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# Amino Acid Sensing Ability by the Mechanistic Target of Rapamycin in Oligodendrocytes

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# AMINO ACID SENSING ABILITY BY THE MECHANISTIC TARGET OF RAPAMYCIN IN OLIGODENDROCYTES

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**Abstract:**

Oligodendrocytes (OLs) are a type of glial cell in the central nervous system that require substantial nutrients such as lipids and amino acids to create the extensive, metabolically expensive myelin sheath surrounding the axons of neurons. The mammalian target of rapamycin (mTOR) is a regulatory kinase that is necessary for the maturation of progenitor OLs through their distinct oligo-lineage phases. Amino acids are vital for the functioning of cells. The focus of this study was to determine if mTOR activity is dependent on the availability of leucine, methionine, and alanine. This was assessed by examining the phosphorylation level of the downstream targets of mTOR, specifically S6K, through western blot analysis. The changes in phosphorylation of S6K will be indicative of the effect of amino acids on mTOR activity because mTOR is the only kinase that phosphorylates this protein. The results showed an increase in phosphorylation upon stimulation with leucine after a 22-hour silencing period, indicating that mTOR activity is dependent on the presence of extracellular leucine in oligo-lineage cells.

## Introduction

### *General*

Throughout evolution, there have been many adaptations that have allowed for different species to come into existence. The myelin sheath is a very important component contributing to the evolution of large and highly complex vertebrates. The myelin sheath is a lipid membrane that insulates the axons of neurons in order to maximize the propagation of signals. The presence of myelin as well as the diameter of axons are two critical factors allowing for rapid conductance of action potentials (Fundamentals of anatomy and physiology, 2018). Increasing axonal diameter aids in propagation of impulses because the resting potential of membranes are less affected by the leakage of potassium ions. In unmyelinated axons, nerve impulse moves down the neuron via continuous propagation (Fundamentals of anatomy and physiology, 2018). In myelinated axons, nerve impulse "jump" from node to node (the gaps in myelin) allowing for faster transmission; this type of propagation is referred to as saltatory conduction (Fundamentals of anatomy and physiology, 2018).

The axons of many invertebrates are ensheathed by glial cells, however, these coverings are not particularly compact and therefore are not considered myelinated (Zalc, 2006). Consequently, the propagation of neural impulses is not as efficient. However, this slower conductance is adequate for small invertebrates. An increase in the diameter of axons has been a beneficial adaptation for larger invertebrates in order to increase the rate of conductance. However, this is not feasible for vertebrates due to physical constraints such as skull size (Zalc, 2006) and therefore, alternative evolutionary adaptations have occurred to allow for sufficient and timely communication in the bodies of larger species. It is proposed that myelin forming cells are evolutionarily related to the "ensheathing glial" cells that are present in many

invertebrates (Zalc, 2016). Yet not all vertebrates possess myelinated axons and some invertebrate do. It is postulated that the acquisition of myelin was coupled with appearance of the hinged jaw. Although these traits are potentially unrelated, fossil records indicate that the presences of myelin dates back to the Devonian period based on preserved features such as the size of the foramen in the skulls of organisms (Zalc, Goujet, Colman, 2008).

Oligodendrocytes and Schwann cells are specialized cells in the central nervous system and peripheral nervous system respectively, which create the myelin sheath. Myelination is a highly complex process that integrates a plethora of regulatory signals that influence the trajectory and fate of these cells. They require a certain composition of nutrients, growth factors, lipids, and amino acids. Cells have an incredible capability to sense their surrounding and take appropriate action – whether that means going through mitosis, apoptosis or simply maintaining. The cellular mechanisms involved in this sensing ability remains a prevalent are of research today. There are many proteins and pathways within cells that are tuned into the surroundings and nutrient availability that control the fate of the cell (Boulanger and Messier, 2014). In recent years, researchers have been particularly interested in the Mammalian (aka. Mechanistic) Target of Rapamycin (mTOR) as a key player in cell growth and potentially nutrient sensing.

*Oligodendrocytes*

The extensive membranous projections require ample nutrients to build and sustain their structures in order to aid in the propagation of action potentials (Figure 1).

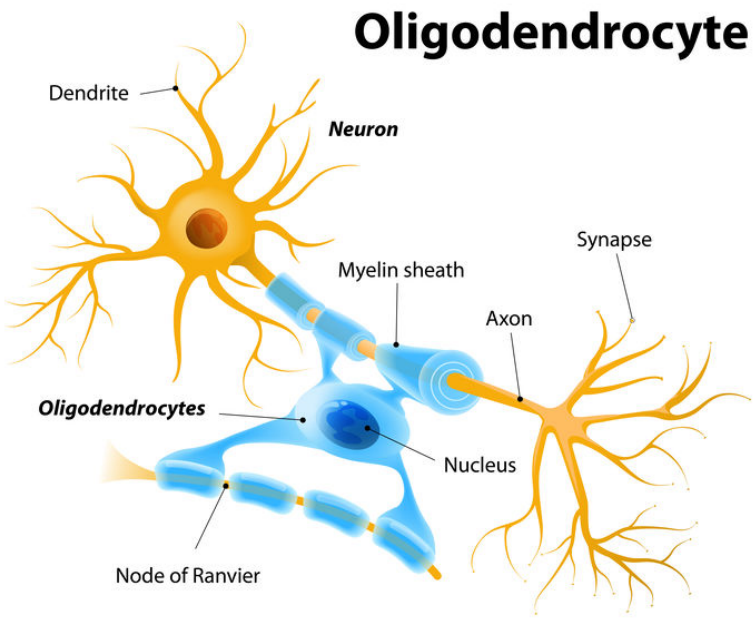



Figure 1. Depicts an oligodendrocyte with projections to more than one neuronal axon creating the extensive myelin sheath insulation (Myelination Assay, n.d.).

Oligodendrocytes go through distinct stages that exhibit discrete morphological and functional changes. There are many intrinsic and extrinsic factors that are involved in this progression (Boulanger, Messier, 2014). In addition, specific antigens are expressed on the surface of the cells at landmark stages in the progression (Armstrong, 1998). The presence or absence of antigens can thus be used to determine which stage the cell is in.

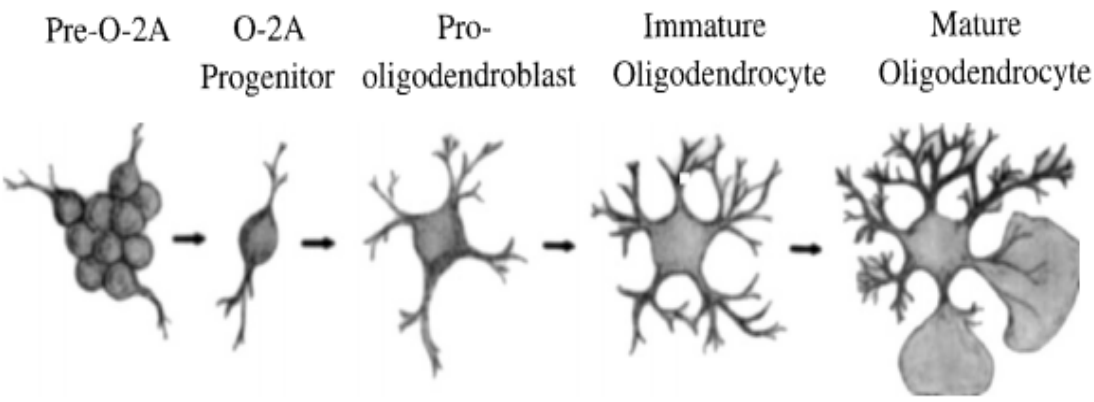



Figure 2. Demonstrates the changes in antigen expression and morphology during the development of oligodendrocytes (Armstrong et. al., 2012).

