Exploring Alternative Therapeutic Interventions For The Treatment Of Leigh Syndrome

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EXPLORING ALTERNATIVE THERAPEUTIC INTERVENTIONS FOR THE TREATMENT OF LEIGH SYNDROME

by

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Bachelor of Science
University of South Carolina, 2015

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DEDICATION

I would like to dedicate this thesis to my parents, who have always encouraged me to push farther and harder than I believed possible. Thank you for supporting me in everything through all of these years. I would also like to dedicate this to my amazing fiancé for being so thoughtful and helping me through the process of obtaining my master’s degree.
ACKNOWLEDGEMENTS

Thank you so very much to my incredible friends and family for pushing me and always believing in me. I would also love to thank my incredible mentor, Dr. Norma Frizzell, for everything that she has done for me over these past two years, always making me strive to become a better scientist. I could not possibly think of a better mentor. You have helped me to accomplish more than I thought possible. Holland Smith, Dr. Gerardo Piroli, Richard McCain, and Josh Wang, thank you for your help in the lab. I would not have been able to do this without you. Finally, I want to thank Dr. Gerardo Piroli and Dr. Jack Goldsmith for serving on my committee.
ABSTRACT

Leigh syndrome is the most common mitochondrial disease, affecting 1:40,000 live births. It manifests with symptoms including ataxia, cognitive impairment, motor difficulties, and stroke. Leigh syndrome is a genetic condition caused by mutations in either the nuclear DNA or mitochondrial DNA that affects the assembly of the electron transport chains. Death typically occurs around 2-3 years of age, due to respiratory failure, as the body cannot produce sufficient ATP for survival. The genetic basis of Leigh syndrome means that there is no cure, and current treatments of the symptoms have proven ineffective. This thesis proposes two different therapeutic approaches for Leigh syndrome. The Ndufs4 knock-out (KO) mouse is an animal model of Leigh syndrome, and is the result of a mutation in complex I of the electron transport chain. This leads to symptoms almost identical to human patients including necrotizing lesions, seizures and motor impairments. The pathology in this model is associated with oxidative stress, protein succination, and inflammation. The therapeutic interventions selected are aimed at reducing these factors and the role that they play in disease progression.

N-acetylcysteine amide (NACA) is a precursor for glutathione, a powerful cellular antioxidant involved in the reduction of oxidative stress. In this study we administered NACA for 14 days and examined the effect on motor coordination in the mice. In addition, we examined the levels of a protein modification (protein
succination) that is derived from the reaction of fumarate with protein thiols and is increased in the affected brain regions. We observed an improvement in the fine motor coordination in the mice receiving NACA for 14 days, highlighting the potential utility of this treatment; however, we did not detect reductions in oxidative stress markers or protein succination in association with this improvement.

Patients with Leigh syndrome display increased neuroinflammation and, in a similar manner, the Ndufs4 KO mouse also displays increased immune cells, particularly microglia, congregated in and around these lesions. PLX5622 is a microglial depletion drug and we hypothesized that decreasing the microglial content would decrease the inflammation, and contribute to improved survival and behavioral improvements. We also proposed that it may reduce protein succination in the brainstem as increased succination has recently been associated with macrophage activation. Overall, PLX5622 administration did not appear to be beneficial for the course of the pathology in the Ndufs4 KO mice, perhaps in part due to the severity of their symptoms and the need for some functional microglial presence to remove dying neurons.
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<tbody>
<tr>
<td>2SC</td>
<td>S-(2-succino)cysteine</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATPase</td>
<td>Complex V</td>
</tr>
<tr>
<td>BG</td>
<td>Basal ganglia</td>
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<td>BS</td>
<td>Brainstem</td>
</tr>
<tr>
<td>CB</td>
<td>Cerebellum</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CoQ10</td>
<td>Coenzyme Q10</td>
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<tr>
<td>ETC</td>
<td>Electron transport chain</td>
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<tr>
<td>Fh KD</td>
<td>Fumarase knock-down</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidized glutathione</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible transcription factors</td>
</tr>
<tr>
<td>HNE</td>
<td>4-hydroxynonenal</td>
</tr>
<tr>
<td>Iba-1</td>
<td>Ionized calcium binding adaptor molecule 1</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>KSS</td>
<td>Kearns-Sayre syndrome</td>
</tr>
<tr>
<td>Leber’s</td>
<td>Leber’s hereditary optic neuropathy</td>
</tr>
<tr>
<td>LS</td>
<td>Leigh syndrome</td>
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MD ................................................................. Mitochondrial disease

MELAS .......................................................... Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes

MERRF ........................................................ Myoclonus epilepsy with ragged red fibers

MRI ................................................................. Magnetic resonance imaging

mtDNA .......................................................... Mitochondrial DNA

mTOR ........................................................... Mechanistic target of rapamycin

NAC .............................................................. N-Acetylcysteine

NACA ............................................................. N-Acetylcysteine amide

nDNA ............................................................. Nuclear DNA

NDUFS4 ....................................................... NADH:Ubiquinone oxidoreductase subunit S4 (gene)

Ndufs4 ......................................................... NADH:Ubiquinone oxidoreductase subunit S4 (protein)

OB ................................................................. Olfactory bulb

OCR .............................................................. Oxygen consumption rate

OXPHOS ........................................................ Oxidative phosphorylation

P(#) ............................................................... Postnatal day (#)

PLX ............................................................... PLX5622

ROS ............................................................. Reactive oxygen species

Scr ............................................................... Scrambled

ShRNA ......................................................... Short hairpin RNA

S-OH ............................................................ Sulfenic acid

VHL ............................................................. Von Hippel-Lindau factor

VN ................................................................. Vestibular Nucleus

WT ............................................................... Wild-type
CHAPTER I
INTRODUCTION

1.1 Mitochondrial Respiration

Mitochondria are the “power-houses” of the cell, providing the body with ~90% of cellular ATP via oxidative phosphorylation (OXPHOS) in all nucleated cells (1, 2). OXPHOS describes electron transfer through a series of oxidoreductase reactions known as the electron transport chain (ETC), coupled to the phosphorylation of ADP to ATP (Figure 1.1) (3, 4). The ETC consists of 5 complexes that are embedded in the inner mitochondrial membrane, with electrons entering through complex I and/or II via NADH and/or FADH$_2$, respectively. The electrons entering through complex I and/or II are transferred through the chain to reduce O$_2$ to H$_2$O at complex IV (3). Protons are simultaneously pumped at complexes I, III and IV from the matrix into the intermembrane space, creating an electrochemical gradient. Complex V (ATPase), also known as ATP synthase, uses the energy from protons re-entering the matrix, via the ATPase, to produce ATP from ADP and inorganic phosphate (3, 5).

Mitochondria are also responsible for the production of reactive oxygen species (ROS). Under normal physiological conditions, the mitochondria produce
a basal level of ROS that can be involved in cell signaling, however, a disturbance in the ETC can cause an increase in the level of ROS produced, leading to oxidative stress (6). The reactive oxygen species hydrogen peroxide (H$_2$O$_2$) and superoxide are not necessarily toxic due to the presence of the antioxidant defense enzymes glutathione peroxidase and superoxide dismutase. However, when present in excess, they can react with metal ion catalysts and are further converted into hydroxyl radicals that react rapidly with intracellular proteins, lipids, and DNA, causing protein aggregation, lipid peroxidation, and DNA hydroxylation (7, 8). Free radical induced lipid peroxidation has been shown to also produce a variety of cytotoxic aldehydes, including 4-hydroxynonanal (HNE), whose adducts on proteins are commonly examined biomarkers of oxidative stress (7-9). Protein carbonylation refers to protein oxidation by ROS, with amino acid side chains forming reactive aldehydes, further reacting to cause aggregation and crosslinking (10). Intracellular stress caused by carbonylation may result in biomolecular malfunction and increased toxicity, leading to apoptotic cell death (11, 12). The accumulation of carbonylated proteins has been associated with affected brain regions of certain neurological disorders, particularly Alzheimer’s disease (12).

1.2 Mitochondrial Respiratory Chain Disorders

Mitochondrial respiratory chain disorders refer to conditions that are due to mutations in either nuclear DNA (nDNA) or mitochondrial DNA (mtDNA) that impact the efficiency of OXPHOS, and occur in a wide range of encephalopathies and myopathies (13, 14). Mutations in the mtDNA can be the result of point mutations, deletions, or large-scale rearrangements (15). While they are rare
individually, together these diseases occur with a prevalence of ~1:5000 (16). A mutual feature is a failure to thrive due to the lack of energy production in cells, specifically in organs that require more energy, such as muscle, brain, and the heart (17). Seizures are one of the most widely observed symptoms and occur in approximately 30-60% of the individuals with biochemically confirmed mitochondrial diseases (MDs), due to mitochondrial epilepsy (18-20). This is usually part of a larger, multi-system disorder, occurring in both early- and late-onset MD (18, 21).

The well characterized MDs are Leber’s Hereditary Optic Neuropathy (Leber’s), Kearns-Sayre Syndrome (KSS), Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes (MELAS), Myoclonus Epilepsy with Ragged Red Fibers (MERRF), and Leigh Syndrome (LS). Leber’s, also known as LHON, is a maternally inherited mtDNA disease that can cause blindness (22). Leber’s is commonly due to primary or secondary mitochondrial dysfunction that affects retinal ganglion cell survival (23). Classically, symptoms begin suddenly around 15 to 35 years of age with the onset of central visual loss (24). The disease occurs predominantly in males, with loss of vision typically beginning in one eye and moving on to the second (bilateral) within a year (24, 25). There are three point mutations in mtDNA that are believed to lead to 90-95% of the cases of LHON known as ‘primary’ LHON mutations (26).

Kearns-Sayre Syndrome (KSS) is one of three phenotypes caused by single-deletions or duplications in mtDNA (27). As a multi-system disorder, KSS displays progressive external ophthalmoplegia and pigmentary degeneration of
the retina, with ~50% of cases displaying cardiac involvement (28). Cardiac conduction blocks occur in varying degrees of severity, with polymorphic ventricular tachycardia being the most common (29).

Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome is one of the most common MDs. It is maternally inherited and has most commonly been attributed to a transition of adenine to guanine in nucleotide 3243 of mtDNA (2, 30, 31). MELAS patients show signs of dementia, muscle weakness, and epilepsy (2). The dementia is caused by both the cortical injuries and stroke-like episodes, resulting in neural difficulties including problems with language, perception, and memory function (2, 32).

