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Mitochondrial Structure and Function in Oligodendrocytes; a Preliminary Study

Dimosthenis Sampatakos
dsampata@trincoll.edu

TRINITY COLLEGE

MITOCHONDRIAL STRUCTURE AND FUNCTION IN OLIGODENDROCYTES; A PRELIMINARY STUDY

BY

DIMOSTHENIS SAMPATAKOS

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Honors Thesis Committee

Approved:

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Hebe Guardiola-Diaz, Advisor

_____

Robert Fleming

_____

Daniel Blackburn

Date: _____

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Abstract

Oligodendrocytes are glial cells responsible for the creation of myelin, a fatty substance that maximizes axon potential efficiency by creating a sheath around axons. The process of creating myelin is called myelination and is exclusively performed by oligodendrocytes. Myelination requires a lot of energy output to cover the needs of creating myelin. Oligodendrocytes have numerous mitochondria that help them meet that energy requirement fulfill their function. Oligodendrocyte progenitors are cells that differentiate to become myelinating oligodendrocytes. Not a lot is known about progenitors' mitochondria and their mitochondrial metabolism. Differentiation is necessary to replenish oligodendrocytes that degrade. If the levels of differentiation are low, there are insufficient myelinating cells to ensure efficient axonal function, ultimately raising the risk of neurodegenerative diseases like multiple sclerosis to occur. This thesis study aims to investigate the difference in mitochondrial structure and function between rat neonatal telencephalon oligodendrocytes and oligodendrocyte progenitors in culture. Function is addressed in terms of energy systems used by each cell through lactate determination assays as well as the importance of mitochondrial division by inhibiting it via the mitochondrial division inhibitor Mdivi-1. Mitochondrial structure is investigated with the use of Transmission Electron Microscopy which allows for length measurements of the organelle. While mitochondrial division inhibition seemed to not be detrimental to oligodendrocyte progenitor differentiation, lactate production hinted towards a trend of change when the inhibitor was introduced. Contrary to previous hypotheses, mitochondrial length and shape is similar between oligodendrocytes and their progenitors. As this is a preliminary investigation sample sizes need to be improved to make more conclusive statements about the metabolism and mitochondria of oligodendrocyte lineage cells.

Introduction

Oligodendrocytes (OLs) are glial cells present in the central nervous system (CNS) responsible for creating myelin through the process of myelination (Miller, 2002). Myelin consists of the extended and altered parts of the OL cell membrane and is the substance responsible for the creation of a sheath around mammalian axons so nerve signals travel more efficiently (Hirano & Dembitzer, 1967). The creation of myelin is an energetically expensive process in the form of lipid and protein synthesis (Harris & Attwell, 2012). OLs use multifaceted ways to cover their metabolic needs. In postnatal rat oligodendrocytes, *in vitro*, prioritize oxidative phosphorylation to produce ATP (Rao *et al*., 2017). The rate of ATP production was not changed in OLs under metabolic stress conditions of low or no glucose (Rao *et al*., 2017). When myelinating OLs die, the axons corresponding to them degrade so nerve signals do not get delivered, ultimately leading to axonal degeneration (Campbell *et al*., 2014). There is a high abundance of OLs in the CNS and each is responsible for creating myelin for up to 20 and 60 axons, with myelin internodes ranging from 20 to 200 μm in length (Chong *et al*., 2012). OLs have especially high energy demands that make the understanding of the underlying energy systems in their cell lineage important.

Mitochondria are necessary to meet energetic and biosynthetic demands, particularly in cells like myelinating oligodendrocytes, because these organelles are highly involved in metabolic processes and in the making of macromolecule precursors and metabolites (Chandel, 2021). To perform these processes, mitochondria are characterized as dynamic, undergoing fusion and fission constantly so they can easily alternate the primary functions on which they focus (Chen *et al*., 2005). With loss of fusion, cells are deprived of their oxidative processes (Chen *et al*., 2005), while mitochondrial fission is known to partake in the maintenance of

mitochondrial health removing damaged organelles which ultimately maintains bioenergetic systems (Twig *et al*., 2008). The oxidative process performed by mitochondria is called oxidative phosphorylation (OXPHOS). OXPHOS happens thanks to the electron transport chain (ETC) on the inner mitochondria membrane in aerobic conditions (Lehniger, 2005). In terms of ATP production, OXPHOS is the metabolic pathway that produces energy most efficiently.

Proliferative and cancerous cells have been shown to undergo aerobic glycolysis, the effect of using glycolysis as the primary energy producer even in the presence of oxygen, also referred to as the Warburg effect (Warburg, 1927 & Chandel, 2021). Warburg's initial idea was that mitochondria were damaged in cancerous cells; however, it has since been shown that mitochondria are involved in macromolecule precursor pathways instead of the making of ATP during that cell division period of the cell cycle (Fantin et al., 2006). An example of mitochondrial biosynthetic function is glutamine uptake's importance in using glucose-derived carbon and citric acid cycle intermediates as biosynthetic precursors (DeBerardinis, *et al*. 2007). A characteristic biproduct of this effect is lactate in the cytoplasm, so glycolytic reactions can continue. It is important to note that there is evidence for the lactate produced from aerobic glycolysis to be used as fuel for oxidative phosphorylation (Sonveaux *et al*., 2008). To investigate the potential for aerobic glycolysis in proliferating oligodendrocyte lineage cells, I will be using lactate concentration as an indicator of metabolic processes followed by these cells.

Mitochondria's connection with metabolism is not only functional, but their structure has a role in metabolic processes as well. Mitochondria have an important role in the differentiation and cell fate of neural stem cell types by changing the primary metabolic pathways they follow (Beckervordersandforth, 2017). In rat optic nerves *in vivo*, OLs have been shown to contain short, fragmented mitochondria that release lactate and produce little

ATP (Rinholm, 2016). On the contrary, *in vivo* rat optic nerve oligodendrocyte progenitor cells (OPCs) and developing oligodendrocytes contain long and tubular mitochondria, shown to consume lactate instead (Tondera *et al.*, 2009). Long and tubular mitochondria actively utilize the oxidative phosphorylation (OXPHOS) pathway (Tondera *et al*., 2009). When myelinating oligodendrocytes are unable to use OXPHOS, they are unable to myelinate, while there is evidence of no necessity for OXPHOS to be used once myelination is over (Fünfschilling *et al*., 2012).

