THE ROLE OF CREATINE IN PROMOTING OLIGODENDROCYTE SURVIVAL AND MODULATING AXONAL MITOCHONDRIA IN THE CNS

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THE ROLE OF CREATINE IN PROMOTING OLIGODENDROCYTE SURVIVAL AND MODULATING AXONAL MITOCHONDRIA IN THE CNS

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ABSTRACT

Oligodendrocytes maintain neuronal integrity in the central nervous system (CNS). Chronic oligodendrocyte loss, a feature of the demyelinating disorder multiple sclerosis (MS), contributes to axonal dysfunction. Current therapies reduce MS severity, but do not prevent disease progression, characterized by accumulated chronic demyelination and neurodegeneration. Pharmacological compounds that promote oligodendrocyte survival and maintain neuronal integrity would be beneficial for MS. One such candidate is creatine, a cytoprotective organic acid involved in ATP buffering. As the creatine-synthesizing enzyme guanidinoacetate-methyltransferase (Gamt) is most highly expressed in oligodendrocytes in the CNS, these cells are likely the primary source of creatine in the brain. Intriguingly, human creatine deficiencies are characterized by severe neurological deficits, indicating that neurons are also heavily reliant on creatine. Therefore, the overarching goals of this dissertation were to investigate how creatine-mediated modulation of mitochondrial function affects 1) oligodendrocyte lineage cell proliferation, progression, differentiation, and survival under basal and injury conditions and 2) neuronal morphology and mitochondrial dynamics.

In this study, creatine directly increased mitochondrial ATP production in purified mouse oligodendrocyte lineage cell cultures, and exerted robust protection of oligodendrocytes by preventing cell death in both naïve and lipopolysaccharide (LPS)-treated mixed glia. Moreover,
lysolecithin-mediated demyelination in *Gamt*-deficient mice did not affect oligodendrocyte precursor cell (OPC) recruitment, but resulted in exacerbated apoptosis of regenerated oligodendrocytes in CNS lesions. Remarkably, creatine administration into mice with demyelinating injury reduced oligodendrocyte apoptosis, thereby increasing oligodendrocyte density and myelin basic protein (MBP) staining in CNS lesions. Creatine did not affect the recruitment of macrophages/microglia into lesions, suggesting that creatine affects oligodendrocyte survival independent of inflammation. These results demonstrate a novel function for creatine in promoting oligodendrocyte viability during CNS remyelination.

This work also demonstrates a crucial role for creatine in modulating neuronal mitochondria. *Gamt*-deficient neurons exhibited aberrant neuronal respiration and axonal mitochondrial dynamics, which likely contributed to their reduced morphological complexity. Alternatively, addition of creatine or oligodendrocyte-derived secreted factors lowered the density of stationary axonal mitochondria, suggesting reduced energetic burden in treated neurons. Overall, this work demonstrates that creatine treatment may be beneficial for normalizing mitochondrial function in order to protect CNS cells in neurological disease.
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LIST OF ABBREVIATIONS

CNS: Central nervous system

MS: Multiple Sclerosis

OPC: Oligodendrocyte progenitor cell

PCR: Phosphocreatine

E: Embryonic

P: Postnatal

NOR: Nodes of Ranvier

PLP: Proteolipid Protein

CNP: 2’3’-Cyclic-nucleotide 3’-phosphodiesterase

MCT1: Monocarboxylate Transporter 1

BDNF: brain-derived neurotrophic factor

NGF: nerve growth factor

NT-3: neurotrophin-3

CHAT: choline acetyltransferase

GDNF: glial cell line-derived neurotrophic factor

IGF1: insulin-like growth factor type-1

RRMS: relapsing-remitting multiple sclerosis

SPMS: secondary progressive multiple sclerosis

Ig: immunoglobulin

PPMS: primary progressive multiple sclerosis

BBB: blood brain barrier

LINGO1: leucine rich repeat and immunoglobulin-like domain-containing protein 1
EAE: autoimmune experimental encephalomyelitis
APP: amyloid precursor protein
NFH: non-phosphorylated neurofilament heavy chain
NAWM: normal appearing white matter
NAGM: normal appearing gray matter
MtCK: mitochondrial creatine kinase
PCr: phosphocreatine
CKBB: creatine kinase, brain
AGAT: L-arginine:glycine amidinotransferase
GAA: guanidinoacetic acid
GAMT: guanidinoacetate methyltransferase
CrT: creatine transporter
SLC6A8: solute 6 carrier family 6, member 8
GPA: guanidinopropionic acid
CSF: cerebrospinal fluid
$^1$H-MRS: proton magnetic resonance spectroscopic imaging
LPS: Lipopolysaccharide
Olig2: oligodendrocyte transcription factor 2
GFAP: glial fibrillary acid protein
CD11B: integrin alpha-M/beta-2
MACS: magnetic activated cell sorting
OCR: oxygen consumption rate
TUNEL: Terminal deoxynucleotidyl transfer dUTP nick-end labeling
EdU: 5-ethynyl-2'-deoxyuridine