Myoclonus epilepsy with ragged red fibers (MERRF) syndrome is an inherited MD with a prevalence of approximately 1:400,000 (33, 34). 80-90% of these cases are caused by an A8344G mutation in the mtDNA tRNA\textsubscript{Lys} gene (35-37). Affecting mainly the nervous system and skeletal muscles, MERRF is known to cause myoclonus, muscular atrophy, epilepsy, and ataxia (38, 39). The defining characteristic of this disease is the ragged-red fibers in the muscles, containing subsarcolemmal aggregations of abnormal mitochondria; however, these muscle fibers have been seen in other MDs, such as KSS and Leigh Syndrome. Ragged-red fibers are not typical of muscle and are seen as “myopathic”, with patients diagnosed with MERRF exhibiting ~12-13% ragged-red fibers in their muscles (39, 40).
1.3 Leigh Syndrome

The most common MD is Leigh Syndrome (LS), occurring in approximately 1 in 40,000 live births (41). Linked to over 75 gene mutations, LS is a devastating neurodegenerative disorder (42, 43). LS was discovered by British neuropathologist Denis Leigh in 1951, when a 7 month old infant was admitted with encephalomyopathy and respiratory difficulties. Symptom onset began approximately six weeks prior to death and caused intense drowsiness, blindness, deafness, and spasticity of his limbs. During autopsy, the child’s brain showed lesions, vascular overgrowth, acute cellular swelling, and increased microglial presence (44). The onset of symptoms in LS normally occur in infancy, however, there have been cases of adult onset reported (45). Very few patients survive past the age of 10 years-old (43). Soufu et al. described a median onset age of 7 months, with 80.8% of patients displaying symptoms by 2 years-old. Predictors of poor survival were seen as symptoms occurring at or under 6 months of age, failure to thrive, brainstem (BS) lesions, and requirement of intensive care (45). LS is now characterized by bilateral necrotizing lesions in the basal ganglia (BG), thalamus and BS, as well as respiratory failure, seizures, ataxia, cognitive impairment, and stroke (46). Biochemically, LS results in lactic acidosis, ensuing from the accumulation of pyruvate due to elevated levels of NADH. There have been reported increases in lactate and lactate:pyruvate ratios in blood, urine, and cerebrospinal fluid of patients (43). This increase in lactate has been displayed in all organs in the body with mitochondrial deficiency, particularly in the liver (47-49).
Increased lactate in cerebrospinal fluid has been associated with a severe disease course (44).

LS is most frequently the result of a mutation that leads to the impaired assembly of one of the five complexes of the ETC, but most commonly complexes I or IV (50). There have also been a number of cases reporting dysfunction in coenzyme Q or the pyruvate dehydrogenase complex as the cause of LS (43). Complex I is the largest complex of the OXPHOS system, with 45 different subunits folding together to create a large (980kD), L-shaped component (3, 51). This complex removes electrons from NADH to transfer them to ubiquinone, where they continue through the ETC. Approximately 30% of the defects in complex I are caused by a mutation in the mtDNA, most frequently MTND1, MTND3, and MTND5 (52). Nuclear DNA mutations also contribute to LS. Assouline et al. described NADH:Ubiquinone oxidoreductase subunit S4 (NDUFS4) mutations that result in fatal, early-onset mitochondrial disease, specifically Leigh syndrome (53). Ndus4 is a relatively small (18kD), nDNA-derived assembly protein that is believed to assist in the establishment of the tertiary structure of complex I, once Ndufs4 is phosphorylated (53-55). Loss of Ndufs4 causes complex I to form a smaller (~830 kD) subcomplex that lacks NADH dehydrogenase (Figure 1.2) (56, 57). This is detrimental for optimal complex I activity, decreasing the amount of NADH that can be oxidized to NAD^+. This subsequently reduces the levels of ATP generated by the ETC, as well as potentially increasing the number of ROS generated (57).

The Ndufs4 knock-out (KO) mouse is used as an animal model of LS as it exhibits many of the same characteristics as the human disease (58-60). The
mouse was first described by Kruse et al. with a deletion of exon 2 causing a frameshift mutation halting the synthesis of the mature protein (58). The complete KO of complex I activity would most likely lead to embryonic death, yet the Ndufs4 KO mouse survives for a limited lifespan, therefore the OXPHOS system must be able to compensate for this defect. Calvaruso et al. demonstrated that the Ndufs4 KO mouse was able to survive due to the formation of a novel supercomplex. This supercomplex is formed between the partially assembled complex I and the fully functioning complex III, causing complex I to stabilize and allowing electrons to be transferred to ubiquinone (56). The phenotype of the KO mice was distinguishable from their wild-type (WT) littermates beginning at approximately postnatal day 21 (p21), and the KOs die ~p50 (58). Quintana et al. verified that these mice experienced cognitive impairment, ataxia, respiratory distress, and defects in vision, all of which are symptoms of LS in humans (56). Light microscopy and electron microscopy have been used to visualize the pathological changes in brain regions that correspond to the deteriorating health of the mice, namely the BS and cerebellum (CB). The degree of gliosis and lesion development in these regions is similar to humans with LS. A significant difference in the mouse model and the human disease is that the basal ganglia (BG) appears to be unaffected in the KO mice (59).

1.4 Mechanisms Contributing to the Pathology of Leigh Syndrome

In addition to the reduced production of neuronal mitochondrial ATP, oxidative stress has been described to be increased in the fibroblasts of patients with LS due to a mutation in complex I. These fibroblasts had significantly
increased levels of H$_2$O$_2$, as well as superoxide anion radicals in the mitochondria. There was also evidence of increased protein carbonylation in these fibroblasts, indicating cumulative oxidative damage. These patients were further observed to have decreased antioxidant protection with lower levels of the enzymes catalase and superoxide dismutase 1 and 2 (61). Mitochondrial epilepsy has been linked to both ATP depletion in the brain, as well as oxidative stress. Epileptogenesis is characterized by neuronal hyperexcitability, which may be triggered by increased ROS production above what is required for synaptic signaling (62).

Beyond the detection of lactic acidosis biochemically, the diagnosis of Leigh Syndrome is often performed via magnetic resonance imaging (MRI) which indicate bilaterally symmetric lesions, particularly in the BG, BS, and CB, before moving to genetic testing (63, 64). Microglia have been discovered to congregate at the periphery of these lesions, often in large numbers (44, 65). Microglia are the immune cells of the brain, comprising roughly 12% of cells, predominantly in the gray matter (66, 67). In a typical brain, these cells are at rest unless they are activated by events such as environmental stress or immunological challenges. Once activated, they undergo morphological and functional transformations to perform a diverse number of functions, such as phagocytosis of dying neurons and facilitating repair at the sight of inflammation and/or injury (67-69). However, in neurological disease, microglia can become over-activated and have been implicated in leading inflammation-mediated neuronal damage. These cells create a neurotoxic environment through the excess production of pro-inflammatory cytokines, as well as cytotoxic factors, such as superoxide (67, 68). This leads to
inflammation, particularly where the microglia are congregated around the lesions (44, 70). Quintana et al. demonstrated that complex I dysfunction in the Ndufs4 KO is associated with the activation of glial cells, and further proposed that this leads to oxidative stress, cytokine release by microglia, and ultimately neuronal death via the extrinsic apoptotic pathway (59).

S-(2-succino)cysteine (2SC) formation, also termed protein succination, refers to the reaction of fumarate with the thiol group on protein cysteine residues, generating an irreversible chemical modification (Figure 1.3) (71). Fumarate levels must be significantly heightened for this reaction to occur, and increased fumarate and protein succination has been described in adipocytes and obesity, as well as hereditary leiomyomatosis and renal cell cancer syndrome (72-76). Interesting, Barshop et al. reported increased fumarate and malate levels in the urine of patients with mitochondrial diseases (77), suggesting that elevated fumarate and protein succination may also have a role in the pathology of LS progression. As the ETC cannot function properly, NADH cannot be oxidized to NAD$^+$, leading to an accrual of NADH and shortage of NAD$^+$, inhibiting NAD$^+$-dependent dehydrogenases and leading to the accumulation of fumarate and other Krebs cycle metabolites (14, 78). Pirolí et al. demonstrated that protein succination is increased predominantly in the BS, olfactory bulbs (OB) and CB of the Ndufs4 KO mice (Figure 1.4). This was particularly increased in the vestibular nucleus (VN), a region of the brainstem that plays a significant role in motor coordination (Figure 1.4) (14). Protein succination has been shown to decrease the functionality of the
proteins modified (79, 80), suggesting that succination in the brain of the KO mice may also cause further damage to the already compromised mitochondria (14).

Overall, in addition to the existing bioenergetics defect, the combined insults of oxidative, metabolite and inflammatory stress appear to be significant contributors to the progression of neuropathology in this model of LS. Despite this, the precise mechanisms linking the bioenergetics defect to neuronal cell death remain to be fully elucidated.

1.5 Therapeutic Treatments for Leigh Syndrome

There are over 75 different gene modifications that are responsible for LS, resulting in variable patterns of disease progression and symptom severity. Since there is no cure for this disease, physicians often face difficulty in generating an effective treatment plan. The typical treatment approach revolves around a cocktail of vitamins and supplements, such as coenzyme Q10 (CoQ10) and its derivatives (81, 82). CoQ10, or ubiquinone, is an electron carrier from complex I and II to complex III of the ETC (82, 83). In a patient with limited complex I activity, CoQ10 may be administered in an attempt to bypass complex I via reducing equivalents entering the ETC through complex II and the oxidation of succinate, followed by transfer to complex III via CoQ10 (82, 84, 85). In its reduced form of ubiquinol, this directly acts as an antioxidant by preventing the initiation or propagation of lipid peroxidation (84). There are also indirect antioxidant properties of this coenzyme as it regenerates vitamins C and E, both of which may act as antioxidants (85). While the idea of ubiquinone as a treatment is practical, studies have shown
controversial results of CoQ10 as a treatment of mitochondrial disease (85-87). García-Corzo et al. demonstrated a water-soluble formulation of CoQ10 that reduced oxidative damage and increased body weight in Coq9<sup>x/x</sup> mice, but did not completely rescue the encephalopathic phenotype (85).

In addition to CoQ10 treatments, riboflavin (B<sub>2</sub>) and folinic acid (B<sub>9</sub>) are frequently administered to LS patients. Riboflavin is believed to be useful in complex I deficiency due riboflavin being present in flavin mononucleotide (FMN). FMN is a prosthetic group of complex I, and has been documented as lacking in the muscle of patients with complex I deficiency (88, 89). Folinic acid is given to patients who suffer from central nervous system (CNS) manifestations (90). It is a reduced form of folic acid, and involved as a cofactor in many metabolic reactions (91).

The common treatments for LS are not necessarily effective, nor do they provide a long term cure, therefore, new, alternative therapeutic practices are being studied. Rapamycin is an inhibitor of the mechanistic Target of Rapamycin (mTOR), and has been proven to increase lifespan in multiple models of different organisms (92-95). mTOR has functions in 2 complexes, mTORC1 and mTORC2, with rapamycin reacting with mTORC1. One of the main functions of mTORC1 is the promotion of mRNA translation and protein synthesis, with mTORC1 also helping to regulate autophagy. However, the activity of this pathway decreases as age increases. It is known that inhibition of mTORC1 increases autophagy, allowing the body to dispose of aged cells, therefore, its inhibition is believed to assist in slowing the aging process (94). A study by Johnson et al. investigated the
effects of rapamycin in Ndufs4 mice in order to distinguish if the longevity given by rapamycin would assist the KO mice past their normal maximum age of ~P50. Daily intraperitoneal injections were administered to the mice, allowing for an extension of life from ~P50 up to a maximum of P269. The treated mice did not display the signature lesions that age-matched, vehicle-treated mice did, nor did their behavior worsen as quickly with age. Unfortunately, this treatment does display some adverse effects, including immunosuppression, hyperlipidemia, and slow wound healing, all of which could prevent its use (96).

Recently, Jain et al. used a Cas9-mediated knock-out screen to examine ~18,000 genes in an effort to find a genetic factor for suppressing MD (81). Von Hippel-Lindau factor (VHL) was identified as the most effective factor in both severe and moderate diseases. VHL is a key factor in the regulation of the hypoxic pathway; a ubiquitin ligase that targets hypoxia-inducible transcription factors (HIF) for degredation. When VHL is knocked out, HIF is stabilized and the cells can bypass cellular oxygen sensing mechanisms. VHL-knock-out cells were found to be more resistant to complex I and complex V ETC dysfunction. To further translate this, Ndufs4 KO mice were treated with chronic moderate environmental hypoxia (11% O₂), preventing development of many of the symptoms of LS, as well as extending life-span (81). This study was expanded upon and found that while their average normoxia-treated KO died ~P60, hypoxia-treated KO mice survived until ~P270 (97). The primary disadvantage to this treatment option is that hypoxia must be chronic, as intermittent hypoxia of 10 hours a day does not yield the same results. Furthermore, once mice are returned to normoxia from hypoxic
conditions, they die within a few days (97), therefore the practical application of hypoxia treatment for LS patients remains under investigation. This thesis will focus on two alternative treatments for LS that may have a more practical application, and may reduce or prevent some of the biochemical contributors to the pathology discussed above.