Poor remyelination from OLs can lead to neurodegeneration. The most widely known neurodegenerative disease is Multiple Sclerosis (MS) that is caused by the accumulation of axonal degradation because of poor remyelination (Narine & Colognato, 2022). Alterations in the metabolic pathways used from OL lineage cells are considered potential reasons behind poor myelination in MS patients. For example, the production of ATP (adenine triphosphate) through lipid metabolism has been described in MS and through short-chain fatty acid supplementation, neurodegeneration was successfully altered (Chen *et al*., 2019). Efforts to establish how glucose is improperly used in MS have been made. Citric acid cycle and glycolysis enzymatic levels have been described to be elevated in MS patients (Nijland, 2015). Additionally, lactate has been associated with the progression of MS (Amorini *et al*., 2014). In MS oligodendrocytes, cytosolic pyruvate is found to be used to make lactate which indicates a switch from the OXPHOS pathway to aerobic glycolysis (Schipers *et al*., 1997**).** Therefore, the OXPHOS pathway is not the only bioenergetic pathway affected in MS (Nijland *et al*., 2015).

To prevent neurodegeneration due to low remyelination levels, our nervous systems have pools of OPCs present and ready to differentiate to become myelinating OLs. These cells have been found to proliferate during demyelination *in vivo*, while their proliferation levels significantly drop when OLs are able to remyelinate (Di Bello *et al*., 1999). However, the percentage of differentiating cells that ultimately become myelinating glial cells drops

dramatically the further the rat body ages (Neumann *et al*., 2019). Neumann *et al*. (2019) compared the rate of differentiation between neonatal and adult rats to find that the differentiation levels from available OPCs were significantly reduced as the animals grew older. Additionally, they were able to show that alternate day fasting in older rats was the most effective method to bring the rates of differentiation back up to a comparable level to the neonatal rat OPCs (Neumann *et al*., 2019). By changing their diets, the data suggest that the OPC pool remained high in adult rats, so the adult rat system failed to differentiate. Postnatal rat brain OLPs in culture utilize the oxidative phosphorylation pathway to produce ATP, a phenomenon that does not change in metabolic stress conditions (Rao *et al*., 2017). It has also been shown that OL lineage cells undergoing differentiation are susceptible to apoptosis if they do not receive the proper survival signals or if they fail to integrate with their environment (Kawai *et al*., 2009). To achieve higher remyelination rates in demyelinated areas, a higher number of myelinating OLs needs to exist. To get to this higher number of OLs, the pools of OPCs present, need to differentiate to myelinating OLs and differentiation rates need to remain high to combat demyelination that can lead to neurodegeneration. We need to further our understanding about the metabolic pathways utilized in OPCs before and during differentiation as well as the mitochondrial involvement in this process.

Mitochondria are dynamic organelles and they constantly undergo fission, or else division, and fusion to change their function when necessary. Inhibiting one of the two processes is a way of investigating how the dynamic life of mitochondria can affect cellular functions. This study targets fission as it has been inhibited in oligodendrocyte lineage cells, but not in the metabolic approach. The first work done with the Mitochondrial Division Inhibitor 1 (Mdivi-1) was performed with the hopes of regulating apoptotic levels *in vitro* and was suggested as a potential therapeutic for neurodegenerative diseases (Cassidy-Stone *et al*., 2008). It was reported that Mdivi-1 inhibits Drp1 GTPase phosphorylation, therefore inhibiting

mitochondrial fission (Cassidy-Stone *et al*., 2008). It has been assumed that Mdivi-1 inhibits Drp1 GTPase, with multiple studies focusing on using it *in vitro* as a mitochondrial division inhibitor in yeast and mammalian cells for its potential therapeutic purposes. Challenges to this claim, however, have been made. In a more recent study, Mdivi-1 was shown to specifically inhibit the function of Complex I in rat neuron cultures at concentration 50 μM, while partially affecting Drp1 in primary cortical neurons (Bordt *et al*., 2017). This paper claimed that there is weak evidence showing the effect of Mdivi-1 on Drp1. The authors make this claim due to the faulty assumption that Mdivi-1 has the same effect as siDrp1 inhibition (Bordt *et al*., 2017). For example, a study performed on β pancreatic cells under hypoxic conditions *in vitro* was able to replicate the effect of siDrp1 with the introduction of Mdivi-1 to mitigate apoptosis as well as reduce mitochondrial damage (Zhang *et al*., 2018). The connection of Mdivi-1 effects and the effect of suppressing Drp1 is what lead Bordt *et al.* (2017) to investigate the exact mechanism of action of Mdivi-1 which is still a question today. More recently, studies have been following the assumption that Mdivi-1 is a Drp1 GTPase inhibitor (Xu *et al*., 2020). Mdivi-1 has been shown to halt OPC differentiation in optic nerve derived oligodendrocyte progenitors *in vitro* therefore suggesting that mitochondrial fission precedes mitophagy, the selective degradation of mitochondria by autophagy, in stressful conditions (Yazdankhah *et al*., 2021). Additionally, when exposed to Mdivi-1, oligodendrocytes show mitochondrial depolarization and sensitivity to oxidative stress (Ruiz *et al*., 2020). There is evidence to support that mitophagy plays an important role in oligodendrocyte differentiation (Yazdankhah, *et al*., 2021). To understand the mitochondrial mechanisms involved in differentiation, I take the approach of inhibiting mitochondrial fission with the use of Mdivi-1.

In this study, I investigated the metabolism and length of OPC mitochondria in enriched oligodendrocyte lineage cell cultures derived from neonatal rats and compare them to differentiated OL mitochondria. To investigate the potential usage of aerobic glycolysis as a

metabolic process, I performed media lactate concentration assays to quantify the lactate produced by OPCs and OLs. I used Transmission Electron Microscopy (TEM) to visualize and make comparisons between OPC mitochondria and differentiated OLs. I also addressed the significance of mitochondrial division in the differentiation process by exposing OPCs to Mdivi-1. When inhibiting mitochondrial division, I hypothesized the OLs will not be able to mature in the same rate as they would not have the sheer number of mitochondria necessary to meet their high energy demands through OXPHOS, a process that takes place inside the mitochondria. I also predicted that differentiation would be reduced since it requires high energy production. This preliminary study is important to begin investigating the mitochondrial mechanisms involved in differentiation both metabolically and structurally.