PDL: poly-d-lysine

PFA: paraformaldehyde

PBS: phosphate-buffered saline

TBS: Tris-buffered saline

DIV: days in vitro

TMRE: tetramethylrhodamine, ethyl ester

FCCP: carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone

UPLC: Ultra-Performance Liquid Chromatography

PI: Propidium Iodide

cDNA: complementary DNA

RT-PCR: reverse transcription polymerase chain reaction

Mg-Gr: magnesium green
CHAPTER I: INTRODUCTION
Oligodendrocytes are glial cells of the central nervous system (CNS) that extend multilayered fatty membranes, known as myelin sheaths, around neuronal axons. Myelination first evolved in vertebrates, but has since appeared in several independent phylogenetic taxa (Castlefranco and Hartline, 2015). This remarkable example of convergent evolution emphasizes the advantageousness of nervous system myelination, of which the most well described function is electrical insulation. Myelin increases the speed of neuronal communication by allowing for rapid signal propagation along axons up to one meter in length. Defects in myelin development and/or function have dire consequences in human diseases such as multiple sclerosis (MS) and leukodystrophy. However, why oligodendrocytes become dysfunctional and how to protect them in disease is not well understood, emphasizing a need for further research in this area.

A. OLIGODENDROCYTE FUNCTION IN THE CENTRAL NERVOUS SYSTEM
Following neurogenesis, oligodendrocyte progenitor cells (OPCs) arising from several ventricular germinal zones begin to proliferate and migrate throughout the CNS. In mice, this process begins around embryonic day (E) 12.5 and peaks during early postnatal (P) development (Sauvageot and Stiles, 2002). In humans, myelination begins in the brainstem and progresses to the cerebellum followed by the cerebrum; myelination proceeds quickly from birth to 9 months of age and continues into adulthood (Aubert-Broche B, 2008). Once in the developing gray and white matter, multiple molecular cues stimulate OPCs to differentiate into mature oligodendrocytes (for review see Emery, 2010), which myelinate axons all the way into adulthood (Dimou et al., 2008; Rivers et al., 2008; Yeung et al., 2014). Interestingly, OPCs remain highly abundant in the adult brain and maintain a homeostatic density by coordinating their symmetric proliferation with differentiation of a nearby OPC or in response to
oligodendrocyte death (Hughes et al., 2014). OPCs maintain the ability to differentiate into myelinating oligodendrocytes, serving as a rare example of regenerative capacity in the adult brain.

I. Axonal Myelination
During myelination, oligodendrocytes spiral a lipid-rich membrane extension around axons, extruding cytoplasm between layers to form the compact myelin sheath (Snaidero et al., 2014; Fields RD, 2014). A single oligodendrocyte can myelinate up to 40 different axonal segments (internodes), in between which are intermittent gaps of unmyelinated axon, termed nodes of Ranvier (NOR). This stereotyped process permits restriction of voltage gated sodium channels to NOR and voltage gated potassium channels to areas called juxtaparanodes (Pedraza et al., 2001; Rasband, 2008, 2010). This precise localization of channels enables saltatory conduction of action potentials, which greatly increases the speed of axonal signaling (Morell and Quarles, 1999).

Demyelination disrupts ion channel segregation (Wolswijk and Balesar, 2003; Coman et al., 2006), such that sodium and potassium channels redistribute along the axon, causing overlap of paranodal and juxtaparanodal protein domains (England et al., 1990; Scherer and Arroyo, 2002; Craner et al., 2004; Podbielska et al., 2013). The diffusion of ion channels and increased membrane capacitance from myelin loss cause an impedance mismatch in demyelinated axons, producing inefficient conduction (Waxman, 2006). In MS, impaired impulse conduction manifests as functional and/or cognitive disabilities, highlighting the importance of intact axonal
myelination for mediating efficient communication between neural networks (Charil et al., 2003).

II. Metabolic and Trophic Support of Axons

Oligodendrocytes play a critical role in maintaining axonal integrity, a function that appears to be independent of myelin itself (Griffiths et al., 1998; Lappe-Siefke et al., 2003; Nave and Trapp, 2008; Edgar et al., 2009; Nave, 2010; Saab et al., 2013). For example, mice harboring mutations in major protein components of myelin, including proteolipid protein (PLP) and 2’3’-Cyclic-nucleotide 3’-phosphodiesterase (CNP), exhibit impaired axonal transport (Edgar et al., 2009, 2010) and axonal degeneration (Lappe-Siefke et al., 2003), respectively, despite structurally normal myelin sheaths. Additionally, targeted ablation of oligodendrocytes produces axonal pathology prior to myelin degeneration (Traka et al., 2010; Oluich et al., 2012).