Chapter 2 will investigate the utility of N-Acetylcysteine Amide (NACA), a precursor to the cellular antioxidant glutathione, in the treatment of LS. I propose that NACA will decrease oxidative stress by increasing the amount of glutathione in the cells, thereby decreasing ROS generation and protein oxidation. I also expect a decrease in succination, as the thiol group of NACA may react directly with available fumarate. Therefore, NACA may be dually beneficial in preventing both oxidant and metabolite derived cellular stress.

Chapter 3 will investigate the use of microglial depletion in the Ndufs4 KO brain via PLX5622 (PLX). As the KO mice have an increased level of microglia compared to their WT littermates, I hypothesize that reducing the quantity of microglia will diminish the inflammation in KO brains. This will lessen the neurological symptoms, resulting in a longer lifespan, as well as increased motor coordination.
Figure 1.1 Electron Transport Chain (ETC). Flow of electrons and protons through the ETC from complex I through complex V (ATPase) (4).
Figure 1.2 Complex I assembly. A) Displays the portion of the complex that provides the NADH dehydrogenase function. This portion includes Ndufs4. B) Assembly of the different subcomplexes contained in complex I. Ndufs4 is located in the final subcomplex, allowing for the finalized structure of complex I and its NADH dehydrogenase function (57).
**Figure 1.3 Formation of S-(2-succino)cysteine (2SC).** Protein thiols react with excess fumarate, forming an irreversible protein modification, S-(2-succino)cysteine (2SC).
Figure 1.4 Detection of Protein Succination in the Vestibular Nucleus (VN) of the Brainstem. VN extracts of Ndufs4 WT and KO mice was probed with anti-2SC antibody to display a significant increase in succination of the VN of KO mice versus WT, particularly in VDAC1, VDAC2 and α-tubulin (14).
CHAPTER II

N-ACETYLCYSTEINE AMIDE TO COMBAT OXIDATIVE STRESS IN NDUFS4 KNOCK-OUT MICE

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2.1 Introduction

Leigh syndrome (LS) is a mitochondrial respiratory chain disorder characterized by bilateral necrotizing lesions in the basal ganglia, thalamus and brainstem (BS), as well as seizures, ataxia, cognitive impairment, and stroke (44, 45). Biochemically, lactic acidosis results from the accrual of pyruvate, as well as elevated levels of NADH, with increased lactate in cerebrospinal fluid associated with a severe disease course (43). As LS is commonly due to a mutation in complex I of the electron transport chain (ETC), the Ndufs4 knock-out (KO) mouse has been developed to model LS, displaying many of the common features of the human disease, including bilateral necrotizing lesions, ataxia, and respiratory distress (59). NADH:Ubiquinone oxidoreductase subunit S4 (Ndufs4) is an assembly protein that participates in the formation of the tertiary structure of complex I. The Ndufs4 KO mouse removes the NDUFS4 gene, resulting in the abolishment of the NADH dehydrogenase activity of complex I (56).

Mechanistically, the mitochondrial dysfunction observed is associated with increased oxidative stress, as evidenced by an increase in hydrogen peroxide (H₂O₂) and superoxide anion radicals in the mitochondrial matrix of fibroblasts from patients with confirmed LS (61). The fibroblasts from these patients were also observed to possess decreased antioxidant protection, including lower levels of enzymes involved in the “neutralization” of the reactive oxygen species (ROS) (61). While Kayser et al. did not notice significant changes in the concentration of ROS
in Ndufs4 KO mice vs WT (98), Koopman et al. displayed an increase in ROS of patients with a complex I deficiency, furthering this via a secondary experiment exposing increased ROS in Ndufs4 KO mice (99, 100).

In addition to oxidative stress, we have recently described increased protein succination in the BS, olfactory bulbs (OB), and CB of the Ndufs4 KO mouse (14). Succination is derived from the spontaneous reaction of fumarate with the thiol group on protein cysteine residues, resulting in an irreversible chemical modification to form S-(2-succino)cysteine (2SC) (71, 72). Amplified levels of the metabolites fumarate and malate have previously been described in the urine of patients with mitochondrial diseases (73). In the Ndufs4 KO mice, this increase in fumarate is driven by the accumulation of NADH due to impaired complex I function, resulting in the inhibition of NAD\(^+\)-dependent enzymes in the Krebs cycle (14).

The tripeptide glutathione (GSH) functions as a potent antioxidant, as well as being necessary for the synthesis of other antioxidants, and exists in both reduced (GSH) and oxidized (GSSG) forms (101-103). Evidence has suggested that dysfunctional GSH homeostasis is implicated in various diseases including neurodegeneration and brain aging (104). N-acetylcysteine (NAC) has been used as a precursor to endogenous GSH, and has been established to have beneficial effects on diseases initiated by oxidative stress (105-109). However, NAC has low lipid solubility and tissue distribution, failing to provide significant antioxidant effects in vivo due to the loss of a proton by its carboxylic group at physiological pH, producing a negative charge (109, 110). In order to combat this, N-
acetylcysteine amide (NACA) was developed, replacing the carboxyl with an amide group. This modification allows NACA to cross membrane barriers, such as the blood brain barrier, and be more effective at lower concentrations (111).

Recently, Liu et al. described an increase in oxidative stress and lipid accumulation as early disease markers in Drosophila models of mitochondrial dysfunction. To counter this, they demonstrated that NACA administration offered therapeutic benefit in both the Drosophila models and Ndufs4 KO mice. NACA administration for 7 days, beginning at postnatal day 21 (p21), delayed the onset of symptoms and reduced their severity throughout the lifespan of the Ndufs4 KO mice (105).

Fumarate reactivity with GSH has previously been described in models of fumarate hydratase deficiency where fumarate levels are significantly elevated and this has, in turn, increased production of ROS (112-114) Considering that excess fumarate may also directly react with the cysteine of NACA, we propose that NACA may offer additional benefit by decreasing protein succination in addition to reducing oxidative stress. Therefore, we hypothesized that extended, 14 day NACA treatment of 150 mg/kg/day in the Ndufs4 KO model might improve motor co-ordination in the mice, and reduce markers of mitochondrial stress including both oxidant and fumarate stress.

2.2 Results

Both NAC and NACA have previously been shown to increase the glutathione (GSH) content in cells during cellular stress (108-111). To confirm that
NACA may act in a cytoprotective manner, we exposed mature cortical neurons to dimethyl fumarate (DMF) for 5 hours. DMF rapidly induces a mild oxidative stress through the covalent modification of reduced GSH, which in turn activates several antioxidant response element genes (111, 115). Figures 2.1A & 2.1C demonstrate a pronounced decrease in the reduced GSH content following 5 hours of exposure to 100 µM DMF. Pre-treatment with either 5 mM NAC or NACA for 24 hrs completely protected the cells from the DMF induced depletion of GSH (Figure 2.1A, 2.1C, (p<0.001)). Oxidized glutathione (GSSG) levels were also reduced upon DMF treatment, but not in the cells treated with NAC or NACA (Figure 2.1B, 1D, (p<0.001)). These results confirm that DMF directly reduces cellular GSH content, leading to lower total levels of both GSH and GSSG. In addition to potentially increasing the GSH content, the pre-treatment with NAC and NACA would provide available thiols that DMF directly modifies, potentially sparing cellular GSH from direct modification.

Previously, a 7 day intervention with NACA in Ndufs4 knock-out (KO) mice versus wild-type (WT) littermates was shown to reduce the severity of clinical symptoms, as well as a delayed latency to fall from the rotarod (105). We hypothesized that extending NACA administration for a total of 14 days might further improve motor co-ordination skills in the Ndufs4 KO mice. Following weaning at 21 days of age, habituation and testing were performed between 23 and 28 days of age. The mice were subsequently administered daily intraperitoneal injections of NACA for 14 days. The body weights of the KO mice decreased in association with the disease progression over the study period versus WT control
mice, and there was no significant effect of NACA treatment on body weight (Figure 2.2).

Fine motor co-ordination skills were evaluated on an inclined balance beam, with the untreated KO mice demonstrating significant impairment in their latency to traverse the beam between initial tests and those performed at the end of the study (KO Pre vs Post, Figure 2.3A, (p<0.001). Following the intervention period, KO mice were approximately three times slower than WT or WT + NACA in the average time to cross the beam, (WT vs KO, Post, Figure 2.3B, (p<0.001)), confirming a loss of fine motor skills. In contrast, KO + NACA mice did not display a significant variation in their speed (KO + NACA Pre vs Post, Figure 2.3A). The NACA treated KO mice traversed the beam significantly faster than the saline injected KO mice (KO vs KO + NACA, Post, Figure 2.3B, (p<0.05)).

The number of paw slips (for any paw) that occurred while traversing the beam were also counted (Figure 2.3C). Both the KO and KO + NACA mice both had a significantly increased number of paw slips in comparison to the WT and WT + NACA groups, indicative of impaired ability to balance. However, the KO + NACA mice had significantly fewer paw slips compared to the untreated KO mice (KO vs KO + NACA, Post, Figure 2.3C, (p<0.001)), indicating that NACA treatment also improved this aspect of subtle coordination and balance.

The co-ordination and endurance of the mice was also assessed in their latency to fall from the rotarod. Figure 2.4A demonstrates the difference in time to fall from the accelerating rod for each group of mice over the course of the study.
The KO and KO + NACA treated mice both fell from the rotarod significantly faster at the end of the study versus the initial testing period (KO pre vs post (p<0.001), KO + NACA pre vs post (p<0.001), Figure 2.4A). During the final trials, KO mice were only able to remain on the wheel for half the time of the WT mice (KO vs WT (p<0.001), KO + NACA vs WT (p<0.05), Figure 2.4B). However, unlike the balance beam, a significant improvement was not detected for the KO mice receiving NACA on the rotarod performance compared to untreated KO.

Since NACA treatment preserved neuronal GSH content \textit{in vitro} (Figure 2.1), an increase in the amount of reduced GSH in the olfactory bulb following NACA administration \textit{in vivo} for 14 days was predicted. There was not a significant difference between the levels of GSH in WT versus KO mice (Figure 2.5A), although there was a slight trend for decreased GSH. This included a decrease between WT control mice and KO control, as well as KO + NACA mice having a lower concentration of GSH than KO control mice. There was also a trend for the GSSG:GSH ratio to be increased in the KO mice versus WT (Figure 2.5B). Unexpectedly, the KO + NACA olfactory bulbs contain less GSH than KO control (Figure 2.5A, p<0.05). GSH protein adducts in the BS of the mice were also assessed by non-reducing SDS-PAGE (Figure 2.6A). Densitometry was performed to determine the GSH adducts compared to amount of protein (Coomassie, Figure 2.6B). KO + NACA mice had a significantly decreased number of GSH protein adducts compared to untreated KO mice.

As our lab previously demonstrated that protein succination is associated with the site of neuropathology as the disease progresses in the Ndufs4 KO model
(14), NACA was hypothesized to reduce protein succination in the KO mice via the thiol group directly reacting with fumarate. Figure 2.7 demonstrates a significant increase in 2SC protein adducts in the KO and KO + NACA mice versus the WT. NACA treated KO mice did not appear to have a significant reduction in the concentration of 2SC compared to KO controls. There was no change in the concentration of HNE protein adducts in the BS of WT mice as opposed to their KO littermates, signifying that there was not an increase in oxidative stress in the KO mice.