Methods

Mixed Glial Cell Cultures

For this procedure, we prepared mixed primary cultures from old neonatal rats (P1-3). We used 12 animals for each round of experiments. Animals were sacrificed by means of quick decapitation, as approved by the Trinity College Institutional Review Board. We retrieved telencephalons by cutting through the skull and exposing the brain. We placed the brains in HEPES (Ca^{2+} and Mg^{2+} free HEPES-buffered 20 mM Earle's Balanced Salt Solution 1X, HE) to avoid dehydration before removing and discarding the hindbrain and the olfactory bulb. We separated the two hemispheres and subsequently rolled them on sterile filter paper to remove the meninges. To separate cells, we first used razors and tweezers to chop the tissue in chunks as fine as possible, followed by controlled digestion in trypsin-HE (0.025% bovine pancreatic trypsin) solution with agitation at 110 rpm for 20 minutes before adding SBTI (Soybean meal Trypsin Inhibitor in HE, 7.8 mg/mL) to terminate this reaction. To digest extracellular DNA, we added $MgSO_4$ (3 mM) and DNase (20 μg/mL) for a 5-minute incubation. We centrifuged the tissue suspension and triturated the pellets in trituration DNase (80 μg/mL DNase in HE) to mechanically separate cells. We then centrifuged through a 4% BSA (Bovine Serum Albumin) and resuspended the cell pellets in fresh cell culture media of 5% FPD (Filter-based Protein Digest). Finally, we seeded 20-30 million cells, determined with the use of a hemocytometer, per T-75 cell culture flask that we coated with Poly-L-Lysine (0.025 mg/mL in HE). We changed the media feeding these mixed glial cell cultures every 3 to 4 days, until day 11 when we followed the procedure described in the next section to obtain enriched oligodendrocyte lineage cultures. All surgical tools and solutions were autoclaved and sterilized, and the procedure took place under a fume hood following aseptic techniques.

Oligodendrocyte Lineage Cultures

As first described by McCarthy and de Vellis (1980), to obtain enriched oligodendrocyte progenitor cultures, 11 days after the establishment of the mixed glial cultures, we added L-Leucine Methyl Ester (1 M, in Phosphate Buffer Saline [PBS]) to each T-75 flask to kill all macrophages and followed this with orbital shaking at 200 rpm in 5% FPD medium. We obtained enriched cultures after differential adhesion of contaminating cells in plastic petri dishes. OL progenitors were seeded in poly-ornithine coated wells in 5% FPD medium. We allowed cells to attach to those surfaces 3-6 hours before exposing them to the experimental solutions in defined N2 medium (insulin 5 μM, T3 15 nM, biotin 10 nM, sodium selenite pentahydrate 30 nM, BSA 0.05%, pyruvate sodium 110nM, penicillin G, hydrocortisone 10 nM, human transferrin 50 μM).

Treatment with Regulators

OPCs will enter a default differentiation program in N2 medium. To maintain OL progenitors in culture, growth factors FGF2 (fibroblast growth factor 2) and PDGF (platelet-derived growth factor) at 10 ng/mL are added as needed (McKinnon *et al*., 1990). I grew these cells in the media: four wells were provided N2 media containing growth factors, and the other four wells were provided N2 media without growth factors for a total of 4 days in culture. Lactate measurements were taken on days 2 and 4. To inhibit mitochondrial division, I exposed the cells to Mdivi-1 for 2 days in culture. In the Mdivi-1 treatment experiments I made controls with and without growth factors (n=4 for each). Mdivi-1 (10 μM) was added in cells exposed to both N2 with growth factors and N2 without growth factors (n=4 for each). This concentration was used based on the experiments performed by Yazdankhah *et al*. (2021). Additionally, I exposed cells without growth factor media to 25 μM of Mdivi-1 (n=2). I took 200-500 μL samples of each well on day 2 and day 4, with day 0 being the first day of exposure to the respective treatment. I kept these samples in -20^{o}C to avoid any of the lactate being

consumed by potential LDH (lactate dehydrogenase enzyme) in the solution produced by the cells. For the metabolic processes experiment, on day 2 and day 4, I collected 200 μL samples of media that I immediately placed on ice so I could preserve the lactate produced and I changed the media on day 2 to repeat the process. In the Mdivi-1 treatment experiments, I allowed cellular growth until day 2, when I fixed the cells and collected all the media. All media samples collected were used for determination of lactate levels using light spectroscopy at λ=490 nm and λ=650 nm. I used 96-well plates for these measurements I diluted every sample in in 1:1 and 1:10 dilutions in DMEM (Dulbecco's Modified Eagle Medium), each in triplicates. To initiate the reactions with the samples, I added 50 μL of the reaction mix (iodonitrotetrazolium chloride, INT, 1.5 mM, phenazine methosulfate, PMS, 40 μM, nicotinamide adenine dinucleotide, NAD^+, 500 μM, LDH 1 μL/well, in Tris Base 0.1 M pH=8) to 50 μL of the diluted sample. The reaction required a 1 hour incubation at room temperature and was stopped via addition of 50 μL 0.1 M Glacial Acetic Acid in the wells. The 96 well plate spectrometer SpectraMax M2 recorded values at wavelengths λ=490 nm and λ=650 nm, using the SpectraMax M2 Plate Reader application. I took measurements before and after the acetic acid addition. For my data analysis, I created standard curves that included 5 lactate concentrations 0-1250 μM made by serial dilution. From the equation that represents the line of best fit, set to cross the axis origin, I calculated the mean lactate concentrations of my 1:1 and 1:10 diluted samples as well as the standard deviation for the biological replicates among all experimental wells. This procedure was first used by Schmiedeknecht *et al*. in efforts to quantify L-lactate produced by cancer cells in culture (2022).

Fluorescent Phalloidin Staining

The fluorescent phalloidin stain was purchased from Invitrogen Detection Technologies. The stock solution was dissolved in 1.5 mL of methanol to create a 6.6 μM stock solution

(equivalent to 200 units/mL). I fixed every well with 4% PFA (paraformaldehyde) and permeabilized cells with 0.1% Tergitol in HE for 4 minutes. After the removal of all solution from the wells, I exposed each well to 18 μL staining mix of phalloidin 1.5 μM (equivalent to 1 unit/well) and Hoechst (1:500 dilution) in HE for a 20-minute incubation in the dark to create cellular outline and nuclear staining. I washed the cells with HE in between each step of this process. I followed the same coverslip procedure as with the immunofluorescence. I stored the wells wrapped in plastic membrane and tinfoil at -20^{o}C. For cell stage identifications, I classified cells based on their appearance under fluorescence using the Zeiss Axiovert 40 CFL fluorescent microscope with the LD Plan-NEOFLUAR 20x magnifier. I used the Hayear 34MP Microscope Camera for the photographs of this experiment.