Precisely how oligodendrocytes maintain axonal integrity remains unclear. It has been hypothesized that physical insulation of myelinated axons elicits a dependency on oligodendrocyte-derived metabolic and trophic support (Edgar et al., 2009; Nave, 2010). Numerous studies have demonstrated increased axonal mitochondrial density following CNS demyelination, suggesting a role for oligodendrocytes in regulating axonal energy metabolism (Mutsaers and Carroll, 1998; Kiryu-Seo et al., 2010; Traka et al., 2010; Zambonin et al., 2011; Campbell et al., 2012). Indeed, downregulation of the lactate transporter, monocarboxylate transporter 1 (MCT1) in oligodendrocytes leads to neuronal dysfunction and degeneration (Lee et al., 2012), illustrating that transport of oligodendrocyte-derived lactate to neurons is crucial for maintaining neuronal health and survival (Lee et al., 2012; Fünfschilling et al., 2012).
Oligodendrocytes may also regulate neuronal survival via production of neurotrophic factors. Oligodendrocytes express mRNA transcripts for brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin-3 (NT-3) (Gonzalez et al., 1990; Byravan et al., 1994; Dougherty et al., 2000; Dai et al., 2003; Zhang et al., 2014). Moreover, oligodendrocyte secretion of BDNF and NT-3 enhance the function of basal forebrain neurons in vitro, by increasing expression of choline acetyltransferase (ChAT) (Dai et al., 2003). Oligodendrocytes also express glial cell line-derived neurotrophic factor (GDNF) and \( \text{IGF1} \), both of which enhance survival of cortical neurons (Wilkins et al., 2003; Zhang et al., 2014; Wilkins et al., 2001; Zhang et al., 2014; Wilkins and Compston, 2005; Wilkins et al., 2001). As the majority of these studies were performed in vitro, significantly more work is needed to determine the role of oligodendrocyte-derived neurotrophic factors in mediating axonal integrity in the CNS.

B. OLIGODENDROCYTE INJURY AND REGENERATION IN MULTIPLE SCLEROSIS

MS is an auto-immune disease affecting more than 2.3 million people worldwide (Milo and Kahana, 2010; Compston and Coles, 2011). For reasons that remain poorly understood, individuals with MS undergo bouts of chronic CNS inflammation characterized by the destruction of oligodendrocytes and their associated myelin membranes. Most patients with MS are diagnosed in the relapsing-remitting (RRMS) phase, in which periods of myelin degeneration (relapse) alternate with periods of spontaneous remyelination (remission). While remyelination is remarkably prolific in early MS (Franklin 2002, Goldschmidt et al., 2009; Patrikios et al., 2006), it becomes less efficient as the disease progresses (Goldschmidt et al., 2009; Franklin 2002). Failure of remyelination is associated with neurodegeneration and accrual of permanent disability (Franklin, 2008); combined occurrence of these features marks the transition into
secondary progressive MS (SPMS). Notably, although current immunosuppressive therapies reduce the number of relapses, they do not prevent patients from progressing (Carrithers, 2014; Rice, 2014).

I. Heterogeneous Patterns of Injury in Multiple Sclerosis

Irrespective of the initial cause, MS pathology gives rise to heterogeneous lesions that can be classified by four different histopathological patterns. Pattern I and II lesions are characterized by perivenous distribution and large demyelinated plaques, however only pattern II lesions contain immunoglobulin (Ig) deposition and activated complement (Lucchinetti et al., 2000). Pattern III lesions, which are difficult to demarcate, show early signs of oligodendrocyte apoptosis and are associated with a short relapsing-remitting clinical course. Pattern IV lesions, which are characterized by perivenous distribution of demyelination and extensive loss of oligodendrocytes (Lucchinetti et al., 2000). These lesions are found exclusively in patients with primary progressive MS (PPMS), the least common form of MS, in which neurological function worsens from the onset of disease symptoms without clear relapses and remissions. MS lesions can also be characterized by distinct stages of inflammatory demyelination: active, chronic active, and chronic inactive (Bo et al., 1994; Bruck et al., 1994; Bruck et al., 1997; Ferguson et al., 1997; Trapp et al., 1998; Valk and De Groot, 2000). Active includes both ‘preactive’ lesions, which exhibit white matter abnormalities without demyelination, and active lesions, which exhibit inflammation in the presence or absence of demyelination. Chronic active includes lesions with maintained inflammatory activity but no demyelination. Finally, chronic inactive includes lesions with and without demyelination that do not exhibit inflammatory activity.
Complicating the issue of lesion heterogeneity, symptomatic lesions are rarely fatal, making it difficult to assess the underlying pathological mechanisms. However, an analysis of nine cases of rapidly deteriorating MS resulting from fatal brainstem lesions demonstrated the earliest pathological change common to all cases was oligodendrocyte apoptosis associated with early microglial activation (Barnett and Prineas, 2004). This group hypothesized that Pattern III lesions may not be a distinct type of lesion, but rather the earliest stage of lesion formation for many, if not all, lesions.