We used an in vitro model to determine if thiol supplementation could decrease excess fumarate and the amount of 2SC protein adducts. A fumarase knock-down (Fh KD) cell line was generated following transduction of N1E-115 neuroblastoma cells with a lentiviral vector containing a fumarase shRNA. These cells were then differentiated into a neuronal phenotype and the effects on protein succination and mitochondrial respiration were characterized. The top panel of Figure 2.8A (2SC) displays the pronounced increase of 2SC in the Fh KD cells compared to scrambled control (Scr) cells, occurring alongside a significant decrease in the levels of fumarase protein. Synaptophysin confirms the successful differentiation of the knock-down cells into neurons (Figure 2.8A). Quantification of fumarate levels from both Scr and Fh KD cells demonstrated a ~17 fold increase in Fh KD compared to Scr cells, confirming that the increase in succination was driven by increased fumarate levels (Figure 2.8B (p<0.001)).

Mitochondrial respiration was assessed in the Fh KD cells to determine the impact of reduced fumarase protein and increased succination on mitochondrial
function. Figure 2.9A illustrates that the Fh KD cells had impaired oxygen consumption versus the Scr control. There were significant decreases in the basal respiration, ATP production, and maximal respiration in the Fh KD cells versus the Scr control (Figure 2.9B, \( p<0.001 \)), see Appendix A for a representative mitochondrial respiratory stress profile. Considering the large increase in fumarate levels, the Fh KD and Scr control cells were treated with 5 or 10 mM NAC for 5 days during differentiation in an effort to reduce succination. While 2SC levels were significantly increased in Fh KD cells compared to Scr control after 5 days, this was not altered with the addition of NAC at either concentration, suggesting that fumarate reactivity with NAC thiols is limited (Figure 2.10A) (Scr vs Fh KD, Fh KD + 5 mM NAC, and Fh KD + 10 mM NAC, Figure 2.10B, \( p<0.005 \)).

While the NACA treated mice had significant improvements in time required to transverse the balance beam, as well as number of paw slips, this was not associated with a visible decrease in protein oxidation or succination, nor an improvement in reduced glutathione levels. Therefore, we hypothesized that early intervention may be more important to this model, given the severity of the disease phenotype. Liu et al. treated Ndufs4 KO mice for 7 days with 150 mg/kg/day beginning at p21, and observed improvement in clinical signs, including a delay of symptom onset (105). In contrast, we treated the mice for longer (14 days), but did not initiate NACA treatments until ~p27-32. In order to determine how protein oxidation is affected, protein sulfenylation was measured via a sulfinic acid (S-OH) antibody. During oxidative stress, cysteine can undergo a reversible oxidative modification to S-OH via ROS, including H\(_2\)O\(_2\). Therefore, the level of
protein S-OH modifications function as a biomarker of redox signaling and oxidative stress (116, 117). BS and OB were removed from WT and KO mice at p21 (young) and p45 (old) and placed directly into a dimedone-containing buffer to stabilize the modification. Figure 2.11A displays a decrease in the amount of S-OH present in the BS of old KO mice versus young. Due to the variability in the densitometry of the bands, while there is not a significant change, the visual decrease in the sulfenylation of old KO mice versus young (Figure 2.11B).

2.3 Discussion

Both NAC and NACA have been used successfully in several models to decrease oxidative stress (105-110). NACA offers improved utility over NAC for the treatment of neurological disorders since it has an improved ability to cross the blood brain barrier (110). The cysteine of NAC/NACA is a component of the tripeptide antioxidant, GSH, and we confirmed that NACA treatment in vitro enhanced the GSH content in neurons responding to DMF induced oxidative stress, similar to NAC (Figure 2.1A, 2.1C). Our lab has formerly shown that DMF directly modifies cysteine residues on proteins throughout the treated cells (115).

To date, only one previous study has shown that NACA administration specifically offers promise for the treatment of MDs (105). NACA administration for 7 days in weaned Ndufs4 KO versus controls reduced the clinical severity of the disease. This was proposed to be related to the concomitant reduction in triglyceride accumulation in the VN and OB of the brain, regions in which triglycerides congregate around the characteristic necrotizing lesions of Leigh
syndrome (LS). Therefore, lipid droplet accumulation was proposed to be linked to the early presence of oxidative stress (105).

In the current study, NACA administration for 14 days offered significant benefit in some behavioral parameters. The performance of the mice on the inclined balance beam exhibited the most promising results, with significant differences between the untreated KO and the KO + NACA groups in both the time to cross the beam and the number of paw slips (Figure 2.3). The success of the mice on the balance beam following NACA administration demonstrated that this treatment offered benefit in preserving fine motor control. However, while NACA had a positive effect on the fine motor skills, it did not increase their speed or endurance on the rotarod (Figure 2.4). This contrasts with the work of Liu et al., who found that the rotarod performance was improved at p30 following 7 days of NACA administration (105), but did not offer continued benefit at p40 or p60 after the treatment was stopped. Since our tests were performed at ~p24-28, it is possible that our studies were not performed within a suitable time frame to see beneficial effects of NACA treatment on this behavioral parameter. In addition, our measurements would also benefit from a larger sample size and we plan to perform further studies to support this. Overall, NACA administration appears to confer some benefit in preserving motor skills even as the neuropathology progresses, and should be considered further as an additional treatment to delay symptoms of LS in patients.

Leigh syndrome has been linked to increased, chronic oxidative stress in the affected cells (61, 98-100). We hypothesized that reduced glutathione (GSH)
levels would be lower in the olfactory bulbs of the KO mice, and that NACA administration might restore or preserve GSH similar to what we had observed in neurons \textit{in vitro} (Figure 1A, 1C). We observed a trend for a decrease in GSH content in KO olfactory bulb versus WT control, as well as an increase in GSSG:GSH for these groups (figure 2.5). In contrast to our expectations, we observed a further decrease in GSH levels in the NACA-treated KO mice versus the untreated KO mice (Figure 2.5A). The ratio of GSSG:GSH was decreased in NACA treated mice, as were the presence of GSH protein adducts (Figure 2.5B, 2.6), suggesting that NACA conferred some benefit in reducing protein oxidation, but does not drive large increases in GSH production in the KO mice. NACA administration did not result in a decreased production of HNE protein adducts in the brainstem (Figure 2.7); however, these were not detectably increased in the untreated KO brainstem versus WT, similar to our and other previous observations (14, 98).

Our lab previously documented that protein succination (2SC) is increased in the brainstem of KO mice versus WT due to excess fumarate (14). We hypothesized that the administration of NACA would provide a source of free thiols that may react with fumarate and preserve protein thiols in the neuronal cells, offering additional therapeutic benefit. However, there was no difference in the 2SC levels of KO mice versus KO + NACA (Figure 2.7). To further examine if NAC/NACA would directly reduce protein succination, we employed an \textit{in vitro} model using fumarase knock-down (Fh KD) N1E-115 neurons. Fh KD neurons versus scrambled controls had an \textasciitilde{}17 fold increase in fumarate and subsequently
2SC levels (Figures 2.8). Notably, these cells also had significant decreases in mitochondrial respiration, as evidenced by decreased basal respiration, ATP production and maximal respiration (Figure 2.9). When treated with 5 mM and 10 mM NAC for 5 days, there was no reduction in protein succination (Figure 2.10). Therefore, it is possible that fumarate is not sufficiently reactive with the thiols of NAC/NACA in vitro and in vivo to provide sufficient protection. Endogenous fumarate is known to react with low pKₐ thiols (118), however, the pKₐ of NAC/NACA is ~9.6 and 8.5 respectively (119-121). Therefore, NACA may not be sufficiently reactive with fumarate to provide sufficient protection in this model. Previous work in fumarase deficient cancer cell lines, where fumarate is elevated to milimolar levels, did demonstrate some reactivity of fumarate with reduced glutathione (122, 123), however, the extent of the modification was low and may only occur when fumarate levels are dramatically elevated.

Ndufs4 KO mice suffer a severe pathology, developing symptoms around p21 with death occurring at ~p50 (58). Considering that Liu et al. started NACA treatment at p21 (106), whereas we began treatments at ~p24-28, we hypothesized that the ~1 week later initiation of treatment may have a large impact on the ideal window for NACA intervention. In order to test the concept that the mice must start treatment early to reduce oxidative stress-related pathology, we used the brainstems of p21 (young) and p46 (old) mice to detect the levels of sulffenic acid, a product of protein thiol oxidation. We predicted that the younger KO mice might have increased levels of protein sulfenylation versus the older KO mice, and Figure 2.11 demonstrates a pronounced difference in several proteins.
in the young and old KO mice, with substantial decreases in select protein in the older mice. This suggests that increased protein oxidation occurs early in the brainstem of the Ndufs4 KO mouse, and may be contributing to the neuropathology that these mice develop, even if changes in oxidative stress markers e.g. HNE cannot be detected later. Therefore, our results suggest that NACA intervention in the Ndufs4 KO model of Leigh Syndrome will offer maximal benefit if administered early and for longer periods across the mouse lifespan. This also emphasizes the importance of early confirmed diagnosis in human patients so that advantageous therapeutic strategies can be prescribed earlier in the course of the disease.
Figure 2.1 Glutathione Fluorescence Assay performed on neuronal cells treated with DMF, NAC, and NACA. A,C) Amount of reduced glutathione in neuronal cells normalized to protein content. B,D) Amount of oxidized glutathione normalized to protein content. A and B were treated with 5 mM NAC for 24 hours followed by 100 µM DMF for an additional 5 hours. C and D were treated with 5mM NACA for 24 hours with a further treatment of 100µM DMF for 5 hours prior to scraping (DMF n=5, Control, NAC, NACA, NAC + DMF and NACA + DMF n=6, **p<0.001 compared to DMF; statistics via one-way ANOVA, ± SEM.).
Figure 2.2 Body weight of mice. Average body weight of the mice in each experimental group, where week 0 is the weight at the beginning of testing, and the final weight (week 3) was measured prior to the termination of the study (WT n=11, WT + NACA n=4, KO n=7 and KO + NACA n=7, ± SD).
Figure 2.3 Balance beam testing performed on wild-type (WT) and knock-out (KO) mice before and after NACA injections. A) The average amount of time taken for mice (by treatment group) to cross the inclined balance beam before and after drug administration (**p<0.001 vs pre KO). B) Average period of time for mice to transverse beam after treatment (*p<0.05 vs WT, **p<0.01 vs WT, #p<0.05 vs KO). C) Number of paw slips by each treatment group during one run across the inclined beam after NACA or saline treatments (*p<0.05 vs WT, **p<0.01 vs WT, ### p<0.001 vs WT; n=11, 4, 7 and 6 for WT, WT + NACA, KO and KO + NACA respectively; statistics via two-way ANOVA, ± SEM.).
Figure 2.4 Rotarod testing performed on NACA injected mice. A) The average amount of time for the mice to fall from the rotarod before and after drug administration (maximum of 300 seconds) (**p<0.01 for pre of same treatment group). B) The final times for mice to fall, averaged by their treatment group (* p<0.05 vs WT, **p<0.01 vs WT; n=11, 4, 7 and 7 for WT, WT + NACA, KO and KO + NACA respectively; statistics via two-way ANOVA, ± SEM.)
Figure 2.5 Glutathione Fluorescence Assay of olfactory bulbs following NACA treatment. A) Reduced glutathione concentration in the OB of Ndufs4 mice normalized to protein content (*p<0.05 compared to KO mice). B) The ratio of oxidized (GSSG) to reduced GSH (statistics performed via one-way ANOVA. WT n=8, KO n=5, and KO + NACA n=6, ± SEM).
Figure 2.6 Glutathione analysis of mouse brainstem following treatment. 
A) 40 µg of protein from the BS was run on a gel under non-reducing conditions and probed with the GSH primary antibody (top panel). Coomassie displays loading of the protein (bottom panel). B) Densitometry analysis, via Image J software, of the GSH adducts normalized to the Coomassie; demonstrating normalization to protein content (*p<0.05 compared to KO mice, statistics performed via one-way ANOVA, ± SEM.).
Figure 2.7 Protein analysis of brainstem from mice after NACA. 30 μg of BS protein run via SDS-PAGE. The top blot, 2SC, presents the intensity of the modification, in a multitude of proteins, as it increases in Ndufs4 KO mice versus WT, without exhibiting a change between KO and KO + NACA BS samples. HNE displays no significant change between the groups (HNE panel). The Coomassie denotes equal loading of BS protein.
Figure 2.8 Effects of Fh knock-down (KD) on protein succination, neuronal differentiation, and intracellular fumarate concentration. A) 20 µg protein from scrambled (Scr) and Fh KD N1E-115 differentiated cells probed with anti-2SC (2SC panel). Increased succination is observed for a large number of proteins in the Fh KD cells. Confirmation of the fumarase shRNA effectiveness to decrease fumarase expression is also shown (fumarase panel). Differentiation of the cells to a neuronal phenotype was confirmed by the expression of the presynaptic marker synaptophysin (synaptophysin panel). Even loading of the lanes was confirmed by Coomassie staining (Coomassie panel). B) Fumarate concentration in extracts from Fh KD is increased more than 17-fold compared to Scr N1E-115 cells. (n=5, ***p<0.001 vs Scr control, ± SD).
Figure 2.9 Effects of Fh knock-down on mitochondrial respiration. 
A) Respiratory profile of Fh KD and Scr N1E-115 neurons measured using the Seahorse XF analyzer. Compounds were added at the indicated arrows to assess mitochondrial function (**p<0.01 vs Scr). 
B) Basal respiration, proton leak, oxygen consumption coupled to ATP production, maximal respiration, spare capacity, and non-mitochondrial respiration were calculated following the addition of oligomycin, FCCP, and rotenone/antimycin A (Rot + Ant). (**p < 0.01 vs. Scr control, n=5, ± SD) (See Appendix A for determination of respiratory profile).
Figure 2.10 2SC levels of fumarase knock-down cells treated with NAC.  