TEM Sample Preparation and Visualization

For the preparation of TEM (Transmission Electron Microscope) samples, I used cell cultures in T-25 flasks that were fixed with Karnovsky's fixative (2% paraformaldehyde, 3% glutaraldehyde in cacodylate buffer ($Na(CH_3)_2AsO_2$) pH adjusted between 7 and 7.74) for 20 minutes. The two T-25 flasks represented the OLP and OL group of cells to visualize mitochondria of differentiated cells and OL progenitors. In these flasks, osmium tetroxide was added to stain lipids from the samples before EtOH dehydration (sequential steps of 50%, 70%, 85%, 95%, and 100%). After removing the EtOH, I added 5mL of N-butylglycidyl ether to scrape and detach the sheet of cells from the bottom of the flask until the cells evidently float. I spun the sheet of cells in a tube where I exposed them to acetone and Spurr's (low-viscosity epoxy resin embedding medium) in a continuous manner where in each interval the ratio of Spurr's to acetone increased (0: 1, 1: 3, 1: 1, 3: 1, 1: 0). In order to solidify the mix with the sheet of cells, I left the mix in an incubator set at 37^{o}C overnight. For the thin slices of sample, I used the Ultracut E ultramicrotome, from Reichert Jung, equipped with a diamond knife to

cut the slices as thin as 90 nm. To visualize these samples, I worked with accelerating voltage of 60 kV and magnifications between 15000x and 19500x using the CM 12 Philips Transmission Electron Microscope. To quantify, I used mitochondria that looked the biggest in the cross sections accounting for their tubular shape, assuming that their entire length was revealed. I chose specific mitochondria from each cross section because the slicing process is random, and mitochondria are three-dimensional organelles. My lack of experience with the microtome did not allow me to create three dimensional visualizations of the mitochondria of these cells. I consulted the scale bars provided by the AMT Capture Engine EM photograph application for the mitochondrial length measurements.

Results

This study produced results for all questions aimed to be tackled. Proliferative OPC metabolic requirements *in vivo* represent different functions than OLs (Rinholm, 2016 & Tondera *et al,* 2009). Here I ask if this is replicated in the controlled environment of cell cultures by treating enriched oligodendrocyte lineage cells with FGF2 and PDGF to keep them in a proliferative state as OPCs and kept them in N2 media without growth factors in a differentiating state. The findings are summarized in this section by addressing metabolism by focusing on lactate release, to answer the question of what metabolic processes do oligodendrocyte lineage cells undergo.

Measuring lactate is a simple way to answer the question of metabolic systems in oligodendrocyte lineage cells. With the aim of determining whether cells in culture undergo aerobic glycolysis, I measured their lactate concentrations via spectrophotometry at days 2 and 4 in differentiating oligodendrocytes and proliferating progenitors. In our system, OPCs have been growing *in vitro* for 11 days prior to creation of enriched oligodendrocyte lineage cell cultures. By exposing the cells to N2 media, enriched oligodendrocyte lineage cell cultures they enter a default differentiation program. By day 2, most of the cells in culture are differentiating and can be identified as OLs, and by day 4, the vast majority of cells are identified as OLs.

Differentiating oligodendrocytes (Figure 1, blue bars) in N2 media did not show a difference in lactate release from day 2 to day 4. This was true for proliferating oligodendrocytes (Figure 1, red bars) in N2 + growth factors. Additionally, there was no difference in lactate release between the two types of oligodendrocyte lineage cells at day 2 or at day 4. When comparing the two types of cells to each other, we can infer from the data that neither cell type is undergoing aerobic glycolysis.

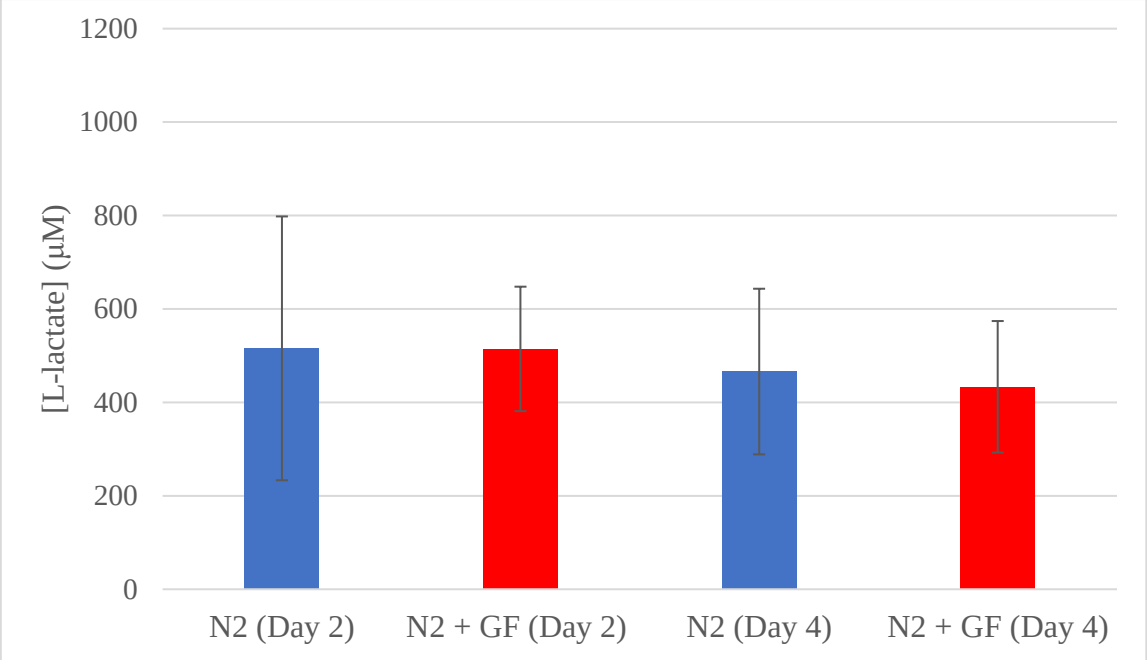

Figure 1: Effect of differentiation on lactate production of oligodendrocyte lineage cells in culture at Days 2 and 4. Enriched oligodendrocyte lineage cell cultures were maintained in N2 media (blue) and N2 media + Growth Factors FGF2 and PDGF (GF) (red). Data shows that there is no significant difference in lactate production between differentiated and proliferating cells or between time points. Error bars represent the standard deviations of the average L-lactate concentrations detected. Total number of samples is n = 4 for each condition at each time point.