II. Mechanisms of Oligodendrocyte Injury

Currently, there are two competing theories to explain the pathological hallmarks of inflammation and demyelination in MS. The “outside-in” theory proposes that primary immune dysfunction causes T-cells in infiltrate the CNS and attack oligodendrocytes. Alternatively, the “inside-out” theory proposes that primary oligodendrocyte dysfunction causes cell death and demyelination, leading to blood brain barrier (BBB) breakage and infiltration of peripheral T-cells. Recently, the inside-out theory has gained significant traction with the discovery that nascent lesions contain apoptotic oligodendrocytes with little infiltration of peripheral immune cells (Lucchinetti et al., 2000; Barnett and Prineas, 2004), suggesting that oligodendrocyte apoptosis may occur early in the disease. Strong support for a potentially causative role of oligodendrocyte death in initiating an adaptive immune response comes from a recent paper demonstrating that diphtheria toxin-induced oligodendrocyte ablation leads to T-cell infiltration into the CNS (Traka et al., 2010; Traka et al., 2015). Additionally, experimental induction of oligodendrocyte cell death in mice has been shown to produce rapid demyelination and axonal damage (Caprariello et al., 2012; Pohl et al., 2011; Traka et al., 2010).
Although the precise timing and cause of oligodendrocyte death remains contentious (Lumsden CE, 1970; Raine et al., 1981; Prineas et al., 1984; Lucchinetti et al., 1999; Lassmann H, 1983), it is generally accepted that oligodendrocyte death is a significant contributor to MS pathology (Macchi et al., 2015).

In prephagocytotic lesions, dying oligodendrocytes co-localize with activated caspase-3 (Prineas and Parratt, 2012). The presence of cleaved caspase-3 indicates activation of mitochondrial-dependent cell death. Depending on the inducer and subsequent mitochondrial-response, oligodendrocytes may die in the form of apoptosis or necrosis (Casaccia-Bonnefil, 2000). In apoptosis, cytoplasmic proteins bind to the mitochondria and signal release of cytochrome c to initiate a downstream signaling cascade for apoptosome formation and subsequent DNA destruction (Duprez et al., 2011). Necrosis is primarily characterized by loss of plasma membrane integrity as well as severe mitochondrial impairment (Casaccia-Bonnefil, 2000). It is believed that oligodendrocytes primarily undergo apoptosis when mitochondrial respiration is not severely impacted, such as during transient activation of death receptors. However, during a prolonged attack, such as long-lasting exposure to inflammatory cytokines in chronic MS lesions, mitochondria become irreparably damaged and oligodendrocytes take on a more necrotic appearance (Casaccia-Bonnefil, 2000). Indeed, chronic MS lesions contain functionally significant alterations in respiratory chain proteins and mitochondrial DNA (Mahad et al., 2009). Interestingly, many oligodendrocytes appear to survive the initial demyelination in chronic-stage MS, but are lost from lesioned areas gradually (Wolswijk G, 2000), suggesting that their survival is compromised over time.
III. Remyelination Failure

The mechanism of remyelination failure is not well understood. While OPCs continue to be recruited to demyelinated areas in approximately 70% of lesions (Lucchinetti et al., 1999; Chang et al., 2002), their differentiation into re-myelinating cells becomes halted for unknown reasons (Charles et al., 2000, 2002; Back et al., 2005; Mi et al., 2005; Fancy et al., 2010). It was hypothesized that impairment of OPC differentiation contributes to remyelination failure (Wolswijk, 1998; Chang et al., 2002; Kuhlmann et al., 2008; Huang and Franklin, 2011). Additionally, gradual loss of oligodendrocytes in lesioned areas in chronic MS (Wolswijk G, 2000) suggests that reduced oligodendrocyte survival under chronic inflammation may contribute to remyelination failure. As remyelination restores axonal conduction and protects axons in rodent models of MS (Jeffery and Blakemore, 1997; Kornek et al., 2000; Irvine and Blakemore, 2008), pro-remyelination therapies might be beneficial for patients with MS. Numerous intrinsic signaling pathways known to regulate OPC differentiation, including leucine rich repeat and immunoglobulin-like domain-containing protein 1 (LINGO-1), canonical Notch, canonical wingless, semaphorin, retinoid X receptor, and muscarinic signaling, are being investigated as therapeutic candidates for promoting remyelination (for review see Chamberlain et al., 2015).