*mTOR*

mTOR is serine/threonine kinase that belongs to a larger phosphatidylinositol 3-kinase (PI3k) family and has been confirmed to be involved in nutrient sensing. The discovery of the TOR complexes (mTOR complex 1 and mTOR complex 2) came from experiments done in the common yeast *Saccharomyces cerevisiae*. The discovery of this kinase and its action came about by using two immunosuppressive compounds FK506 and rapamycin to block the activation of helper T cells (Heitman et. al, 1991). Rapamycin is now a widely utilized immunosuppressive compound that has been repeatedly confirmed to block mTOR activity. The mTOR complexes 1 and 2 are vital in the survival and regulation of a wide range of cells and these kinases are highly conserved through evolution from yeasts to mammals (Takahara and Maeda, 2013). The mTOR pathway is very complex and influential; as it is a regulatory kinase that is stimulated by nutrients and growth factors but inhibited by stress, lack of nutrients, and rapamycin (Wullschleger et. al., 2006). Specifically, mTORC1 phosphorylates downstream targets such as 4E-BP1 and p70S6K which are influential in mRNA translation for proteins involved in proliferation.

In the past 30 years, researchers have been studying the involvement of the TOR complexes in cellular mechanisms, nutrient sensing, cancer, neurodegenerative diseases, autophagy etc. The vastness of its function consequently means that mTOR is modulated by many stimulatory and inhibitory proteins that will dictate its level of activity. Studying it in other systems has shed light on the involved cellular mechanisms (Wullschleger et. al., 2006; Hall, 2008). The activation of this multimer complex in the presence of the appropriate nutrients and growth factors will upregulate macromolecule synthesis and increase both the mass and size of cells going through mitosis (Wullschleger et. al., 2006) and also inhibit the initiation of autophagy by blocking complexes such as ULK1 and proteins involved in the fusion of autophagosomes with the lysosomes (Kiriyama and Nochi, 2015).

In recent years the interaction between mTOR activity and oligodendrocyte lineage progression has indicated its importance in maturation (Guardiola-Diaz et. al., 2012). When mTOR is inhibited by rapamycin in the early stages of the oligo-lineage (progenitor and immature OLs), they will continue through their life cycle. However, when OLs are treated with rapamycin during its transition from the immature OL to the mature OL stage, they will cease the progression of their cytoskeleton and lead to reduced expression of myelin basic protein (a vital protein during myelination) (Guardiola-Diaz et. al., 2012).

*Nutrient sensing and Leucine*

The reception of growth factors on cellular membrane leads to complex signal transduction. A plethora of steps are involved in signal transduction and different molecules will induce distinct cascades of events that will affect activity of specific kinases, phosphatases, and even gene expression and protein translation. One type of cell signaling is through transmembrane receptor tyrosine kinases (RTKs). RTKs encompass a large range of plasma

membrane receptors that are involved in “enzymatic activity” (Campbell Biology, 2011). Tyrosine kinases “catalyze the transfer phosphate groups from ATP” to the tyrosine amino acid on proteins (Campbell Biology, 2011) which will activate them to carry out different functions.

Nutrient sensing occurs directly when a molecule binds to a sensor or indirectly when a “surrogate molecule” indicates nutrient abundance (Efeyan, Comb, Sabatini, 2015). The nutrient requirements will vary depending on the type of cell and thus the mechanisms involved are also variable. For instance, many unicellular organisms and multicellular plants do not require the same composition in their surrounds because they can synthesize much of their own energy from basic elements such as carbon and nitrogen (Palm and Thompson, 2017). On the other hand, mammalian cells do not have the ability to convert organic substances into energy and thus must acquire various nutrients from their surrounds (Palm and Thompson, 2017) (Figure 3). Mammalian cells that do not have the same function will also not require the same exact composition of nutrients because they are not synthesizing the same proteins in the same quantities, yet the TOR complexes are vital to growth and metabolism in all of these cell types (Wullschleger et al., 2006). For example, the activation of a specific G-protein coupled receptor in of white adipocytes resulted in PI3K/AKT activation and glucose uptake (Efeyan, Comb, Sabatini, 2015), which is the kinase family that mTOR belongs to. Since the main function of adipocytes is to store fat, they will uptake a lot of glucose. Although adipocytes do not function the same as a kidney cells, neurons, or pancreatic cells etc., they all require a certain level of energy to maintain and the presence of amino acids for protein synthesis.

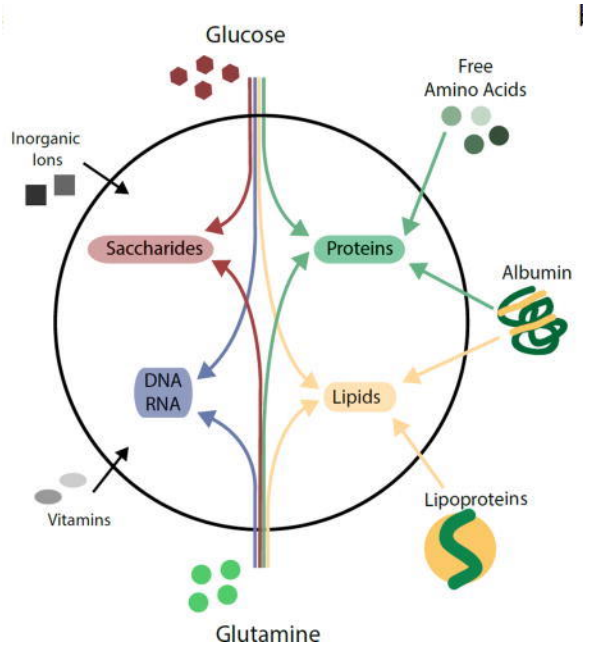



Figure 3. Illustrates the major nutrients / molecules required for mammalian cells to undergo macromolecule synthesis (Palm and Thompson, 2017).

Lipogenesis, the formation of lipids/fats, is essential for the growth of the oligodendrocytes because lipids are the main component of the myelin sheath. The sterol regulatory element-binding protein (SREBP) is the "master regulator of lipo- and sterologenic gene transcription" meaning it is involved in lipids synthesis (Peterson, 2011). An in vitro study done in oligo-lineage cells indicated that SREBP 1 and 2 are expressed in both precursor and differentiating cells. The study also demonstrated that when SREBP processing was inhibited selectively and that there was a downregulation in myelin basic protein as well as a reduction in transcription of SREBP target genes involved in fatty acid and cholesterol synthesis (Monnerie et al., 2017). Interestingly, in liver cells SREBP is indirectly regulated by mTORC1. The inhibition of mTORC1 diminishes SREBP activity (Peterson, 2011). These conclusions are important to note due to the involvement of mTORC1 with proteins that regulate lipogenesis -- a process that is crucial to the formation of the elaborate myelin sheath in oligodendrocytes.