While measuring lactate release in media is important to determine energy system utilization in oligodendrocyte lineage cells, it is also crucial to visualize their mitochondria to make connections between mitochondrial form and the metabolic processes they perform. The longest mitochondria from each field on a section were measured. The method of measurement assumes that the fixed mitochondrial samples were sectioned in the “middle” of the organelle. In addition to the measured and represented mitochondrial sections, there were a lot of short

and circular or short and thin sections that were not measured in the assumption that they were stemming from the periphery of the tubular organelle that is distributed across the three-dimensional space of the cell's cytoplasm (data not shown). Lastly, a qualitative observation was made about the shape of mitochondria in OL samples compared to OPC samples. A total of 10 mitochondria were identified as appropriate for length measurement in OPCs and 14 in OLs.

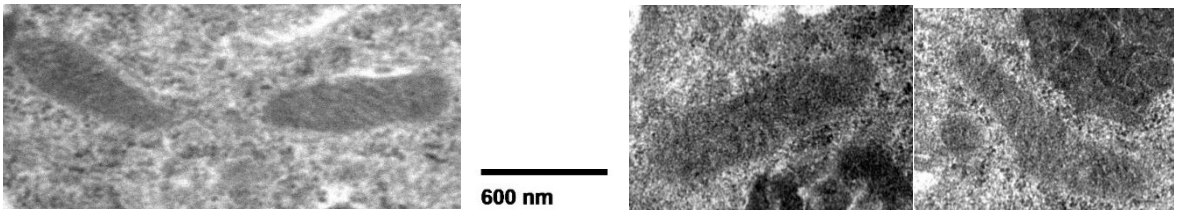

Figure 2: Examples of mitochondria from oligodendrocyte progenitor cells (two mitochondria on the left) and differentiated oligodendrocytes (two mitochondria on the right). They are portrayed as so to make observations of length and width. Mitochondria quantified were assumed to be sectioned longitudinally and in the longest part of the orgenelle. These photos were taken using a TEM at 19500x after 4 days in culture. A scale bar of 600 nm is shown for comparison purposes.

The visual similarities of these mitochondria at 4 days in culture was reflected in the quantification of their lengths as well. The mitochondria captured with the help of the TEM, seemed long and thin for both OLPs and OLs (Figure 2). The average length of mitochondria observed on the TEM were recorded with the help of legends provided by the TEM camera application, AMT. The legend was used on the scale of the screen which helped measure the length of the mitochondria on the same photograph. The average length for OLPs was 960 nm,

while for OLs it was 920 nm (Figure 3). Most mitochondria from both experimental groups were not counted as there was trouble determining which ones were sectioned at their longest points and which ones at their shortest. Considering the tubular and three-dimensional shape of mitochondria, it was difficult to determine which ones were appropriate for measurement. The data presented comes from mitochondria determined to be sectioned longitudinally and approximately the middle of the organelle is present. It is important to acknowledge that mitochondria of smaller size have been omitted in this process. While estimations of the total mitochondria present, showed that OLs had more mitochondria than OLPs, there is not data to suggest that is true. Nevertheless, it is evident that there are mitochondria of the same length and shape in both proliferating and differentiating oligodendrocyte lineage cells.

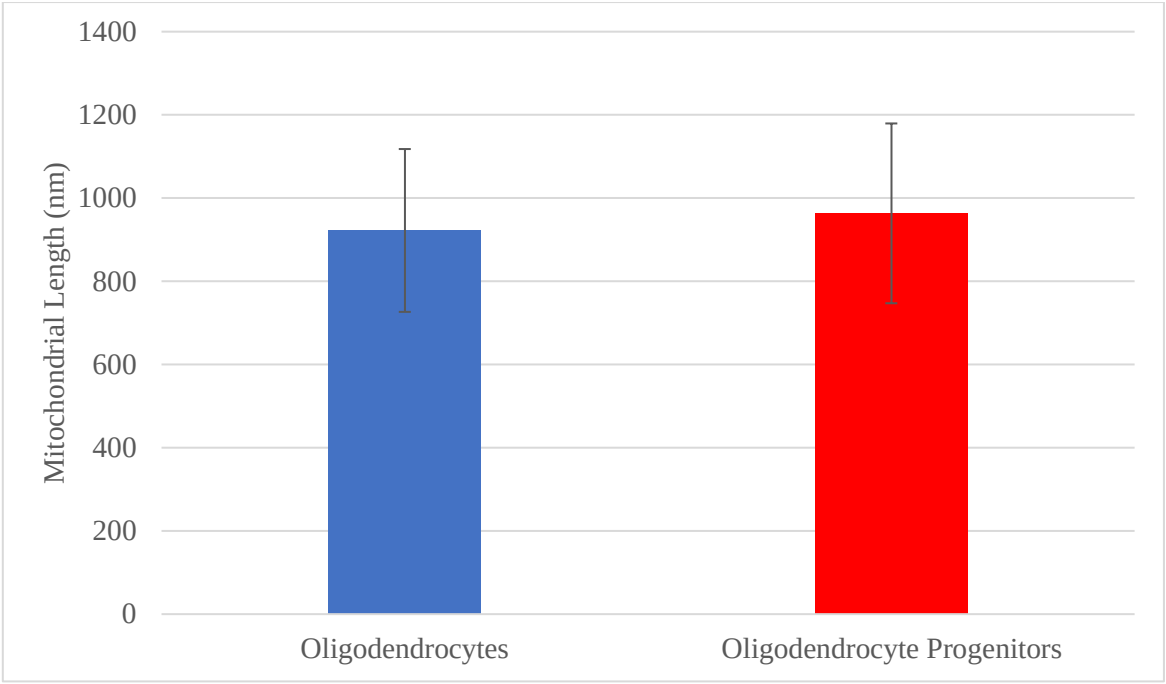

Figure 3: Comparison of mitochondrial length in oligodendrocytes (blue) and oligodendrocyte progenitors (red) after 4 days grown in N2 media (oligodendrocytes) and N2 media + Growth Factors (oligodendrocyte progenitors). Mitochondrial length was not different between the two types of oligodendrocyte lineage cells. Error bars portray the standard deviation from the average length in nm. The total number of appropriate mitochondrial sections used for these measurements were n = 10 for Oligodendrocyte Progenitors and n = 14 for Oligodendrocytes.