IV. Axonal Injury and Neurodegeneration

It is hypothesized that axonal degeneration is the cause of irreversible disability in MS (Wegner et al., 2006; Mahad et al., 2015a). The evidence for axonal damage in MS is robust and comes from clinical, histopathological, biochemical, and imaging studies (for review see Haines et al., 2011; Trapp and Nave, 2008). MS patients with permanent paralysis are estimated to have
between 60-70% axonal loss (Mews et al., 1998; Bjartmar et al., 2000) and this finding is closely recapitulated in the autoimmune experimental encephalomyelitis (EAE) model, in which mice with permanent paralysis exhibit 59% and 43% axonal loss in the cervical and lumbar spinal cord, respectively (Wujek et al., 2002). Histologically, acute axon damage is characterized by the presence of varicosities and spheroid structures (Saxena and Caroni, 2007), which reflect impaired organelle transport in the axon (Williamson and Cleveland, 1999). This impairment results in the accumulation of proteins, including amyloid precursor protein (APP), along the damaged axon. APP accumulation is present in active, remyelinating, and inactive MS lesions (Ferguson et al., 1997; Bitsch et al., 2000). A second marker of neurodegeneration, non-phosphorylated neurofilament heavy chain (NFH), also accumulates in neurons in MS lesions (Petzold et al., 2008; Dziedzic et al., 2010; Schirmer et al., 2011; Gray et al., 2012).

Axon damage occurs in both acute demyelinating lesions and in chronically demyelinated lesions (Ferguson et al., 1997; Trapp et al., 1998; Ciccarelli et al., 2014), however it remains unknown whether this is a consequence of direct inflammatory attack, secreted inflammatory mediators, or secondary to demyelination (Trapp et al., 1998; Haines et al., 2011). Observations of neuronal loss and dendritic atrophy in normal appearing white matter (NAWM) (Trapp et al., 1998; Bjartmar et al., 2000; Wood et al., 2012), and in normal appearing gray matter (NAGM) (Klaver et al., 2015) suggest an early pathogenic mechanism. A growing body of evidence suggests that energetic failure and oxidative stress may be a driving factor of neurodegeneration (Trapp and Stys, 2009), as abnormalities in mitochondrial density, morphology, and activity are consistently found in models of MS (Qi et al., 2006; Mahad et al., 2008, 2009, Witte et al., 2009, 2010, 2014; Kiryu-Seo et al., 2010; Fischer et al., 2012; Campbell et al., 2014; Joshi et al., 2015). The exact
cause of neurodegeneration in MS remains to be seen, however the demonstration that early axonal damage can be reversed with remyelination (Bjartmar et al., 2003; Nikić et al., 2011) strengthens the rationale that remyelination failure contributes to neurodegeneration.

C. CREATINE IN THE CENTRAL NERVOUS SYSTEM

I. Creatine Function in the Central Nervous System

The human cortex alone requires $\sim 3 \times 10^{23}$ ATP/s/m$^3$ (Howarth et al., 2012) with individual neurons estimated to utilize 4.7 billion ATP/s at rest (Zhu et al., 2012). This astonishing energy demand means the CNS utilizes 20% of the entire body’s energy supply (Mergenthaler et al., 2013). The organic acid creatine is hypothesized to mitigate this energy demand by allowing for rapid ATP regeneration in the cytoplasm (Wyss and Kaddurah-Daouk, 2000). It is thought that creatine becomes phosphorylated near sites of ATP production by mitochondrial creatine kinase (MtCK) to form phosphocreatine (PCr), which is then reversibly dephosphorylated by cytoplasmic brain creatine kinase (CK-BB; Wyss and Kaddurah-Daouk, 2000). This interplay between mitochondrial and cytoplasmic creatine kinases would allow for rapid ATP regeneration near sites of high utilization without the need for de novo oxidative phosphorylation. In addition, creatine and PCr are smaller and less negatively charged than ADP and ATP, respectively, allowing comparatively higher buildup and diffusion of creatine and PCr (Wyss and Kaddurah-Daouk, 2000). Inborn errors of creatine metabolism present with severe mental retardation, autistic-like behavior, motor disorder, speech delay, and delayed myelination (Barkovich et al., 2011; Anselm et al., 2006). Although it is clear that creatine is crucial for proper brain
development, the precise mechanisms by which creatine deficiency impairs cell-specific function have not been elucidated.