As noted, the TOR complex being studied here is a serine and tyrosine kinase in the PI3K/AKT family and has been studied in a variety of systems. However, its nutrient sensing abilities have been primarily studied in cancer lines up to this point. The mechanistic target of rapamycin "regulates cell growth and metabolism in response to environmental cues" (Wullschleger, 2006). Amino acids, specifically leucine is thought to promote the addition of GTP to Rag A/B and GDP to Rag C/D subunits which bind to the raptor subunit of mTORC1 (Figure 4). Leucine regulates the activity mTOR by controlling Rheb-GTP, which activates mTORC1. The binding of Rheb to mTOR is a critical step involved in the activation of mTOR (Avruch et al, 2008). The activation of the complex prompts its translocation to the lysosomal surface and further activation of downstream pathways (Menon, 2014). The identity of the amino acids being sensed by mTOR are still being studied, but leucine is thought to be a major indicator of macromolecules availability. There is a strong link between leucine availability and cancer development. In melanoma tumors for instance, leucine deprivation in combination with an autophagy inhibitor suppressed cancer cell growth and lead to apoptotic cell death (Sheen et al., 2011). The deprivation of amino acids leads to both the inhibition of protein synthesis and activation of autophagy, which are both linked to mTOR due to diminished amino acid availability preventing mTORC1 activity (Lamb et al., 2012).

According to studies done in human embryonic kidney (HEK-293T) cell lines, the removal of the essential amino acid leucine will inhibit mTORC1 signaling (Wolfson et al., 2016). Additionally, it seems as though other amino acids such as alanine facilitate the activation of the TOR complexes in HEK293T cell lines when paired with essential amino acids such as leucine after a silencing period (Dyachok et al., 2016). However, there has not been extensive

research pertaining to the relationship between amino acid sensing and mTOR activity in oligo-lineage cells.

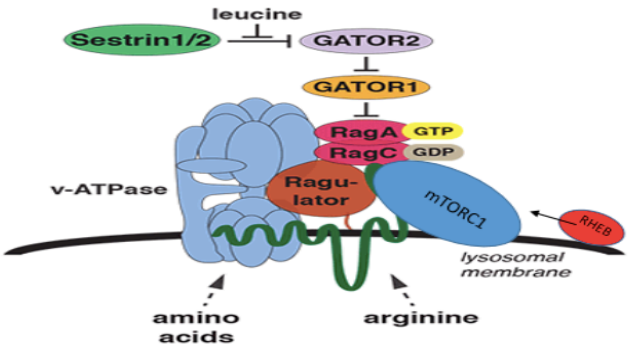



Figure 4. Illustrates the involvement of the heterodimer Rag proteins bound to the mTORC1 which initiates the localization of mTOR to the surface of the lysosomal and resulting activation of the complex. (Wolfson et al., 2016)

*Objective*

The major questions examined of this experiment were: "does leucine serves as a proxy for nutrient availability in oligodendrocytes" and "is there an induction of mTOR activity upon leucine stimulation (back to its "normal" level) after a period of leucine withdrawal?". In the 2018-2019 academic year, Trinity College Biology honors thesis student Sameir Madden conducted experiments in attempts to answer these questions. Previously, the oligodendrocytes were allowed to mature for 4 days, an adequate amount of time to develop into their fully mature oligodendrocyte form and then were placed into a media solution that lacked insulin and the essential amino acid leucine for four hours. Following the four-hour starvation period, leucine was added back into the media for approximately one hour. Analysis through western blotting probed the level of phosphorylation of the specific downstream target of mTOR, S6K. However, Madden was unable to obtain consistent results and thus he was not able to determine significance between the cells that had been starved of leucine and those that were starved and

stimulated with leucine. Therefore, this study was a continuation of the work done by Sameir Madden. The idea of this study was to extend the silencing period in attempts to better silence mTOR activity down to a basal level in order to obtain consistent and significant results in attempts to improve our understanding of the ability of mTOR to sense the absence/presence of leucine in the extracellular environment and test the tolerance to leucine starvation.

Further, the starvation was paired with the nonessential amino acid alanine in to see if the paired stimulation would facilitate the induction of mTOR activity. Additionally, the same experiment done with leucine was also done with methionine so examine the effects of mTOR activity when deprived and then stimulated with a different amino acid. Finally, immunofluorescence microscopy was utilized to observe the integrity and the morphological progression of the cells during amino acid starvation.

**Methods and Materials**

*Cell Culture*

The cells used for these analyses were obtained from 0 to 3 day old neonatal rat pups. The pups were sacrificed following a standard ethical procedure. Under a sterile fume hood, the brain was removed, and the telencephalon was separated from the midbrain and hindbrain. The meninges were removed from each hemisphere and placed into a petri dish over ice in sterile HEPES-EDTA (HE) media buffer. The hemispheres were finely minced with a sterile razor blade and the cells were added to 0.025% Trypsin and shaken for 20 minutes, at 37°C at 110 RPM. After the 20 minute shake, Soy Bean Trypsin Inhibitor at 0.2mL per 10 mL trypsin followed by $MgSO_4$ at 0.2 mL per 10 mL trypsin (final concentration of 3mM) were added to destroy all extracellular DNA. Following the addition of $MgSO_4$, 0.1 mL of DNase per 10 mL trypsin (final concentration 20 ug/mL) was added and incubated for five minutes at room

temperature. After incubation, this solution was transferred to centrifuge tubes and spun down at 2000 rpm for one minute. Next, the supernatant was carefully aspirated from the tubes and then the pellet was resuspended in 5 mL of Trituration DNase (final concentration 80ug/mL) and allowed to settle for approximately five minutes. The cells were then centrifuged at 800rpm for 8 minutes in 4% bovine serum albumin. Following the centrifugation, the supernatant was carefully aspirated, and the pellet was resuspended in 5% fetal bovine serum and seeded in T75 flasks in HE. The media was replaced every 3 days for 10 days.

After 10 days, the flasks were placed in an orbital shaker at 200 rpm and 37 °C to separate the oligodendrocyte progenitors from the astrocyte bed. The oligodendrocyte progenitors were then seeded on 10 cm poly-L-Ornithine plates (25ug/mL; approximately 1 million cell per plate). At day 0 of differentiation, the 5% FPD media (consisting of DMEM and 5% heat inactivated feta) was removed from the plate and replaced with N2 media, serum-free defined media [DMEM with human transferrin (50 ug/mL), bovine pancreatic insulin (5 ug/mL), 3,3,5-triiodo-L-thyronine (10 ng/mL), sodium selenium (30 nM), D-biotin (10 ng/mL), hydrocortisone (10 nM), sodium pyruvate (0.11 mg/mL), penicillin-streptomycin (10 IU/mL and 100 μg/mL, respectively)], and 0.1% BSA. The removal of PDGF marked the beginning of differentiation (day 0) for the oligo-lineage. Media was replaced every other day.

*Experimental Conditions*

Experimental #1: The cells were allowed to grow in the standard, N2 growth media containing insulin, growth factors, and amino acids. At Day 3, the N2 media was removed from the "D3" cellular condition plates and replaced with silencing media for a 22-hour silencing period. The silencing media was made without insulin, leucine, or growth factors. For the one-hour stimulation 5 uL of from the 200X leucine stock was added per 1mL of media to yield the

"normal" concentration of leucine like in the standard growth media. The following day, for cellular conditions that were blocked with rapamycin, 1uL of rapamycin was added for every 1 mL of silencing media 30 minutes prior to leucine stimulation (1uL per 5uL of media from a 200X stock). This acted as the secondary control (Figure 5). The cells in the experimental condition were stimulated with leucine for one hour (Figure 5).

Additionally, at Day 4, the N2 media was removed from the "D4" cellular conditions plates and replaced with the silencing media for approximately 4 hours. The secondary control and the experimental conditions were consistent with the Day 3 cellular conditions (Figure 5).