The TEM images provide evidence of the mitochondrial structure of oligodendrocyte lineage cells in culture at one specific point in time. Mitochondria, however, are dynamic, undergoing fission and fusion constantly, and to address their dynamicity I exposed them to Mdivi-1 in order to inhibit their fission. Cells exposed to Mdivi-1 were stained with fluorescent phalloidin to characterized cell differentiation and their media were used to quantify lactate concentrations released. The fluorescent microscopy with phalloidin data led to the reliance of cell differentiation stage characterization on cell morphology. In the presence of Growth

Factors, very few cells differentiated with or without Mdivi-1 present (Figure 4 and Figure 5 A, C). Additionally, there seemed to be no significant difference of counts between the two biological replicates of Control wells with Growth Factors and the Mdivi-1 (10μM) with Growth Factors (Figure 4). A surprising finding from this experiment was the fact that Mdivi-1 at concentration 10 μM, the same concentration used by Yazdankhah et al. (2021) did not reduce differentiation (Figure 4 and Figure 5 A). This is contrary to the findings from Yazdankhah *et al.*, a study that worked with optic nerve oligodendrocyte lineage cells and showed that Mdivi-1 reduced differentiation levels (2021). While this particular finding was not highlighted in their publication, it is an important one for this thesis. The contradiction in results poses a question about the effect of the dose used on our cells in culture. To address this question, the experiment was repeated with Mdivi-1 25 μM exposure for 2 days and the cells were observed to go through differentiation from light microscopy qualitative observations (data not shown). Therefore, replicating the results from the lower dosage. Unfortunately, the cells exposed to the higher Mdivi-1 concentration were lost during the fixation process.

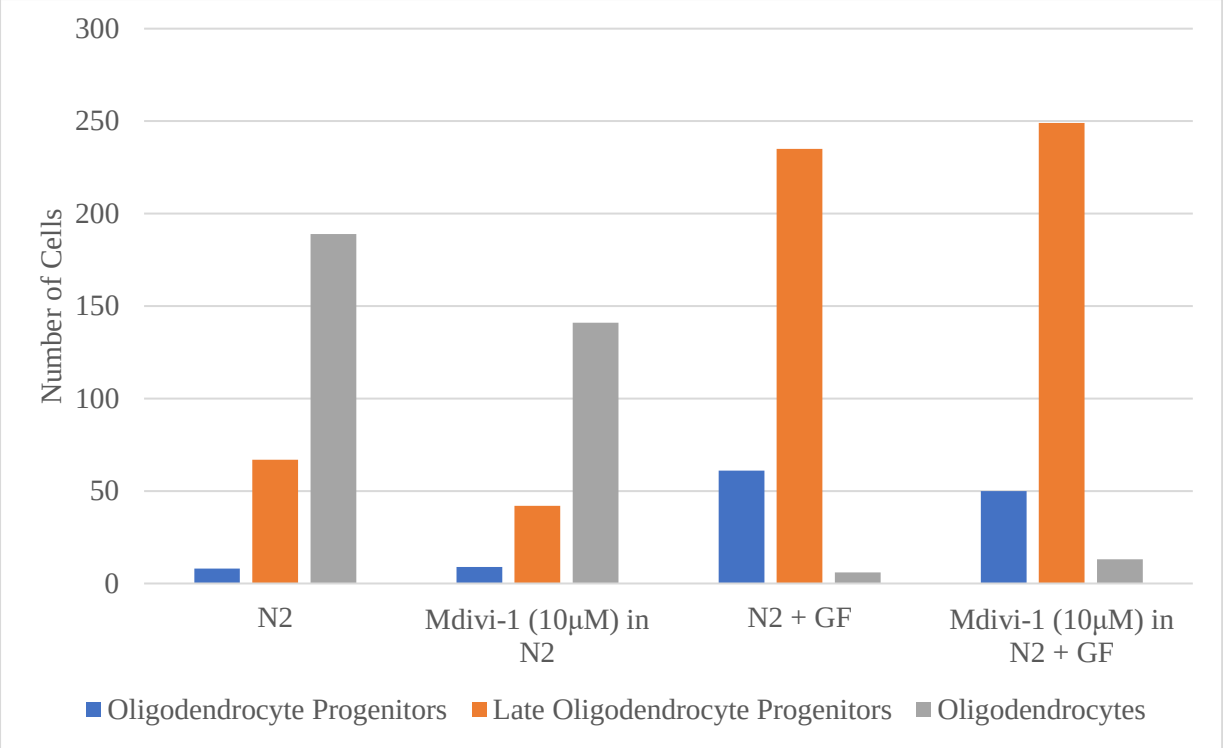

Figure 4: Effect of inhibition of mitochondrial division via exposure to Mdivi-1 (10 µM) on oligodendrocyte lineage cell differentiation after 2 days in culture. Cells were grown in N2 media (differentiation control), Mdivi-1 in N2, N2 + Growth Factors FGF2 and PDGF (GF) (proliferation control of non-differentiating cells), and Mdivi-1 in N2 + GF for 2 days. Cells were staged under fluorescent microscopy using fluorescent phalloidin, by identifying the differentiation status of oligodendrocyte lineage cells in 10 random fields of view at 40x magnification. Three stages of differentiation were used: oligodendrocyte progenitor (blue), late oligodendrocyte progenitor (orange), and oligodendrocyte (grey). Mdivi-1 in N2 did not stop differentiation, while Mdivi-1 in N2 + GF prevented cells from differentiating. Each condition had a total sample number of n =2.