II. Creatine Synthesis and Transport
Creatine comes from both the diet and endogenous synthesis, which occurs in a two-step enzymatic reaction wherein L-arginine:glycine amidinotransferase (AGAT) converts glycine and arginine into guanidinoacetic acid (GAA), which is then converted into creatine via guanidinoacetate methyltransferase (GAMT) (Wyss and Kaddurah-Daouk, 2000). How this reaction actually proceeds in the mammalian body remains enigmatic as mismatched cellular expression patterns and activity levels of AGAT and GAMT indicate a complicated transport system. Currently, it is postulated that GAA, synthesized in the kidney, is transported to the liver for conversion into creatine by GAMT (Wyss and Kaddurah-Daouk, 2000). Once in the bloodstream, creatine enters cells via a Na+/Cl- coupled symporter known as creatine transporter (CrT) or solute 6 carrier family 6, member 8 (SLC6A8) (Betsalel et al., 2012).

A similarly complicated multi-cellular transport system has been suggested in the CNS (Braissant et al., 2001; Tachikawa et al., 2009; Braissant et al., 2010), as oligodendrocytes are the only CNS cells expressing high levels of Gamt (Zhang et al., 2014), indicating that they may be the primary synthesizers of creatine within the brain and spinal cord. In addition to expression of AGAT and GAMT inside the brain, there are several lines of evidence supporting the notion that creatine is endogenously synthesized within the CNS. First, administration of guanidinopropionic acid (GPA), a competitive antagonist of the creatine transporter, has very little influence on creatine and PCr concentrations inside the brains of rats and mice (Holtzman
et al., 1989). Second, in addition to having the highest expression of Gamt (Zhang et al., 2014), oligodendrocytes also have the highest rate of creatine kinase activation (Manos et al., 1991). Lastly, transport of creatine through the BBB in rodents is largely inefficient (Ohtsuki et al., 2002; Perasso et al., 2003), which is in agreement with the observation that treatment of AGAT- and GAMT-deficient patients with high concentrations of creatine leads to slow and only partial replenishment of creatine in the brain (Schulze and Battini, 2007; Stockler et al., 2007). Additionally, despite not being able to transport creatine into the brain, patients with SLC6A8 deficiency have normal creatine levels in their cerebrospinal fluid (CSF) (Cecil et al., 2001; DeGrauw et al., 2002; Salomons et al., 2001). On the contrary, GAMT-deficient patients, who should theoretically be able to transport dietary creatine from the periphery into the CNS, have severely diminished CSF creatine levels (Schulze et al., 1997).

III. Human Creatine Deficiencies

Cerebral creatine deficiency syndromes (CCDS) arise from genetic mutations in either of the creatine-synthesizing enzymes, AGAT and GAMT, or the creatine transport, CrT. Proton magnetic resonance spectroscopic imaging (1H-MRS) of brain creatine levels is the primary method of diagnosis (Mercimek-Mahmutoglu and Salomons, 2009). Intellectual disability and seizures are common to all three disorders. Only 14 patients with AGAT deficiency have been identified and reported. About 40% of GAMT-deficient patients exhibit some form of movement disorder and frequently present with autistic-like behaviors, including self-mutilation (Schulze et al., 1997, 2003; Joncquel-Chevalier Curt et al., 2015). While most GAMT-deficient patients begin to exhibit symptoms between 3 months and 3 years of age, people with CrT deficiency have been diagnosed anywhere from 2 to 66 years old (Mercimek-Mahmutoglu and Salomons,
The range of symptoms in CrT deficiency is more heterogeneous—ranging from mild to severe intellectual disability, speech delay, movement disorder, seizures, and delayed myelination (Anselm et al., 2006; Barkovich, 2011; Joncquel-Chevalier Curt et al., 2015). Creatine deficiencies are treated with high doses of oral creatine monohydrate to replenish creatine levels. GAMT-deficient patients are also treated with ornithine and dietary restriction of arginine. CrT-deficient patients are significantly more difficult to treat as creatine supplementation does not restore cerebral creatine concentrations and therefore has no effect on clinical outcome (Mercimek-Mahmutoglu and Salomons, 2009).

IV. Creatine in the Multiple Sclerosis Brain

Numerous reports investigating creatine levels in MS pathology have been published. Proton magnetic resonance spectroscopic imaging ($^1$H-MRS) studies are in direct conflict: 1. Higher creatine was found in NAWM and focal lesions of patients with PPMS compared to controls and patients with RRMS (Suhy et al., 2000), 2. No differences in creatine levels were observed in the NAWM of PPMS patients compared to controls (Leary et al., 1999), 3. Creatine levels were reduced, albeit not significantly, in all MS patients compared to controls (Davie et al., 1997), and 4. Creatine levels were increased in MS patients (RRMS and SPMS) compared to controls (Vafaeyan et al., 2015). 3D $^1$H-MRS, which provides increased volume resolution, revealed a 22% increase in creatine levels in the NAWM of patients with RRMS compared to controls (Inglese et al., 2003). While not exhaustive, this list illustrates the difficulty in drawing conclusions about how the MS disease course affects creatine concentrations.
CHAPTER II: METHODS
A. MICE

C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and provided food and water *ad libitum*. *Gamt*+- and *MtCK*+- mice were kind gifts from Dr. Dirk Isbrandt (University of Cologne) and Dr. Petras Dzeja (Mayo Clinic). All experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Georgetown University.