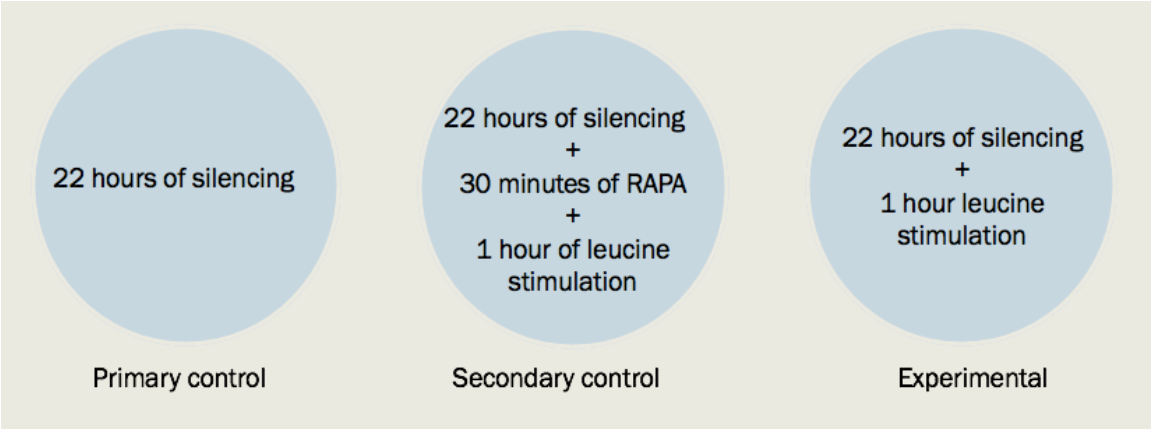



Figure 5. Illustrates the experimental set up for Day 3 oligo-lineage cells undergoing the 22-hour starvation.

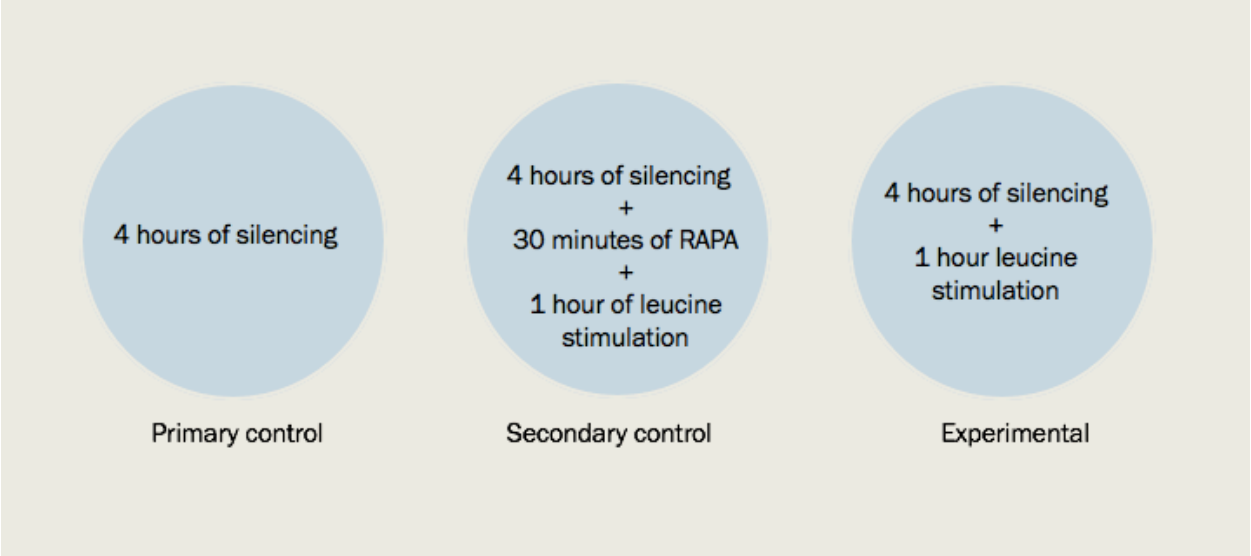



Figure 6. Illustrates the experimental set up for Day 4 oligo-lineage cells undergoing the 4 hour starvation.

Experimental #2: This second experiment was run in a very similar manner to experiment 2, however, this focused on the effects of methionine rather than leucine. Additionally, the cells in this experiment all had 22 hour starvation periods, thus there were no "Day 4" cells. For the one-hour stimulation 5 uL of from the 200X methionine stock was added per 1mL of media to yield the "normal" concentration of leucine like in the standard growth media. Also, the secondary control for this experiment was omitted due to limited availability to cells and consistent results indicated the effectiveness of rapamycin. The cells were allowed to grow in the standard, N2 growth media containing insulin, growth factors, and amino acids. At Day 3, the N2 media was removed from the plates and replaced without methionine, insulin, or growth factors. The experimental cells were stimulated with methionine for one hour after the 24 hour starvation (Figure 6).

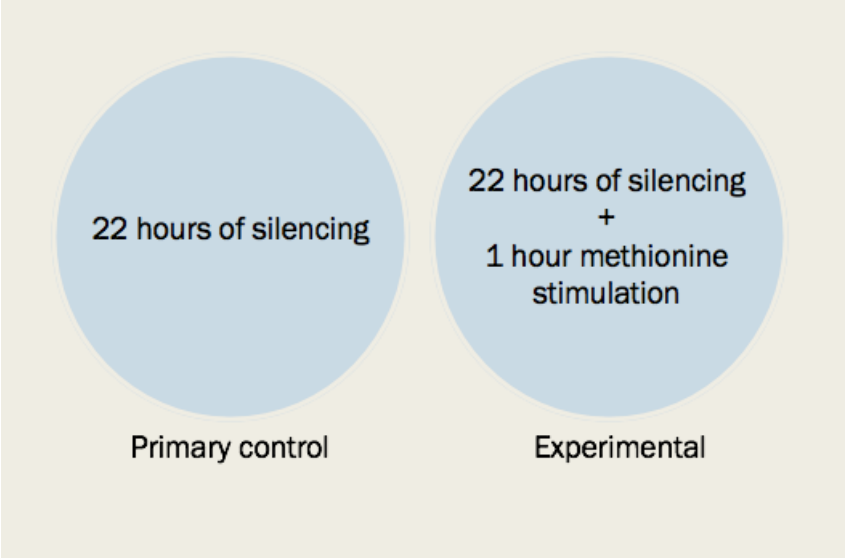



Figure 7. Illustrates the plate conditions for the second experiment.

Experimental #3: The third experiment was designed to measure the effect of leucine when it was combined with the nonessential amino acid alanine. The cells were allowed to grow in the standard, N2 growth media containing insulin, growth factors, and amino acids. The plates were silenced at Day 3 for a 22-hour silencing period with silencing media without insulin, leucine, or growth factors. After the 22-hour silencing period, the "experimental #1" was stimulated with leucine for one hour, the "experimental #2" was stimulated with alanine for one hour, and the "experimental #3" was stimulated with both alanine and leucine for one hour (1uL per 5uL of media from a 200X stock for both alanine and leucine).