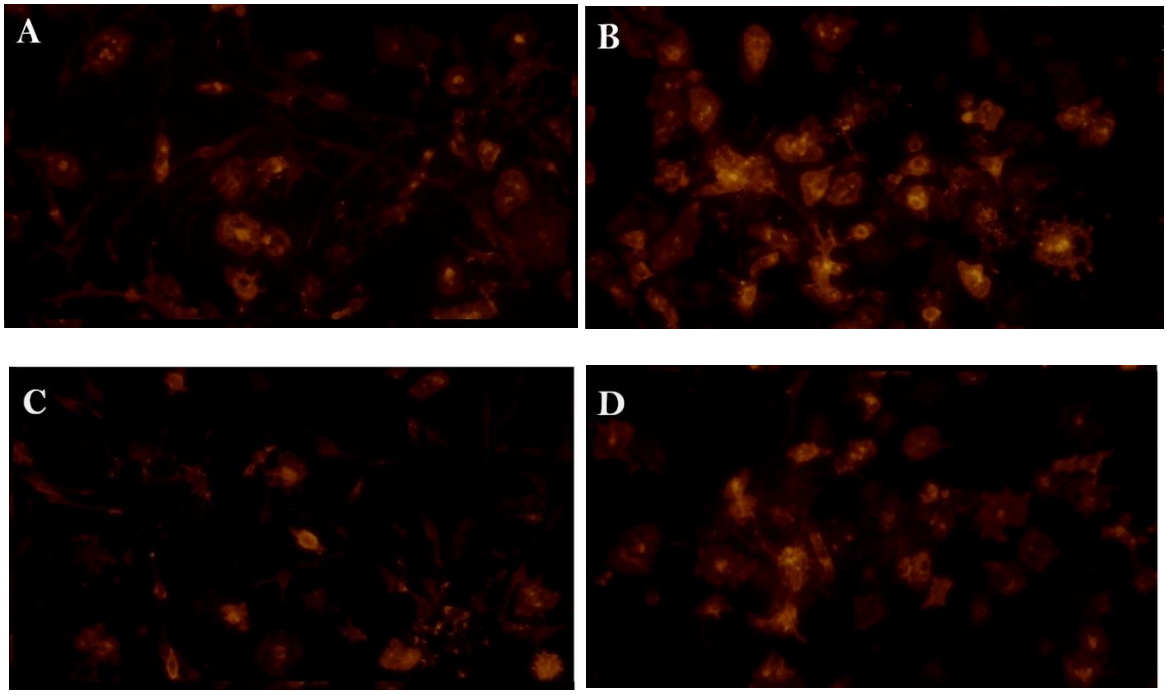

Figure 5: Visual representation of effect of inhibition of mitochondrial division on oligodendrocyte lineage cell differentiation. A. Mdivi-1 (10μM) with Growth Factors (FGF2 and PDGF). B. Mdivi-1 (10μM) without Growth Factors. C. N2 media control with Growth Factors. D. N2 media control without Growth Factors. Cells were grown in these conditions for 2 days. Panels A and C show high prevalence in oligodendrocyte progenitors, while differentiated oligodendrocytes are present in panels B and D. Each condition had a total sample size of n = 2. These cells were stained with fluorescent phalloidin.

Lastly, by inhibiting mitochondrial fission, I am inhibiting a crucial dynamic process of the mitochondria that is involved in metabolism. In the presence of Mdivi-1, differentiated cells (Figure 6, patterned blue bar) at day 2 produced slightly higher lactate than their control (Figure 6, blue, solid). In the presence of Mdivi-1 and growth factors, proliferating oligodendrocyte progenitors (Figure 6, patterned red bar) produced the highest amounts of lactate in this experiment. The higher lactate production numbers in the presence of Mdivi-1

(10 μM) appear, potentially because mitochondrial fission helps with the elimination of damaged mitochondria therefore limiting these cells to utilize their cytoplasmic metabolic processes like glycolysis (Twig et al., 2008). While none of the lactate production data of this experiment is significantly different from one another, there seems to be a trend of higher lactate concentrations in the presence of Mdivi-1 (Figure 6). Given that this study is preliminary and considering the small sample size, this trend is worth investigating further with higher sample sizes.

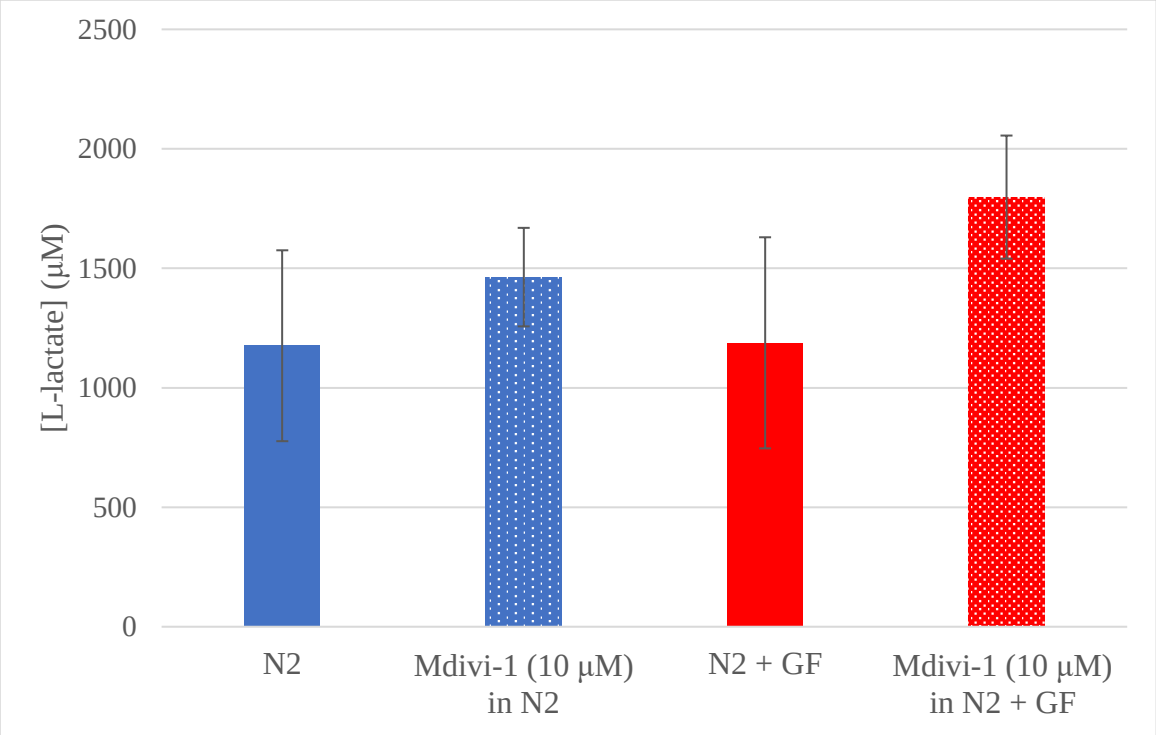

Figure 6: The effect of inhibition of mitochondrial division on lactate production of oligodendrocyte lineage cells. Cells were grown in N2 media (control for differentiating cells, solid blue bar), Mdivi-1 in N2 (patterned blue bar), N2 + Growth Factors FGF2 and PDGF (GF) (proliferation control for non-differentiating cells, solid red bar), and Mdivi-1 in N2 + GF (patterned red bar) for 2 days. Mdivi-1 in N2 + GF showed the highest lactate production and Mdivi-1 in N2 showed the second highest. The production levels are not significantly different,

however. Error bars represent the standard deviation of the average L-lactate concentrations (μM) measured. The total sample number of each condition was n = 4.