A) 30 µg of protein from Fh KD and Scr N1E-115 cells treated with 5 mM and 10 mM NAC was probed with anti-2SC, demonstrating increased succination in the KD cells. Coomassie panel presents protein loading.  

B) 2SC levels were normalized to Coomassie via Image J and graphed with Sigma Plot. This demonstrated significantly increased succination in Fh KD cells, compared to Scr, but no significant change when cells were treated with NAC compared to control (**p<0.01 equated to Scr control, statistics performed via one-way ANOVA, ± SEM).
Figure 2.11 Detection of protein sulfenylation in the brainstems of young and old wild-type and knock-out mice. A) 20 µg of protein from the BS of young and old WT and KO mice run via SDS-PAGE, and probed with anti-S-OH. Actin exhibits protein loading (actin panel). B) S-OH content normalized to actin via densitometry performed on Image J. While there were no significant decreases due to the variability in band intensity, there was a pronounced trend for decreased sulfenylation in old KO mice versus young (± SEM).
CHAPTER III

MICROGLIAL DEPLETION IN A MOUSE MODEL OF LEIGH SYNDROME

3.1 Introduction

Microglia are the principal immune cells of the central nervous system (CNS), and compose approximately 12% of the total cells in the CNS (124). Microglia play a crucial role in limiting damage from injury, for example, phagocytosing aggregating peptides that may be toxic and limiting plaque formation (124-126). In other situations, however, this “beneficial” response may lead to chronic inflammation in the brain, contributing further to the disease (124, 127). During neuroinflammation, the microglia secrete proinflammatory cytokines, including \textit{IL-1\beta}, \textit{IL-6} and \textit{TNF\alpha} that may exacerbate neuronal damage or injury (128, 129). Rice \textit{et al.} induced lesions in the brain of mice and discovered that activated microglia increased the level of inflammation, which was associated with impeded recovery. Once lesions were present, removal of the microglia resulted in improved scores on the elevated plus maze, in addition to reversing the lesion-induced pro-inflammatory release, including \textit{B2m} and \textit{Bax}. While microglia prove beneficial during a diphtheria toxin-induced neuronal lesion, their continued presence also correlated with delayed recovery post-lesion (127).
Colony stimulating factor 1 receptor (CSF1R) is a cell surface receptor located on macrophages, microglia and osteoclasts (130). CSF1, a natural ligand of the CSF1R, is a growth factor that regulates the proliferation, differentiation, and survival of these cell types. Microglia are the only cells in the brain that express CSF1R, and CSF1R knock-out mice are devoid of microglia in the brain (130, 131). Recently, a pharmacological approach was developed that specifically depletes microglia via inhibition of CSF1R signaling (131, 132). Spangenberg et al. eliminated approximately 80% of the microglia in a mouse model of Alzheimer's disease using a CSF1R inhibitor, resulting in the rescue of dendritic spine loss, preventing neuronal loss, reducing neuroinflammation, and improving contextual memory (133). CSF1R inhibitors have also been used to improve radiation-induced cognitive deficits in mice treated with irradiation for brain cancer (134).

PLX5622 (PLX) is a CSF1R inhibitor proven to eliminate up to ~99% of microglia in the nervous system of mice receiving it orally over several days to months (124, 135). Acharya et al. treated mice for 3 days with 1200 mg/kg of PLX, resulting in 95% reduction in microglial number (134). A lower concentration of 300 mg/kg was fed to 3xTg-AD mice, a model of Alzheimer's disease, for 7-21 days and resulted in a 30% reduction of microglial number and improved cognition (124). Results were obtained using C57BL/6J, a common black laboratory mouse, discovering that both the 300 mg/kg and 1200 mg/kg doses of PLX achieved comparable microglial reductions (136). Ndufs4 KO mice have been observed with enhanced microglial activation, particularly in regions suffering from necrotizing lesions. As the gliosis and lesions worsen, the ataxia, hypothermia, and morbidity
increase simultaneously (59). Ionized calcium binding adaptor molecule 1 (Iba-1) is a protein expressed solely in microglia, regulating their function (137, 138). We hypothesized that PLX administration to deplete microglia and reduce neuroinflammation in the Ndufs4 KO mice may reduce microgliosis and the motor co-ordination deficits associated with the neuropathology. In addition, we have recently observed that activated macrophages (lipopolysaccharide-stimulated) have pronounced increases in fumarate and protein succination (unpublished data). Therefore, we hypothesized that PLX-mediated removal of microglia (the macrophages of the CNS) may result in a decrease of protein succination in the KO mice. Alternatively, it may increase neuronal survival and also prevent increased succination in neuronal cells. Thus, we investigated diets containing both the 300 mg/kg (low dose) and 1200 mg/kg PLX (high dose).

3.2 Results

PLX5622 (PLX) is a CSF1R inhibitor demonstrated to eliminate microglia (124, 134-136), and these experiments represent the first use of this compound in a mitochondrial disease model to decrease neuroinflammation. In this study, two trials were performed, one using 1200 mg/kg PLX in the diet for 3 days, and the other with 300 mg/kg PLX for 14 days. Previous work has been conducted in murine models for a duration of 1-3 months, however, shorter exposures (up to 2 weeks) are necessary in the Ndufs4 KO model as they have such a severe disease progression and die by <9 weeks of age (124, 136, 139).
A specific anti-Iba-1 was used for immunoblotting to assess the levels of Iba-1 in both the brainstem (BS) and olfactory bulb (OB) of WT and KO mice (Figure 3.1A). While there was a slight increase in Iba-1 concentration in BS from WT mice versus KO, the OB demonstrated a significant increase in Iba-1 in the KO mice versus their WT littermates, confirming the microgliosis. To determine, more specifically, if the mice would be treated at an age where KOs already contained increased microglia, early (p24-26) and mid (p46) KO mice OB were compared to early (p24-26) and late (p60-63) WT mice OB. Iba-1 probing demonstrated a significant difference in both of the KO age groups versus the WT controls (WT early versus KO early and mid (p<0.05); WT late versus KO early and mid (p<0.001), Figure 3.1 B, 3.1C), confirming that microgliosis is present as early as p24 in the KO mice.

Mice were to be treated with PLX for 3 days (1200 mg/kg) or 14 days (300 mg/kg) beginning at approximately p22-24. When administered the 1200 mg/kg dose, Ndufs4 KO mice suffered rapidly declining health, including respiratory distress and limited-to-no motor movement. This lead to termination of the study at three days and the determination that Ndufs4 KO’s pathology is too severe to survive on the 1200 mg/kg dose of PLX. The 14 day, 300 mg/kg PLX trial also triggered a worsening in clinical symptoms, leading to an early cessation of the experiment. A PLX-treated mouse died 2 days prior to the planned termination of the study (14 days), and resulted in the early termination of the study at 13 days.
Following the PLX treatment of the WT and KO mice, the BS of the 3 day (1200 mg/kg diet) and 13 day (300 mg/kg diet) were examined to determine if Iba-1 levels had been altered by either treatment (Figure 3.2A). While several bands were apparent on the Iba-1 blot, the band ~17kDa appeared to be more intense in the untreated WT group versus the KO mice, which is inconsistent with our previous observations in Figure 3.1, and the work of others (44). PLX treatment of the WT mice appeared to significantly decrease Iba-1 levels (Figure 3.2B, p<0.001). However, since the levels of Iba-1 could not be accurately assessed in the whole brainstem across all groups, despite multiple attempts, we could not use this to determine if PLX treatment had been successful in these mice. Unfortunately, the olfactory bulbs from these mice were used for a separate experiment and were not available for protein analysis.

Due to the fact that activated microglia may show increased fumarate levels and protein succination, we hypothesized that decreasing the heightened population of microglia in KO mice would decrease the levels of 2SC present. Figure 3.3A displays the levels of 2SC in the BS of mice from the 3 day PLX study. There was no significant difference in untreated KO mice, and those receiving PLX (Figure 3.3B). BS protein was also immunoblotted and probed with anti-2SC for the 13 day treated mice (Figure 3.4A). As expected, 2SC levels were again increased in the untreated KO versus WT (p<0.01), however there was no significant change in 2SC levels between treated and untreated KO groups (Figure 3.4B). Note: one of the PLX treated Ndufs4 KO mice was included on the blot and appeared to have reduced succination (lane 9, Figure 3.4A). However, was
subsequently learned that this mouse had died prematurely and had been refrigerated prior to protein calculation. This lane was not included in normalization or statistical analysis. Overall, the data suggested that 2SC levels were not significantly altered in the PLX treated KO mice versus untreated KO; however, the sample size of remaining mice in the study was limited.

Since the mice did not have any improved survival on the 300 mg/kg PLX containing diet for 14 days, we performed a trial on WT mice in order to determine a shorter period of time that would still significantly reduce the amount of microglia. WT mice were given 300 mg/kg PLX chow for 3, 5, or 7 days, and the hippocampus was used for Iba-1 probing. The hippocampus was selected as this region exhibits a change in microglial content when mice are fed PLX (124). Hippocampal Iba-1 levels showed significant reduction for 3, 5 and 7 days (control versus 3, 5 and 7 day PLX, Figure 3.5A, 3.5B, (p<0.001)). While all three treatments times displayed reductions in Iba-1, we decided to perform a 7 day treatment with PLX, as it demonstrated the greatest change from the control mice.