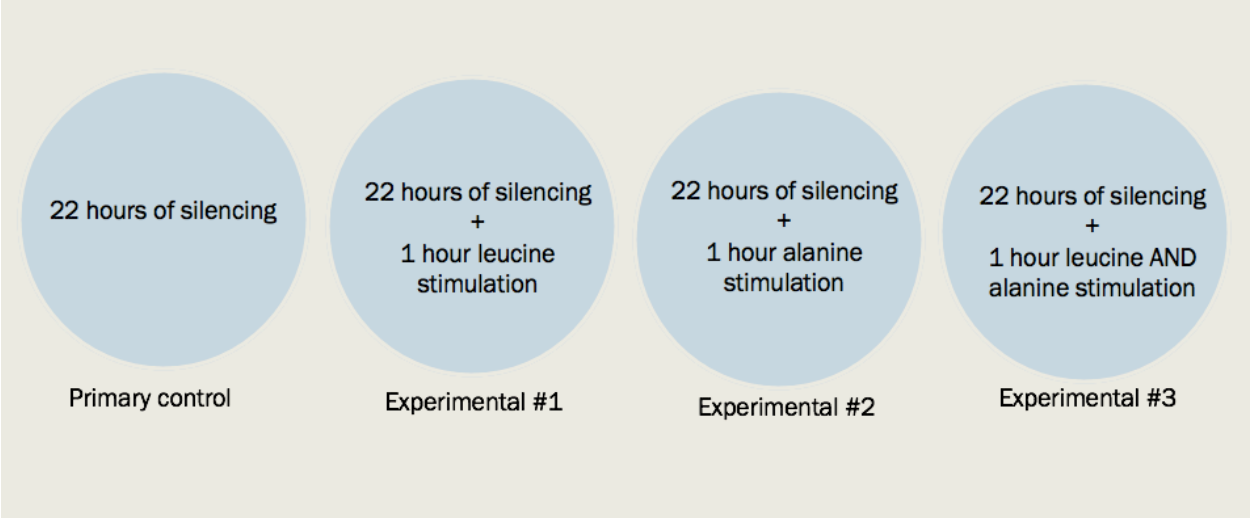



Figure 8. Illustrates the plate conditions for the third experiment.

*Protein Quantification and Western Blotting*

Each 10 cm plate was placed on ice along with two microcentrifuge tubes to collect the contents from plate scraping. The media was removed and 5 mL of non-sterile PBS was added and then aspirated. Following this rinse, 5 mL of PSB with inhibitors (PMSF, L&A, Sodium Ortho-Vanadate, Sodium Flouride, and Sodium Pyro-Phosphate) were added to the plates and aspirated until the plates were fully dry. Finally, 100 uLs of RIPA lysis buffer were added (10mM Tris-HCl, 150mM NaCl, 0.1% SDS, 1% SDS, 1% deoxycholate, 1% NP40, and 1% TX-100, pH 7.4) and spread along the plate and with a scraper. The samples were transferred to a labelled microcentrifuge tube and sonicated for three – thirty second intervals at 50 amps. After sonication, 10 uL were removed and set aside in a microcentrifuge tube for protein quantification. The remaining was used for analysis via western blotting. The samples were stored in a -80°C freezer to preserve the proteins.

The proteins were quantified by using varying volumes of albumin (concentration: 2mg/mL) to create a standard curve and determine the concentration of the unknown proteins. This was done using the BCA Protein Assay Kit. The volume of protein needed to obtain 10ug of

protein per well was calculated based on the quantification results. The protein, SDS buffer, and water were to each well in a total volume between 20 and 30 uL. Using a Invitrogen Novex™ WedgeWell™ 10% Tris-Glycine gel, the samples ran against 5uL of See Blue® Prestained Standard (P/N 100006636) for 30 minutes in approximately 400 mL of 1X Tris-Glycine SDS running buffer. After the gel ran, the Tris-Glycine gel was transferred to nitrocellulose membrane in Tris-Glycine SDS transfer buffer for approximately an hour. Following the transfer, the nitrocellulose membrane rinsed with 100mM TBS-T for 5 minutes and was then blocked at room temperature in 5%Bovine Serum Albumin (BSA) 100mM Tris-Buffered Saline with Tween (TBS-T) for approximately 4 hours. Following the blocking, the three primary antibodies were added; GAPDH, S6K, and S6KP (1:1000 dilution in 5% BSA TBST for each) and incubated overnight at 4°C. The blots were rinsed 4 times in TBS-T the following morning (each wash was 5 minutes). Following the washed, the secondary antibody was added and the blots incubated at room temperature for one hour using horseradish peroxidase-linked (HRP) Anti-rabbit IgG (1:10.000 in 5 % BSA TBS-T). The blots were then washed 4 times with TBST and then the TBS-T was decanted and an ECL reagent (0.1M Tris, 0.2mM PCA, 1.25mM Luminol, hydrogen peroxide 30 wt % in $H_2O$) was added. The Azure-biosystems™ c300 was used to for chemiluminescent antibody detection and then the blot images where then quantified using ImageJ.

As a preliminary experiment, a strand of human embryonic kidney (HEK293) cells sonicated with 1X RIPA buffer and plated in triplets in a 1:5 and 1:50 dilution for protein quantification. The concentration was determined, and the proteins were loaded into a Tris-Glycine gel in three different concentrations (10, 5, and 2.5 ug). After the gel ran, the standard western blot procedure listed above was followed and blocked with GAPDH, S6K, and S6KP.

These data were used to confirm the integrity of both the experimenters' technique and the antibodies (Figure 9).

*Fluorescence Microscopy (IFM)*

Cells were plated and treated in various conditions leading up to the staining. To begin, the plates were placed on ice for two minutes and then the media was aspirated and then blocked with 3% NGS/HE for ten minutes. Following the removal of the blocking solution, 20uL of the primary antibody cocktail was added to each well in the 4 well plate (A2B5 1:25 dilution, O4 1:15 dilution, HPC-7 1:25 dilution in NGS/HE) and incubated on ice for 15 minutes. Following the 15 minute incubation, each well was washed 4 times, for 5 minutes each with HE. The cells were then removed from the ice and fixed with 4% PFA for 15 minutes and then washed 3 times for 5 minutes each with HE. After fixation, 20 uL of the secondary antibody cocktail was added to each well and let incubate for 45 minutes at room temperature (Hoescht 1:200 dilution, uFl 1:50 dilution, gCY3 1:500 dilution in NGS/HE). Next, the wells were washed 5 times for 5 minutes each with HE. The HE was removed and a coverslip was added and the plates were stored in a -30 °C freezer.

**Results**

Unfortunately, due to the COVID-19 pandemic, the figures below only depict the first experimental condition. The proteins from experiment 2 and 3 were obtained, sonicated and stored, but the western blot analysis was not conducted. Additionally, IFM staining was done to categorize the morphology and integrity of the cells in the different experimental conditions but the plates were not counted or pictured prior to the pandemic so these data are also not included. A preliminary experiment was conducted with HEK293 cells to confirm the integrity of the antibodies used in the experiments with the oligo-lineage cell culture as well as the accuracy and

validity of the technique (Figure 9). The GAPDH antibody was used to visualize consistency loading the protein into the gel. The protein concentration was doubled from lane 1 to lane 2 and lane 2 to 3 and so it would be expected that the band intensities would approximately double if loading was consistent.


Figure 9. Demonstrates band intensities of three protein concentrations from HEK293 cells probed with the GAPDH primary antibody. The numeric values associated with the GAPDH arbitrary band intensities were approximately double indicating consistency in loading (3739, 8430, and 16019 respectively) and linearity of response.