Discussion

The process of myelination is energetically expensive and is performed by oligodendrocyte cells (OLs) (Miller, 2002). Myelin covers mammalian axons and needs replenishment to keep axonal integrity. When OLs die, axons lose myelin through demyelination and gain it back from modified extensions of OL membrane in a process called remyelination (Hirano & Dembitzer, 1967). This process can only be performed by differentiated OLs. Oligodendrocyte progenitor cells (OPCs) can replenish OLs that die during their normal life cycle as animals carry pools of the progenitors (Dimou et al., 2008). In order to meet their energetic demands, their metabolism must be efficient, which leads them to use their mitochondrial membranes for oxidative phosphorylation (OXPHOS). Mitochondria are dynamic organelles that undergo fusion and fission, processes necessary for ensuring that mitochondria remain healthy and that the appropriate energy systems are used (Chen et al., 2005). Multiple sclerosis is a disease that occurs when lost myelin is not replenished via remyelination in a fast enough rate that ultimately degrades axons (Kuhlmann et al., 2008). Blocking OPC differentiation has been strongly associated with failure to remyelinate in chronic MS (Kuhlmann et al., 2008). Multiple sclerosis is rising in prevalence across the world and is a disease that needs more research to combat its effects on human populations (Walton et al., 2020). The purpose of this thesis was to investigate the metabolic energy systems used by oligodendrocyte lineage cells and the significance of mitochondrial division on oligodendrocyte lineage cell differentiation and metabolism.

Lactate determinations in proliferating oligodendrocyte progenitors and differentiated oligodendrocytes reveal that there is no change in metabolic systems in different differentiation stages. In our cell culture systems, by day 2, there are mostly differentiated oligodendrocytes, and by day 4 the vast majority of cells are oligodendrocytes. Expanding the discussion on

metabolic processes, I visualized their mitochondria, a crucial metabolic component of every cell, at day 4 to examine potential differences in differentiation stages. There was in fact, no size differences, when length was the metric used (Figure 3). These results tell a different story than the *in vivo* observations performed by Fünfschilling *et al*. (2012), but the morphological observations are not conclusive to suggest that there is no difference between OPC and OL mitochondria. What is certain, however, is that long mitochondria were found in both types of cells, which raises questions about how evident is mitochondrial fusion in oligodendrocyte lineage cells.

Dynamic mitochondria have different ways of controlling their metabolic pathways. When inhibiting their mitochondrial division via the use of Mdivi-1, cellular metabolic functions are interrupted, and mitochondrial dynamicity is hindered (Twig *et al*., 2008). Contrary to previous research, the data suggested that Mdivi-1 did not reduce the differentiation process (Figure 4) (Yazdankhah et al., 2021). Cells exposed to Mdivi-1 in N2 media differentiated regularly. One difference between the experimental set up of this thesis and the Yazdankhah et al. (2021), however, was the duration of exposure to Mdivi-1 10 μM. Yazdankhah et al. (2021) exposed OPCs for 3 days, while I exposed them to 2 days instead. Differentiation is not a reversible process; hence, an additional day of exposure would not have taken the differentiated OLs of this experiment back to the progenitor stage. Additionally, they used optic nerve oligodendrocytes in culture, which seems to be perhaps the only methodological difference of importance between the two studies. It is most logical for OPCs to need mitochondria functioning at optimal conditions to produce the energy and biosynthetic material required for differentiation. However, the data presented in this thesis suggest that there is evidence that mitochondrial division inhibition is not detrimental to the oligodendrocyte lineage cell's differentiation process. There is potential for differentiation to not be heavily reliant on a mitochondrial fission dependent mechanism. This connection,

however, cannot be confirmed with just these experiments performed for the purpose of this thesis. To come to further conclusions, there need to be more repetitions of this experiment exposing OPCs to Mdivi-1 so the visual data can be expanded the lactate data can show if the higher lactate concentration trend suggested is of statistical significance. One more experiment that would be crucial in understanding the link between differentiation and mitochondrial division is the observation and length measurements of mitochondria exposed to Mdivi-1 under the TEM, as the changing of mitochondrial dynamics throughout oligodendrocyte differentiation has not yet been characterized (Gil & Gama, 2023).

One more piece of data presented in this thesis that supports the argument for the independence of mitochondrial division from differentiation is the fact that there were long mitochondria comparable between OLPs and OLs and of the same size (Figure 2 and Figure 3). On the contrary, mitochondrial fission is responsible for the upkeep of mitochondrial integrity (Chen et al., 2005) and by inhibiting the process by using Mdivi-1, the cells showed a trend towards different metabolic pathways being used. This was revealed in the higher lactate productions in the presence of Mdivi-1 that could mean additive metabolic stress in the form of lactate release (Figure 6). Lactate production levels in the presence of Mdivi-1 hint towards a more cytoplasmic energy production than when mitochondrial division is not inhibited. This needs to be investigated further so that sample sizes become larger and this trend can be solidified by statistical significance.

Future work could include a more accurate lactate production rate determination instead of pure lactate production. Oligodendrocyte progenitors are smaller cells than differentiated oligodendrocytes, but progenitor cultures had higher total cell counts, making experiments like a BCA protein quantification assay useful to gather more accurate results about lactate. Another experiment that can help understand the dynamicity of mitochondria and their effect on

metabolism is to visualize mitochondria of oligodendrocyte lineage cells exposed to Mdivi-1. Lastly, it is important to acknowledge the fact that cell cultures for the purposes of this thesis came specifically from neonatal rat telencephalons, because of their sheer size and ease to handle. A more complete image about oligodendrocyte lineage cells can be created by gathering similar data and asking similar questions when investigating different parts of the brain and the central nervous system.

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