Since we observed negative effects on the mice when we attempted to remove all of the microglia over 13 days, we hypothesized that a minimal period of 7 days on PLX, followed by another 7 days for repopulation, would be more beneficial for the KO mice. We considered that repopulation of the affected area with healthy microglia might prove beneficial for the removal of dying neurons in the Ndufs4 KO model, and that the microgliosis that is present may be the result of an impaired ability for microglial turnover.
Behavioral tests were performed the prior to PLX administration (day 0), after PLX administration (day 6-7), and finally after repopulation (day 12-13). The rotarod was used to assess the speed and endurance of the mice, in addition, we considered time spent “cart-wheeling”, in which the mice are not continually running on the wheel, but holding-on and allowing the wheel to spin them around, thereby breaking from the exercise. The time that the mice were able to run on the wheel was recorded, minus time spent cartwheeling. Figure 3.6A displays an average time for the treatment groups during each testing cycle. The WT + PLX mice had a subtle decrease in time spent on the rotarod directly after drug administration, but were able to run longer after the repopulation period. During the repopulation period, there was a significant decrease in time to fall between WT mice versus KO and KO + PLX (WT vs KO and KO + PLX, Figure 3.6B, 3.6C, 3.6D, (p<0.01)). There were no statistical significances discovered between KO and KO + PLX mice at any time point tested.

An inclined balance beam was used to test the speed and fine motor coordination of the mice. The time for mice to transverse the meter-long beam was recorded, along with the number of paw slips during the test. There was a significant increase in the time to cross for WT + PLX following PLX administration (p<0.05) and after repopulation (p<0.01) (Figure 3.7 A, C, and D). There were significant differences between WT mice and both KO and KO + PLX directly after treatment (WT vs KO and KO + PLX, Figure 3.7B, (p<0.05)). WT versus KO mice had a p-value of <0.05, while WT versus KO + PLX had slightly greater significance.
with p<0.01. However, no statistical significance was found between KO and KO + PLX, following the repopulation period.

Paw slips were difficult to determine during the repopulation period due to two mice not being able to keep their paws on the top of the beam, therefore only one mouse in the KO group and two in KO + PLX could be considered. Figure 3.8A demonstrates a continually increasing number of paw slips in the KO + PLX group, while the other treatment groups are significantly lower. There was an increase in paw slips in the KO + PLX immediately post-treatment versus prior to treatment (KO + PLX pre vs post, Figure 3.8C, (p<0.05)). The number of paw slips in the testing period after microglial repopulation was significantly increased over both pre-treatment and post-treatment (pre vs repopulation (p<0.001), post vs repopulation (p<0.05), Figure 3.8C). Immediately after the 7 day treatment, KO + PLX had a significantly increased number of paw slips in comparison to WT mice (WT vs KO + PLX, Figure 3.8B, (p<0.05). The difference between KO + PLX and WT was increased further after repopulation, as well as a significant difference between KO and KO + PLX (WT vs KO + PLX (p<0.001), KO vs KO + PLX (p<0.05), Figure 3.8B, 3.8D). The data suggested that the number of paw slips increased following PLX treatment in the KO mice, but not in WT animals, although the limited sample size is a source of variability in these measurements.

Ndufs4 KO mice are naturally smaller and weigh less than their WT counterparts. WT and WT + PLX mice had significantly increased body weight from day 0 (beginning of PLX) until day 14 (sacrifice) (WT day 0 vs day 14 (p<0.01), WT + PLX day 0 vs day 14 (p<0.01), Figure 3.9A), while KO and KO + PLX mice did
not show significance. During the last four days of the study (day 11 to 14), KO and KO + PLX mice both displayed weight that was significantly lower than their WT counterparts (Figure 3.9B). KO + PLX mice weighed less than control KO mice, and showed a greater significance compared to both WT groups.

Protein analysis was completed on the olfactory bulb of the mice, probing for both 2SC and Iba-1. Probing for microglial marker content in the OB with Iba-1 showed, that while there was a slight increase in KO + PLX mice versus KO, there was no significance (Figure 3.10A, 3.10B). This confirmed that microglial repopulation occurred, however, the limited number of mice in the study prevented a post-treatment analysis of Iba-1 at 7 days. Anti-2SC immunoblotting displayed a significant increase in KO and KO + PLX mice compared to WT mice (WT vs KO and KO + PLX, Figure 3.10C, 3.10D, (p<0.001). However, there was no difference between KO and KO + PLX, suggesting that treatment did not alter succination levels in the newly repopulated microglia.

3.3 Discussion

PLX treatment has been shown to remove up to ~99% of microglia following a 21 day exposure in dietary chow (136). In this study we first confirmed increased microgliosis in the olfactory bulbs of KO versus WT mice (Figure 3.1), however, Iba-1 levels could not be reliably detected with the antibody in the brainstems of the 3 day and 13 day PLX treated mice (Figure 3.2). Unfortunately, the olfactory bulbs were not available for Iba-1 analysis in these mice, as this region appears to be more reliable for the detection and monitoring of microgliosis. Nevertheless,
while we could not reliably confirm that the drug had resulted in microglial clearance, it was evident that the microglial removal did not confer any additional benefit on survival of the KO mice as there was a steep decline in the severity of clinical symptoms. The 3 day treated mice had respiratory difficulties, highly impaired motor coordination and muscle strength, resulting in the necessity of chow being placed beside the mice, as they were not able to feed otherwise. While Ndufs4 KO mice treated for 13 days were slightly healthier than those receiving PLX for 3 days, their condition was significantly harsher than that of untreated mice, ultimately resulting in premature death of one mouse. For this reason the planned 14 day PLX intervention had to be terminated early at 13 days.

Despite the apparent lack of benefit, we were still interested to determine if microglial removal had resulted in altered succination profile in the brain. A recent collaboration with another laboratory highlighted that activated macrophages may have increased levels of protein succination in the brain during neuropathology in the KO mice. To determine if PLX administration and microglial removal reduced succination, we performed immunoblotting for 2SC in the 3 day and 13 day treated mice (Figures 3.3 and 3.4). There was a trend for a slight reduction in protein succination in the PLX treated KO mice versus untreated KO, however, it is difficult to determine if this was due to a limited effect of PLX to remove microglia, or if succination was significantly altered. It is possible that the (dying) neurons contain higher levels of succinated proteins and therefore a decrease in the microglia may not be detectable in the affected brain region. The trend for a decrease does
warrant further investigation in a study where reduced microglial content can be confirmed.

Since 14 days on PLX was confirmed to be too long a period for our mice, a time course was set up to discover if administration for 3, 5, or 7 days would be a better alternative. Using the promising elimination data obtained in Figure 3.5, we further hypothesized that administration of PLX for 7 days, followed by allowing 7 days for ‘new’ microglial repopulation would be a better strategy. This would remove the unhealthy microglia that they have difficulty being cleared from the lesion, and allow new, healthy microglia to surround the lesion and help phagocytose the neuronal debris. We anticipated that this would result in a slightly decreased number of microglia in KO + PLX versus KO control mice, and hoped to see increased motor coordination during behavioral tests if survival was positively impacted.

The test of speed and endurance, rotarod, demonstrated the KO and KO + PLX mice had approximately identical performance through the three testing periods. Both untreated and treated KO mice fell from the rotarod earlier in each subsequent testing period (Figure 3.6). Balance beam also measured speed, as well as fine motor coordination reflected similar results, with both taking longer to transverse the beam (Figure 3.7). The PLX treated KO mice appeared to have more paw slips (Figure 3.8), however, there was a lot of variability in this measurement and we were severely hindered by the small sample size in the experiment. These studies will need to be repeated with a much larger sample size.
Protein analysis of the 7 day on/7 day off PLX mice was performed to determine Iba-1 levels in the brain after the treatment. Unfortunately, we did not have sufficient mice to sacrifice a separate group at 7 days, immediately after the drug had been administered as we hoped to record more positive behavioral changes and focused on this with this particular group of mice. Iba-1 displayed an increase in microglia of KO + PLX versus KO mice, although this was not significant (Figure 3.10). Elmore et al. also experimented with withdrawal of PLX and showed microglia exhibiting a larger morphology versus control at 3 and 7 days post-PLX. They also displayed a higher concentration of microglia at 7 days after treatment, while by 14 days, the population of microglia was almost normal (130). Both increased size and number of microglia could explain the increase in Iba-1 we observed, as well as 2SC levels remaining unchanged between the untreated KO and KO + PLX groups. We proposed that the KO + PLX KO mice would contain fewer succinated proteins because the unhealthy microglia would be absent, along with their heightened level of succination. However, the increased number of microglia may result in a relatively unchanged amount of protein succination, even if the level of succination in individual cells is decreased.