Experiment 1

*MXG1*

Each mixed glial culture was set up slightly different. In the first mixed glial cell culture, only 5 plates of cells were available. Thus, the normal media was removed from three of the plates at Day 3 after release and replaced with leucine-limiting media. Following approximately 22 hours of starvation, one of the plates was treated with rapamycin for 30 minutes. After the 30 minutes passed, the rapamycin plate and one of the other plates were stimulated with leucine for one hour. Therefore, there were three conditions for the D3 cells (control “C”, leucine “L”, and rapamycin followed by leucine “LR”) (Figure 5). With the remaining 2 plates, the D4 cells were starved of leucine for only 4 hours, and one plate was stimulated with leucine for one hour.

There was no secondary control plate with rapamycin inhibition followed by leucine stimulation (Figure 6). Unfortunately, western blot analysis was not done with MXG1.

*MXG2*

In the second mix glial cell culture, there were enough cells to have 6 plates. Three of the plates were used for the longer starvation period at D3 and three were used for the 4-hour starvation period at D4 (Figure 5 and 6).

| | D3 control | D3 Leu | D3 Leu + RAPA |
|---|---|---|---|
| **(P-S6K)/TOTAL Low exposure** | 0.7144105 | 1.40641186 | 0 |
| **(P-S6K)/TOTAL Med exposure** | 0.64136326 | 1.42632764 | 0 |
| **average** | 0.67788688 | 1.41636975 | 0 |
| **STDEV** | 0.0516522 | 0.01408258 | 0 |
| **STERR** | 0.03652362 | 0.00995789 | 0 |

Table 1. Indicates the arbitrary band intensities analyzed through ImageJ at two saturations in the control, leucine, and leucine with rapamycin conditions for cell cultures starved of leucine for 22 hours.

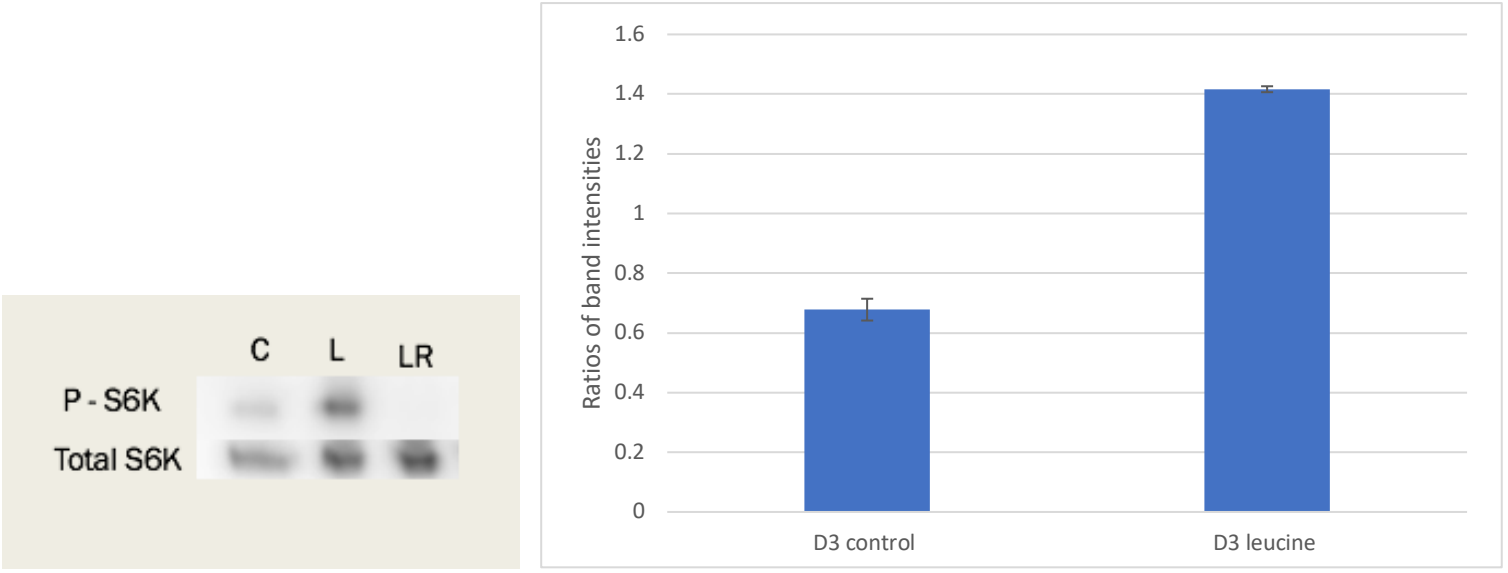



Figure 10. Represents the level of mTOR activity in immature oligodendrocytes starved of leucine for approximately 22 hours (D3 cells). The intensity of the western blot bands is displayed as a ratio of phosphorylated S6K over the total S6K. The control cells demonstrated

less phosphorylated S6K compared to the cells stimulated with leucine. The activity of mTOR was completely inhibited by rapamycin as seen by no band in the lane for phosphorylated S6K.

| | D4 control | D4 Leu | D4 Leu + RAPA |
|---|---|---|---|
| **(P-S6K)/TOTAL** | 0.43082494 | 0.59821 | 0 |
| **(P-S6K)/TOTAL** | 0.32846256 | 0.5512072 | 0 |
| **average** | 0.37964375 | 0.5747086 | 0 |
| **STDEV** | 0.07238114 | 0.033236 | 0 |
| **STERR** | 0.05118119 | 0.0235014 | 0 |

Table 2. Indicates the arbitrary band intensities analyzed through ImageJ at two saturations in the control, leucine, and leucine with rapamycin conditions for cell cultures starved of leucine for 4 hours.

