *GLIA*. http://doi.org/10.1002/glia.22729