Overall, these preliminary studies with PLX demonstrate that profound decreases in microglial number do not appear to positively impact behavior or survival in the Ndufs4 KO model similar to studies in other disease models. This may be related to the severity of the neuropathology in the Ndufs4 KO mice, and the fact that the microglia are performing a necessary function to remove dying neurons in the affected brain regions of these mice.
Figure 3.1. Brainstem versus olfactory bulb and effects of aging on microglial concentration. A) Trial of brainstem (BS) vs olfactory bulb (OB) used 40 µg protein from respective areas to display differences in Iba-1 between WT and KO mice. B) Effects of aging on Iba-1 levels in 40 µg of protein from OB (WT early (p24-26), KO early (p24-26), KO mid (p46) and WT late (p60-63)). C) Normalization of age Iba-1 blot to Coomassie (*p<0.05 vs WT early; ###p<0.001 vs WT late. Statistics performed via one-way ANOVA, ± SEM).
Figure 3.2 Microglial concentration in mice treated with PLX for 3 and 13 days. A) 40 µg BS protein, run using SDS-PAGE and probed for Iba-1 for WT and KO mice treated with PLX for 3 and 13 days (middle band, 17kDa) (lanes 1-5 = untreated WT, lanes 6-9 = WT + 3 day PLX, lanes 10-12 = WT + 13 day PLX, lanes 13-15 = KO + 13 day PLX, lanes 16-19 = KO + 3 day PLX, lanes 20-23 = untreated KO). B) Iba-1 of 3 versus 13 day PLX normalized to Coomassie (**p<0.01 vs WT; ***p<0.001 vs WT; ##p<0.01 vs WT-3; WT n = 5, WT-3 n = 4, WT-13 n = 3, KO–13 n = 3, KO-3 n = 4, KO n = 4; Statistics performed via one-way ANOVA, ± SEM).
Figure 3.3 Differences in succination of the brainstem with 3 day PLX treatment. A) 30 µg BS protein from 3 day PLX treated mice was probed with anti-2SC. B) Normalized 2SC levels to Coomassie (***p<0.001 vs WT; WT n = 2, KO n = 2, KO + 3 day PLX n = 4, WT + 3 day PLX n = 4; Statistics performed via one-way ANOVA, ± SEM).
Figure 3.4 Determination of 2SC content in the brainstem of 13 day treated PLX mice. A) 40 µg protein was run via SDS-PAGE, and probed with 2SC antibody (+ is positive control, Fh KD cell protein). B) Densitometry performed on 2SC with Image J and normalized to Coomassie (*p<0.05; **p<0.01. Statistics via one-way ANOVA, ± SEM). *The third lane in KO + PLX was not used for normalization or statistics, as this mouse died prior to the others, and was not dissected and frozen immediately, the carcass was first refrigerated for several hours.
Figure 3.5 Test for the efficiency of PLX in eliminating microglia over 3, 5, and 7 days. A) 40 µg protein from the hippocampus of WT mice treated with PLX for increasing variables of time was probed with anti-Iba-1 to test for microglial presence. B) Normalized amount of hippocampal Iba-1 to Coomassie (***p<0.001 vs control, ± SEM).
Figure 3.6 Rotarod testing of mice treated with PLX for 7 days followed by a 7 day repopulation period. **A and B** Average time to fall for mice during pre, post and repopulation periods (**p<0.01 vs WT repopulation). **C** Average time to fall for each treatment group at the 3 time points. **D** Average time for mice to fall after the 7 day repopulation period (**p<0.001 vs WT, WT n = 4, WT + PLX n = 4, KO n = 2, KO + PLX n = 3; statistics calculation via two-way ANOVA, ± SEM).
Figure 3.7 Balance beam behavioral testing for mice treated with PLX 7 days on and 7 days off. A and B) Average times of treatment groups for pre-, post- and repopulation periods (*p<0.05 vs WT post-treatment; #p<0.05 vs WT repopulation; ##p<0.01 vs WT repopulation). C) Average time to converse the beam of each treatment group, at the three testing stages (*p<0.05 vs KO + PLX pre; **p<0.01 vs KO + PLX pre). D) Time for the groups to cross the beam during the final repopulation testing (*p<0.05 vs WT; **p<0.001 vs WT, WT n = 4, WT + PLX n = 4, KO n = 2, KO + PLX n = 3; statistics performed in Sigma Plot via two-way ANOVA, ± SEM).
Figure 3.8 Paw slips of mice while crossing the balance beam. A and B) Average number of paw slips during each testing period (#p<0.05 vs WT post; ***p<0.001 vs WT repopulation; $p<0.05$ vs KO repopulation). C) Average paw slips for treatment groups during the 3 tests (*p<0.05 vs KO + PLX pre; ***p<0.001 vs KO + PLX pre; #p<0.05 vs KO + PLX post). D) Paw slips during repopulation testing (**p<0.001 vs WT; #p<0.05 vs KO, WT n = 4, WT + PLX n = 4, KO n = 1, KO + PLX n = 2; statistics via two-way ANOVA, ± SEM).
Figure 3.9 Weight of mice from initial treatment exposure (Day 0) to date of sacrifice (Day 14). A) Body weight of mice (**p<0.01 vs WT Day 0; ##p<0.01 vs WT + PLX Day 0) B) Body weight of mice by individual date (#p<0.05 vs WT + PLX; *p<0.05 vs WT; **p<0.01 vs WT; ***p<0.001 vs WT). (WT n = 4; WT + PLX n = 4; KO n = 2; KO + PLX n = 4; statistics via two-way ANOVA, ± SD).
Figure 3.10 Protein analysis of olfactory bulb samples obtained from 7 day PLX, 7 day repopulation mice. A) 40 µg OB protein was loaded and run with SDS-PAGE and probed with Iba-1 antibody. B) Iba-1 blot normalized to Coomassie using Image J software. C) 30 µg protein was loaded and probed with 2SC using Coomassie for protein loading control. D) 2SC blot normalized to Coomassie via Image J (**p<0.01 vs WT; ***p<0.001 vs WT; statistics performed via one-way ANOVA, ± SEM).
CHAPTER IV

FUTURE DIRECTIONS

Due to the visual difference in sulfenylation of KO young versus old mice, I believe that it would be good to begin administering NACA at 21 days, as performed in Liu et al. We would continue to treat for the full 14 days in an effort to enhance the results that they observed, including a delay in the onset of symptoms, as well as decreasing their severity. Since we believe that the oxidative stress begins early in life, and does not seem as predominant later, treating this early could potentially increase the longevity of the mice, as well as their standard of living.

We proved that there was a significant decrease in the number of microglia when WT mice were treated with PLX for 7 days, thereby predicting that a 7 day period after this would allow new, healthier microglia to repopulate and assist the brain. The next experiment would build on the 7 days on/7 days off experiment we already completed. More mice would be used in order to obtain additional testing groups, as well as adding to the data from the current mice. These groups would include mice both sacrificed directly after the 7 day treatment period and pushed to survive an additional 7 days past the current repopulation period. Protein analysis immediately following microglial elimination would allow us to determine
the proficiency of PLX5622 in this mouse model. In addition, 2SC levels, from all four time points, would be measured to determine if the new microglia are ‘healthier’ in terms of succination. Besides the protein analysis, we would take slices of the BS, particularly the VN, and the hippocampus. Immunohistochemistry of sections from before PLX treatment, immediately post-treatment, after 7 days repopulation, and 14 days repopulation would allow us a visual of the number and size of microglia present, when staining with Iba-1. While immunoblotting allows us to see the amount of certain proteins, this will allow visualization of where they are located and if size or shape has changed due to the treatment. That way we would be able to determine if results observed in Elmore et al. (126) apply to our mice as well, with altered size and cell count throughout the repopulation period.
MATERIALS AND METHODS

Materials. Unless otherwise noted, all chemicals were purchased from Sigma Aldrich (St. Louis, MO). Anti-2SC antibody was prepared by Eurogentec (Fremont, CA). Tween-20 and Criterion™ TGX™ Precast Gels were from Bio-Rad (Hercules, CA). Polyvinylidene fluoride (PVDF) was purchased from GE Healthcare (Fairfield, CT). L-glycine and sodium dodecyl sulfate (SDS) were purchased from Fisher Scientific (Waltham, MA). N-acetylcysteine amide (NACA, (R)-2-Acetamido-3-mercaptopropanamide) was synthesized by Matrix Scientific (Columbia, SC). PLX5622 was generously provided by Plexikkon Inc. in standard chow prepared by Research Diets Inc. (Berkeley, CA) (300 mg/kg D11100406i) (1200 mg/kg D11100404i).

Animal Model. All animal use described in this and other sections was consistent with the guidelines issued by the National Institutes of Health and were approved by the University of South Carolina Institutional Animal Care and Use Committee. NDUFS4 knock-out mice were obtained from Dr. Richard Palmiter (University of Washington, WA). The mice were bred and pups were genotyped at 3 weeks. WT and KO mice in the NACA experiment were treated with intraperitoneal injections of 150mg/kg/day NACA for 14 days, beginning at approximately p26. At the termination of the studies mice were anesthetized by isoflurane prior to decapitation. In the PLX experiment, Ndufs4 KO mice were treated with 1200
mg/kg PLX for 3 days or 300 mg/kg PLX for 7 or 13 days. The mice participating in the 7 day on/7 day off PLX study underwent behavioral testing prior to 7 days of 300 mg/kg PLX, followed by a 7 day repopulation period before being anesthetized by isoflurane, followed by decapitation.

**Behavioral Testing**

**Balance Beam.** Mice underwent training 3 days prior to testing, as well as habituation 30-60 minutes before the test. Mice were placed at the end of a one meter long beam with a width of 7 mm. The beam was placed at a 4 degree angle with a black box at the opposite end containing food and bedding. The mouse was allowed 20 seconds to sit in the box before crossing the beam. Between trials, mice were allowed an additional 15-20 seconds to recuperate in the box. Total number of paw slips and the time taken to cross were both measured during the 4 times the mouse crossed the beam. The three best times were used for statistics.

**Rotarod.** As stated above, mice were trained 3 days before testing, and habituated in the room. The speed on the rotarod was increase 4-40 rpm over a span of 5 minutes. Mice were tested 5 times each, with four of those used for statistics. In PLX experiment, mice were recorded, and cartwheeling time taken into account. Cartwheeling occurs when the mouse holds onto the wheel and circles instead of running.

**Protein Extraction from Brain Tissue.** Brainstem, olfactory bulb, or hippocampus were added to 100-250 μL radio immunoprecipitation assay (RIPA) buffer along
with 2 mM Sodium Fluoride, 2 mM Sodium Orthovanadate, and 1/1000 protease inhibitor cocktail (RIPA+ buffer) and sonicated 2 times for 7 seconds each. The tissue was incubated on ice for 10 minutes and centrifuged at 5000 rpm for 10 minutes at 4°C. A supernatant and pellet were then visible. The supernatant was extracted and acetone (9x volume) was then added to each supernatant sample. The samples were vortexed, allowed to sit on ice for 10 minutes and then centrifuged at 2000 rpm for 10 minutes at 4°C. The acetone was removed completely and the protein pellets were re-suspended in 125-200 µL of RIPA+ buffer and sonicated for 10 seconds each. The protein concentration was determined using the Lowry method (140).

**Cell Culture and Viral Transduction.** The lentiviral vectors were prepared by the University of South Carolina Viral Vector Facility. Briefly, TRC2 Fh1 shRNA, clone-TRCN0000246831 or SHC202 MISSION TRC2 pLKO.5-puro Non-Mammalian shRNA control plasmids (Sigma/Aldrich) were used to generate the lentiviral vectors. The specific vector plasmid packing, envelope and Rev plasmids were transfected into 293T cells. N1E-115 neuroblastoma cells obtained from Sigma were grown in 90% DMEM plus 10% fetal bovine serum (non-differentiation medium). At 80% confluence, the cells were washed with DPBS prior to the addition of the lentiviral vectors for transduction. Selection of transduced cells with puromycin (1.75 g/ml) started 2 days later and lasted for 1 week. At the end of this period, the remaining cells were expanded and differentiated into neurons in the presence of 2% FBS, 1.25% DMSO in DMEM in addition to 1 g/ml puromycin for
5 days. The cells were harvested in RIPA buffer; protein extracts were subjected to immunoblotting.

**Primary Neuron Isolation and Culture.** Primary neurons from newborn mouse brain cortices were isolated and cultured using an adaptation of the method described by Brewer (141). Briefly, postnatal day 1 mice were sacrificed by decapitation, the brains were aseptically dissected and cortices were separated from the rest of the brain in ice-cold Hibernate A medium (Gibco/Thermo Fisher Scientific, Waltham, MA), containing 2% (v/v) B-27 supplement (Gibco/Thermo Fisher Scientific) and 0.5 mM glutamine (Invitrogen/Thermo Fisher Scientific, Waltham, MA). The tissue was minced in fragments of about 1 mm³ with a scalpel, and subjected to digestion with 2 mg/ml papain (Worthington Biochemical Corp., Lakewood, NJ) in the supplemented Hibernate A medium for 20 min at 30°C in a shaker incubator set at 100 rpm. After thorough trituration through a fire polished Pasteur pipette, the tissue was allowed to settle for 5 min and the supernatant was carefully layered on top of a discontinuous OptiPrep (Sigma-Aldrich) gradient prepared in Hibernate A medium; the layers contained 35, 25, 20 and 15% OptiPrep. The gradient was centrifuged at 800 g for 15 min at room temperature, and layers 1 (15% OptiPrep) 8 and 2 (20%) were discarded. Layer 3 (25%) was collected and added a five-fold volume of Neurobasal A medium (Gibco/Thermo Fisher Scientific), containing 0.5 mM glutamine and 2% B-27 supplement. After a centrifugation at 500 g for 5 min, cells were resuspended in Neurobasal A medium containing 0.5 mM glutamine, 2% B-27 supplement, and 5 ng/ml bFGF (Invitrogen/Thermo Fisher Scientific); counted and plated on 24-well plates.
pretreated with 0.01% poly-L-lysine (Sigma-Aldrich) at a density of 200,000 cells/well. Fifty percent of the media was replaced every third day, with the addition of 5 µM AraC from DIV 3 to inhibit glial proliferation. On DIV 8, cells were left untreated or treated with 5 mM NAC or NACA for 24 hours. Following, the cells were either subsequently treated with 100 µM DMF for an additional 5 hours, or left alone, resulting in 6 treatment groups (control, control + DMF, NAC, NAC + DMF, NACA or NACA + DMF).