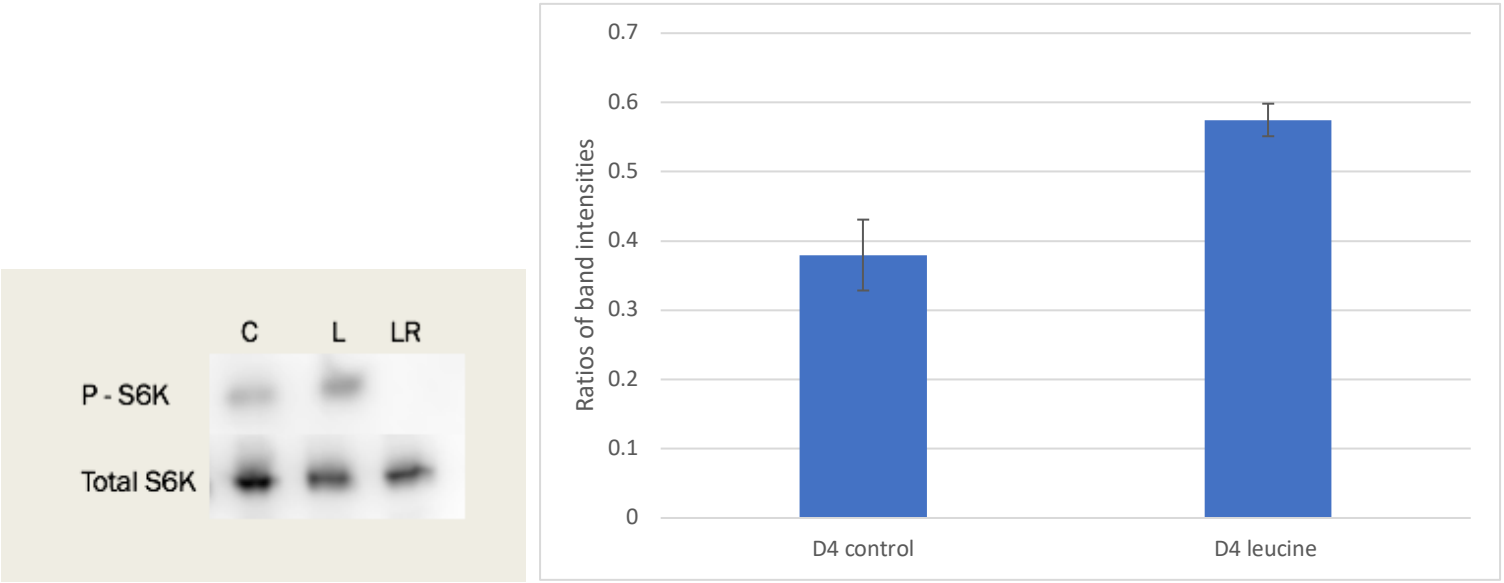



Figure 11. Represents the level of mTOR activity in immature oligodendrocytes starved of leucine for 4 hours (D4 cells). The intensity of the western blot bands is displayed as a ratio of phosphorylated S6K over the total S6K. The control cells had less phosphorylated S6K compared to the cells stimulated with leucine. There was no band for the phosphorylated LR group indicating the activity of mTOR was completely inhibited by rapamycin.

Figure 10 shows the level of mTOR activity is significantly enhanced when leucine was added to cells that were silenced for a longer period of time. This induction was more prominent in the D3 cells compared to the D4 cells that had a shorter starvation period (Figure 11). However, in both D3 and D4 groups, there was a significantly higher ratio of phosphorylated S6 ribosomal protein (P-S6K) in the cells that were stimulated with leucine compared to the primary and secondary

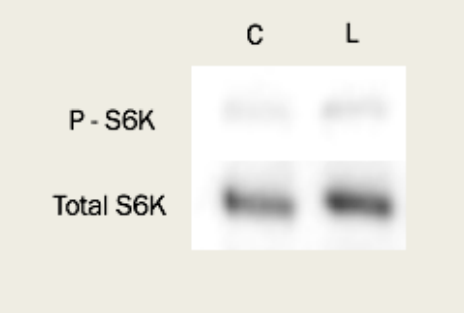
controls. The arbitrary band intensities that were analyzed through ImageJ software. These analyses were performed on two western blot exposures that did not appear to be saturated. The standard error bars were calculated from standard deviations of these varying exposures.

*MXG3*

In the third mixed glial cell culture, there were only enough cells to seed 5 plates. Thus, the normal media was removed from two of the plates at Day 3 after release and replaced with leucine-limiting media. After the 22 hours starvation period, one of the other plates was stimulated with leucine for one hour. There was no secondary control for the D3 cells (Figure 5). With the remaining three plates, the D4 cells were starved of leucine for 4 hours. One plated was stimulated with leucine after the 4 hours passed, and one plate was inhibited with rapamycin for 30 minutes after three and a half hours and then stimulated with leucine for an hour (Figure 6). Therefore, there were only two conditions for the D3 cells (control "C", leucine "L"), while there were three conditions for the D4 cells (control "C", leucine "L", and rapamycin followed by leucine "LR").

| | **D3 control** | **D3 Leu** |
|---|---|---|
| **(P-S6K)/(TOTAL) low exposure** | 1.28782414 | 1.37905478 |
| **(P-S6K)/(TOTAL) Med. exposure** | 1.36777867 | 1.51293559 |
| **(P-S6K)/(TOTAL) high exposure** | 1.29725324 | 1.36841403 |
| **average** | 1.31761868 | 1.4201348 |
| **STDEV** | 0.04369491 | 0.08054375 |
| **STERR** | 0.02522727 | 0.04650196 |

Table 3. Indicates the arbitrary band intensities analyzed through ImageJ at three saturations in the control and leucine conditions for cell cultures starved of leucine for 22 hours.



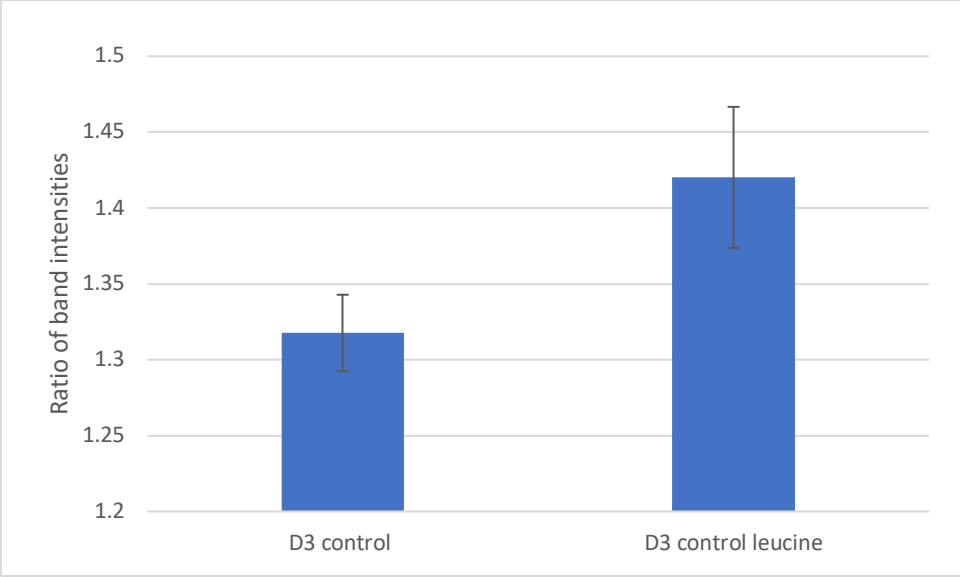
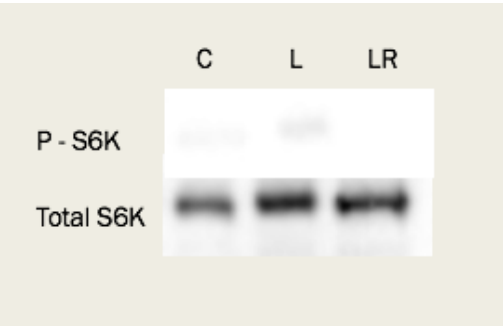




Figure 12. Displays the level of mTOR activity in immature oligodendrocytes starved of leucine for approximately 22 hours (D3 cells). This figure on the right is a ratio of phosphorylated S6K over the total S6K. The control cells demonstrated less phosphorylated S6K compared to the cells stimulated with leucine. There was no secondary control for this group.

| | D4 control | D4 Leu | D4 Leu + RAPA |
|---|---|---|---|
| **(P-S6K)/(TOTAL) low exposure** | 0.63043706 | 0.69747117 | 0 |
| **(P-S6K)/(TOTAL) med exposure** | 0.57683718 | 0.68913271 | 0 |
| **(P-S6K)/(TOTAL) high exposure** | 0.38207538 | 0.54512014 | 0 |
| **average** | 0.52978321 | 0.64390801 | 0 |
| **STDEV** | 0.13069598 | 0.08565433 | 0 |
| **STERR** | 0.07545736 | 0.04945255 | 0 |

Table 4. Indicates the arbitrary band intensities analyzed through ImageJ at three saturations in the control, leucine, and leucine with rapamycin conditions for cell cultures starved of leucine for 4 hours.