B. WESTERN BLOT ANALYSIS

Cerebellum was dissected from mice at various postnatal time points. Proteins were harvested in lysate buffer (150mM sodium chloride, 1% Triton X-100, and 50mM Tris, pH 8.0), separated by SDS-PAGE, and immunoblotted using the following antibodies: rat anti-MBP (1:500; AbD Serotec), rabbit anti-Olig2 (1:1000, Millipore), goat anti-PLP (1:200; Santa Cruz), and rabbit anti-β-Actin (1:1000; Abcam). Proteins were detected using horseradish peroxidase (HRP)-conjugated secondary antibodies and Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific). Oligodendrocyte protein expression was quantified by densitometry (ImageStudioLite) and represented as average protein expression normalized to β-Actin loading control.

C. CHEMICALS AND ANTIBODIES

LPS (Escherichia coli 0111:B4) was obtained from InvivoGen (San Diego, CA). Creatine monohydrate (C3630), 3-Guanidinopropionioic acid (GPA; G6878), and carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; 370-86-5), were obtained from Sigma-Aldrich. MitoTracker® Red FM (M22425), tetramethylrhodamine, ethyl ester (TMRE; T669), and
propidium iodide (PI; P3566) were obtained from Thermo Fisher Scientific. Primary antibodies for immunohistochemistry (IHC): anti-GAMT (1:500, Abcam), anti-Tom20 (1:50, Santa Cruz Biotechnology), anti-VDAC (1:50, Abcam), rat anti-CD11B (1:100; AbD Serotec), rabbit anti-YM1 (1:100; StemCell Technologies), rabbit anti-cleaved caspase-3 (1:100; Cell Signaling), mouse anti-iNOS (1:50; BD Pharmingen), rabbit anti-Olig2 (1:300; Millipore), rat anti-MBP (1:200; AbD Serotec), mouse anti-CC1 (1:300; Millipore), mouse anti-Nkx2.2 (1:100; DSHB). Primary antibodies for immunocytochemistry (ICC): anti-β-Tubulin (1:500, Sigma), anti-SLC6A8 (1:300, Abcam), anti-NeuN (1:100, Millipore), rabbit anti-Olig2 (1:500; Millipore), rat anti-MBP (1:500; AbD Serotec), mouse anti-GFAP (1:500; Sigma), mouse anti-CC1 (1:200; Millipore); rabbit anti-PDGFRα (1:300; BD Pharmingen). Alexa Fluor® 488 or 594 secondary antibodies (Thermo Fisher Scientific) were used at a concentration of 1:500. Primary antibodies for western blot (WB): anti-β-Actin (1:1000, Abcam), anti-GAMT (1:1000, Abcam), anti-MBP (1:1000, AbD Serotec), anti-Olig2 (1:1000, Millipore), anti-PLP (1:1000, Santa Cruz Biotechnology). To label nuclei, cell and tissues were labeled with 1µg/mL Hoechst in PBS for 5min at room temperature (RT) (33342; Thermo Fisher Scientific).

D. PRIMARY MIXED GLIA CELL CULTURE

Mixed glia cultures containing oligodendrocyte lineage cells (Olig2⁺), astrocytes (glial fibrillary acid protein, GFAP⁺), and microglia (CD11B⁺) were prepared from postnatal day (P) 3-5 old mouse cortices as previously described (Daniele et al., 2014) and maintained in DMEM-F12 containing 10% FBS, 1% Penicillin/Streptomycin/Fungizone, and 1% GlutaMAX™ for two weeks (Fig. II.1A,B). Mixed glia cultures were switched to a serum-free medium for 24hr prior to initiating treatments.
Figure II.1. Establishment of mixed glia cultures containing oligodendrocyte lineage cells, astrocytes, and microglia.  

A, Immunostaining of mixed glia cultures at DIV14 showing Olig2$^+$ (magenta) oligodendrocyte lineage cells and GFAP$^+$ astrocytes (green). All nuclei labeled with Hoechst (blue).  