Glutathione Quantification. Cellular protein was washed 3 times with ice cold phosphate buffered saline (PBS) before being scraped and precipitated in 5% sulfosalicylic acid and sonicated, while tissue was placed into the acid and homogenized via glass mortar and pestle. Each solution was then allowed to sit on ice for 10 minutes and centrifuged at 14,000 rpm, +4°C for 10 minutes. The supernatant was removed and diluted 1:5 with assay buffer and then diluted 1:5 with sample diluent buffer supplied by the commercially available kit (Arbor Assay Glutathione Fluorescent Detection Kit, Ann Arbor, MI). Glutathione standards were prepared via adding 50 µL Glutathione Standard into a tube of 450 µL sample diluent and vortexed. This was then serial diluted through seven other tubes using 250 µL of the mixture. 50 µL of samples, standards and a glutathione control were pipetted in duplicate into a 96-well clear bottomed plate. 25 µL of the Thiostar reagent was added to each sample. Total glutathione levels were quantified according to the manufacturer’s instructions (fluorescent emission at 510 nm with excitation at 370 nm). 25 µL of the reaction mixture was pipetted into each well.
before being read again on the plate reader. This allowed for calculations of total and oxidized glutathione concentrations.

The pellet remaining at the bottom of the centrifuged sample was resuspended in RIPA\textsuperscript{+} buffer and protein levels quantified via the Lowry method (140).

**Fumarate Extraction & Quantification.** Fh KD and Scr differentiated N1E-115 cells were washed three times with ice-cold PBS followed by the immediate addition of ice-cold chloroform/methanol (2:1) and scraping. After 10 min on ice, 0.2 volumes of water were added, followed by centrifugation at 3,220 \( g \) for 20 min. The extraction was repeated, and the aqueous supernatants were dried. The protein interface for each sample was removed for protein quantification. After TMS derivatization of the extracts, samples were injected into an Agilent 7890A GC system, coupled to an Agilent 5975C electron ionization mass selective detector. Selected ion monitoring was performed for fumarate and the peak areas obtained were normalized to the added internal standard. Absolute quantitation was performed based on standard curves obtained from the normalized reference standards, and the final metabolite concentrations were normalized to the protein content of the cells. **Note:** Fumarate analysis was performed at DHMRI in Charlotte, NC.

**Determination of the Oxygen Consumption Rate (OCR).** N1E-115 Scr and Fh KD cells were seeded on V7 cell culture microplates coated with 0.2\% gelatin at a density of 10,000 cells/well. After 3 days in culture, the cells were differentiated for
5 days as described above, and treated with 0, 10 or 50 µM DMF for the last 24 hours (n=8/group). The Seahorse extracellular flux analyzer XF-24 (Agilent Technologies, INC., Santa Clara, CA), was used to measure the oxygen consumption rate (OCR), using XF Assay Medium supplemented with 25 mM glucose (142). After measurement of basal respiration, oligomycin (5 µg/mL), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (0.5 µM), rotenone/antimycin A (3 µM/4 µM) were added sequentially to determine ATP production/proton leak, spare respiratory capacity and non-mitochondrial respiration. After completion of the assay, the medium was removed and the cells were immediately washed 3 times with cold PBS. The plate was stored at -70°C prior to the measurement of the total protein content to normalize individual measurements.

**Tissue Sulfenic Acid Stabilization.** Olfactory bulbs and brainstems p21 (young) and p46 (old) mice were harvested and immediately placed into buffer containing 250 mM Hepes and 1 mM DTPA, pH to 7.7 and add 20 mM NEM, 10 mM Dimedone, 1% Triton-X, 2 mM NaF, 2 mM SOV, 1/1000 protease inhibitor cocktail, and 200 U/mL catalase. These were then homogenized using mortar and pestle before being placed on a shaker for 30 minutes prior to centrifugation for 10 min at 14,000 rpm at 4°C. The supernatant was removed to perform the Lowry method (140) and subsequent western blotting.

**Immunoblotting.** 20-40 µg cell lysate protein or 20-40 µg protein from mouse brain tissue was diluted to total volume with nano-pure deionized water and incubated with 4X Laemmli loading buffer at 95°C for 15 minutes. The samples
were centrifuged at 5000 rpm for 10 seconds and then loaded on 7.5%, 12% or 18% gels and electrophoresed at 200 volts for 60 minutes. The protein was transferred to a PVDF membrane in transfer buffer (0.1% Tris Base, 20% methanol, 79.9% nano-pure deionized water) at 250 mA for 100 minutes or 40 mM overnight at 4°C. The membrane protein loading was visualized with Ponceau Red, followed by blocking in 5% bovine serum albumin (BSA) or 5% 134 non-fat milk for 1 hour at room temperature, or overnight at 4°C according to the antibody manufacturer's recommendations. Primary polyclonal antibody to actin (sc-1616) (1:5,000) was from Santa Cruz Biotechnology Inc. (Dallas, TX). Polyclonal antibodies to GFAP (3670) (1:30,000), DM1A Tubulin (3873) (1:15,000) and β3 Tubulin (5666) (1:30,000) were from Cell Signaling (Danvers, MA). The anti-HNE (HNE11-S) (1:5,000) was from Alpha Diagnostic International Inc. (San Antonio, TX). Polyclonal antibody to glutathione (101-A) (1:2,000) was from Virogen (Watertown, MA). Polyclonal sulfenic acid was from EMD Millipore (ABS30) (1:15,000). The polyclonal anti-2SC antibody was prepared as described previously (Nagai et al. 2007) (1:6,000). Polyclonal antibody to IBA-1 (016-20001) (1:1,000) was purchased from Wako Pure Chemical Industries Ltd. (Richmond, VA). Chemiluminescent substrate (Thermo Pierce, Rockford, IL) was utilized followed by detection on photographic film (Denville Scientific, Metuchen, NJ). Immunoblots were stripped (62.5 mM Tris, pH 6.8, 2% SDS and 0.7% (v/v) beta mercaptoethanol) for 20 minutes at 67°C prior to re-probing with new primary antibodies. Image J software (National Institute of Health) was used to quantify
band intensity by densitometry. All immunoblots were normalized to protein content using either actin or Coomassie.
REFERENCES


Appendix A Seahorse Cell Mitochondrial Respiration Stress Test. A) Oligomycin, FCCP, and a combination of rotenone and antimycin A are serially injected into the cells. These measure ATP-linked respiration, maximal respiration, and non-mitochondrial respiration, respectively. Using these measurements, as well as their basal respiration, proton leak and spare respiratory capacity are calculated. B) Denotes the ETC complex that each compound affects (143).
APPENDIX B
BUFFER PREPARATIONS

Radio Immunoprecipitation Buffer
50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton-X. Dissolve in 200 mL with DI water and store at +4. On the day of use, add 2 mM sodium orthovanadate, 2 mM sodium fluoride and protease inhibitor cocktail at 1:1000 dilution.

10x Wash Buffer
200 mM Tris-HCl pH 7.4 in 1 mL DI water. 0.05% Tween-20 was added to 1x wash buffer.

10x Transfer Buffer
250 mM Tris-HCl and 1920 mM glycine dissolved in 1 mL DI water.

1x Transfer Buffer
700 mL DI water, 200 mL methanol and 100 mL 10x Transfer buffer.

10x SDS Running Buffer
250 mM Tris-HCl, 1920 mM glycine and 1% SDS was added to 1 mL water.

Stripping Buffer
Add 3.785 grams Tris-Base to ~400 mL nano-pure water pH 6.8. Dissolve 10 grams SDS and bring the final volume to 500 mL with nano-pure water. Before stripping add 7 µL beta-mercaptoethanol/1 mL stripping buffer used.
**4x Laemmli Loading Buffer**

Dissolve 0.969 grams Tris-Base in 10 mL nano-pure water and pH 6.8. Add 2.468 grams DTT, 3.2 grams SDS, 16 mL glycerol and ~8 milligrams bromophenol blue for color. Store at -20ºC.

**Coomassie Stain**

Stir 400 mL nano-pure water, 100 mL glacial acetic acid and 500 mL methanol for 10 mins. Add 2.5 grams Coomassie Brilliant Blue R-250 (*Do not use G-250*) and stir at least 2 hours at room temperature. Filter staining solution using a 0.45 µm Zap Cap and vacuum aspirator.

**Coomassie Destain**

Mix 100 mL methanol, 830 mL nano-pure water and 70 mL acetic acid.
APPENDIX C

BEHAVIOR PROTOCOLS

Balance Beam Protocol

Set up:

1) Place beam (7mm) on an incline of 4°, with the end of the beam higher than the starting point.

2) Add food and bedding (from the cage being used) to the ending platform.

Habituation:

Transfer mice into testing space 30-60 minutes prior to training/testing to allow for acclimation.

Note: NACA mice did not undergo habituation.

Training:

1) Set first mouse on the platform and place the black box over them, allow to sit for 20 seconds to acclimate.

2) Take the mouse out of the box and place on beam approximately 2-3 inches from the passage into the box, allowing the mouse to enter on its own accord.
3) Repeat the training at 1/3 of the way down the beam, 1/2 and 2/3 before placing mouse at the starting point on the beam and having them walk/run into the box (allow mouse~15 sec inside box to recuperate).  

**Note:** Keep something under KO mice (hands, cage lid, etc) in case of fall.

4) Repeat training until the mouse is able to transverse the beam without assistance.

**Testing:**

1) Allow mouse 30 seconds in the box with bedding and food for acclimation.

2) Place mouse at the starting point on the beam, beginning timing and counting of paw slips, ending when its nose reaches the black box.

3) Permit the mouse time to recuperate in the box (WT: 15-20 sec, KO: 30-40 sec) before starting the next trial.

4) Repeat 4x per mouse, with the 3 best times used in statistics.

5) Use alcohol to clean the balance beam in between mice.

6) When switching to a mouse from a different cage, also switch the bedding to maintain a familiar scent for the mice to run towards.

**Rotarod Protocol**

**Set up:**

1) Set the removable platforms at an angle, they will click into place, in order for the machine to time each mouse.
Habituation:

Transfer mice into testing space 30-60 minutes prior to training/testing to allow for acclimation.

Note: NACA mice did not undergo habituation.

Training:

1) Place one mouse in between each set of partitions, allow to sit for one minute, before removing them.
2) Set the rotarod to a fixed speed at 4 rpm, place the mice back onto the machine and start the wheel, letting it rotate for one minute.
3) Set the rotarod to accelerated mode, with the minimum at 4 rpm and max at 40 rpm, this will also be for 1 minute. The mice should always be placed on the wheel while it is stopped.

Testing:

1) Using the accelerating function, set the minimum at 4 rpm and the max at 40 rpm, constantly accelerating for 5 minutes.
2) Place mice on wheel, making sure the platforms are up, and begin testing.
3) When a mouse falls from the wheel and hits the platform, the time from start to fall will be displayed on the screen.
4) Repeat this process with the next cage. One run should be performed by each cage before beginning on the second run. Rotating through the
cages in this way should allow mice a resting period of ~20-30 minutes, dependent upon the number of cages

5) Mice are to be tested four times each.

6) In PLX study, each run was recorded and later analyzed for cartwheeling. This provided the amount of time the mice spent running versus holding on to the wheel.
APPENDIX D

ARTICLES

Peer-reviewed journal article


Article in preparation to be submitted