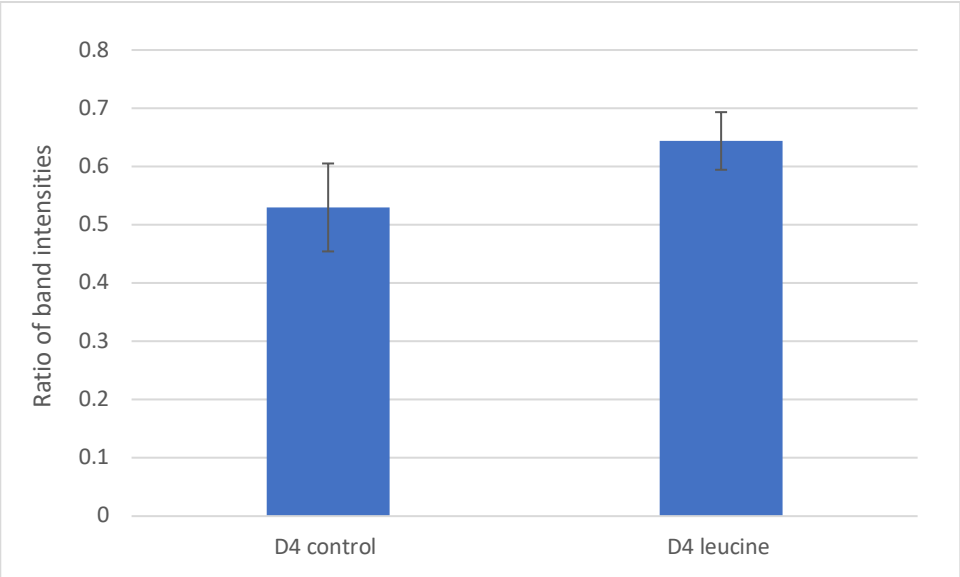




Figure 13. Depicts the level of mTOR activity in immature oligodendrocytes starved of leucine for 4 hours (D4 cells). The band intensity is displayed as a ratio of phosphorylated S6K over the total S6K. There appeared to be less phosphorylated S6K in the control cells compared to the those stimulated with leucine, but it was not significant. There was no band for the phosphorylated LR group which suggests the activity of mTOR was completely inhibited by rapamycin.

Figure 12 shows a significant increase in the level of phosphorylation of S6K after stimulation with leucine indicating an upregulation in mTOR activity after 22-hours of silencing. Figure 13 demonstrates an increase in phosphorylation between the control cells and those stimulated with leucine, although the induction in mTOR activity did not appear to be significant. The arbitrary band intensities were obtained through analysis with ImageJ software on two western blot exposures. The standard error bars were calculated from standard deviations of these varying exposures.

*MXG4*

The fourth mixed glial cell culture was set up exactly like the second mixed glial cell culture. Six plated were seeded with cells. Three plates were treated starved of leucine for 22 hours, one plate was treated with rapamycin for 30 minutes, and then the plate with rapamycin as well as one

other plate were stimulated with leucine for approximately an hour and a half (Figure 5). The other three plates were starved of leucine for four hours and then one plate was treated with rapamycin for 30 minutes. The plate that was treated with rapamycin as well as one of the other plates were stimulated with leucine for approximately an hour and a half (Figure 6). Unfortunately, western blots were not run with MXG4.

Experiment 2:

*MXG5*

The fifth mixed glial culture had only two plates. In this experiment there was not a secondary control because rapamycin was consistently eliminating mTOR activity and phosphorylation of S6K. The silencing media in this experiment lacked methionine rather than leucine. At D3, the growth media was removed and replaced with methionine limiting media for approximately 22 hours and then stimulated with methionine for one hour (Figure 6). Unfortunately, western blots were not run with MXG5.

Experiment 3:

*MXG6*

The sixth mixed glial culture had 4 plates. In this experiment, all 4 plates were replaced with leucine limiting media at D3 for approximately 22-hour starvation. One plate acted as a control and was left alone. The second plate acted as a secondary control and was stimulated with leucine for an hour. The third plate acted as another secondary control and was stimulated with alanine for one hour. The third plate was stimulated with both leucine and alanine for an hour. The purpose of this was to see if there was a different between the induction of mTOR activity with leucine and alanine, compared to just with leucine to see if alanine amplified the phosphorylation of S6k. Unfortunately, western blots were not run with MXG6.

Fluorescence Microscopy (IMF):

IMF was used to verify the morphology of the oligodendrocytes and the integrity of the nuclei. Fragmented nuclei suggest that cells are going through apoptosis and can be used to indicate the oligodendrocytes tolerance to prolonged amino acid starvation. Additionally, IMF was done to confirm the biological markers at the distinct stages with the use of Hoescht and Phalloidin antibodies. Cell staining was done one cells starved of methionine and cells starved of leucine. A cell count is needed to examine the number of cells in the progenitor versus the immature versus the mature oligodendrocyte form in the cultures with a 22-hour compared to a 4-hour leucine withdrawal period. Unfortunately, the cells were not pictured or quantified.

**Discussion**

The goal of this thesis was to examine the effects of leucine on mTOR activity by looking at the changes in the phosphorylation of S6K. Inconsistent results were obtained from previous research using a four-hour starvation period; therefore, a longer starvation time was implemented. The phosphorylation of S6K was quantified via ImageJ software after the obtaining images of the western blot with different saturations. Proportions from the arbitrary band intensities were calculated from the phosphorylated S6K over the total amount of the S6K. This was done for the primary control cell cultures where leucine was withdrawn for 22 hours and compared to the experimental cell cultures where leucine was withdrawn for 22 hour and then added back into the media for one hour. The prolonged starvation yielded consistently better results than the 4-hour starvations. However, both indicated that there is an induction of mTOR activity upon leucine stimulation (Figures 10 through 13). This suggests that mTOR does sense extracellular leucine and adjust its activity depending on its presence or absence. Additionally, the data indicate that rapamycin completely inhibited mTOR activity in the presence of leucine

(Tables 1, 2, and 4) (Figures 10, 11, and 13), which further confirms that the leucine effects on the phosphorylation of S6K are mediated by mTOR. It is important to note that the induction in mTOR activity in our control cultures may not be equivalent to the basal activity of mTOR in cells grown in N2 media, as this media contains leucine and insulin.

Unfortunately, the completion of the study was not possible due to unforeseen circumstances (MXG1, MXG4, MXG5, and MXG6 were not analyzed). However, the results from the experiments that were examined provided consistent and encouraging results for future directions. First, the cell cultures that were starved and then treated with methionine must be analyzed. If there is not a prominent induction in mTOR activity in these cultures it could potentially indicate that mTOR is more sensitive to leucine and leucine could potentially serve as a proxy in oligodendrocytes. Additionally, the analysis of the cell cultures with leucine and alanine must be run in order to confirm is alanine facilitates the induction of mTOR activity with leucine. Finally, it will be important to quantify the number of cells based on their expressed antigens as well by the morphology in cell cultures deprived of leucine (or methionine) for 22 versus the control. This is crucial for the validity of the experiment to confirm that the cell cultures had an equal dispersion of cells in each stage and that the starvation did not inhibit their progression through the oligo-lineage. If there is a significant difference in this, it could potentially affect the interpretation of the data because it could mean that the induction in mTOR activity was due to the stage of maturation rather than a better silencing to “basal level”. Furthermore, more experiment on the effect leucine on mTOR activity in oligodendrocytes is needed to make a confident conclusion about its significance.

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