B, Immunostaining of mixed glia cultures at DIV14 showing CD11B$^+$ (magenta) microglia. Scale bars represent 20µm. Brightness and contrast adjusted for visualization.
E. PRIMARY MIXED CORTICAL NEURON CULTURE

Primary mouse neurons were cultured from P3-5 WT mice after papain dissociation and O4+ magnetic activated cell sorting (MACS) (Miltenyi). Neurons were plated in poly-d-lysine (PDL)-coated plastic well plates or 35mm glass-bottom dishes and maintained in co-culture media (DMEM-F12, N2, B27, P/S, Insulin, and T3) described here (Dincman et al., 2012). Co-culture media supports growth of cortical neurons (Fig. II.2A,B) as well as glial cells, including astrocytes (Fig. II.2C) and oligodendrocyte lineage cells (Fig. II.2D).

F. PRIMARY OLIGODENDROCYTE CULTURE: MAGNETIC ACTIVATED CELL SORTING

Primary oligodendrocyte lineage cell cultures (Fig. II.3) were obtained from P3-5 mouse cortices utilizing magnetic activated cell sorting (MACS) with the Neural Tissue Dissociation Kit (P; 130-092-628) and anti-O4 microbeads (130-096-670) according to the manufacturers protocol (Miltenyi). Primary oligodendrocyte lineage cells were expanded in growth media (DMEM-F12 with N2, B27, P/S, BSA, FGF and PDGF) and then differentiated using defined media (DMEM-F12, N2, B27, P/S, Insulin, and T3) as previously described (Dincman et al., 2012). The resulting cultures contain 94% Olig2+ cells, of which 7% differentiate into mature, myelin basic protein (MBP) positive oligodendrocytes following 3 days of defined media.

G. CELL LINE CULTURE

OLN-93 cells (Richter-Landsberg and Heinrich, 2013), originally from Dr. Christiane Richter-Landsberg (University of Oldenburg), were kindly provided by Dr. Wendy Macklin (University of Colorado) and were maintained in DMEM containing 10% FBS and 2mM Glutamine. All
Figure II.2. Establishment of mixed cortical cultures containing neurons, astrocytes, and oligodendrocytes. A,B, Immunostaining of mixed cortical cultures at DIV7 showing β-Tubulin+ (magenta) cortical neurons with 3-5 primary branches at 10X (A) and 60X (B). Scale bars represent 200µm and 50µm, respectively. C-D, Mixed cortical cultures at DIV22 showing astrocytes positive for glial fibrillary acid protein (C, GFAP, green) and oligodendrocytes positive for myelin basic protein (D, MBP, green). Scale bars represent 100µm. Brightness and contrast adjusted for visualization.
Figure II.3. Establishment of purified oligodendrocyte lineage cell cultures via magnetic activated cell sorting (MACS). A, DIV10 oligodendrocyte lineage cells cultured via MACS. OPCs are Olig2⁺ (green) and mature oligodendrocytes are double positive for Olig2⁺ and MBP⁺ (magenta). Scale bar represents 100µm. B, Greater than 94% of the nuclei (Hoechst⁺, blue) are also Olig2⁺, indicating highly pure cultures of oligodendrocyte lineage cells. Scale bar represents 100µm. C, Mature oligodendrocytes extend flat, expansive MBP⁺ membranes. Scale bar represents 50µm. Brightness and contrast adjusted for visualization.
cultures were treated with 100ng/ml creatine, 100ng/ml 3-Guanidinopropionic acid, and/or 1µg/ml LPS for 24-48 hours.

H. SPINAL CORD DEMYELENATION

Focal demyelination was induced by injection of 1.0% lysolecithin (Sigma-Aldrich Company Ltd, Dorset, UK) diluted in sterile PBS into the spinal cord ventral funiculus of 9-12 week-old mice. Creatine monohydrate (25ng) or PBS was co-injected along with 1.0% lysolecithin. The animals were euthanized for analysis at 5, 10, or 20 days post-surgery (Fig. II.4).

I. IMMUNOCHEMISTRY

Mice were perfusion-fixed with 4% (w/v) PFA (Sigma) in PBS. Spinal cord tissue was dissected and postfixed for 45min in 4% PFA at RT. Tissue was cryoprotected in 20% (w/v) sucrose (Sigma) in PBS at 4°C overnight before freezing in OCT on the surface of dry ice. 12 µm frozen spinal cord sections were collected on SuperFrost®Plus slides (Stellar Scientific) using a cryostat and were dried for 30min prior to storage at -80°C. For in vitro experiments, cells were fixed with 4% (w/v) PFA and 120mM sucrose for 20min and then washed with PBS. Sections/cells were then incubated in blocking solution (0.01% [v/v] Triton X-100 and 5% goat serum in PBS) for 1 hour at RT. Primary and secondary antibodies were diluted in PBS containing 0.01% (v/v) Triton X-100 and 1% (w/v) BSA and applied to sections/cells overnight at 4°C. Tris-buffered saline (TBS) was substituted for PBS when immunolabeling tissue sections. For detection of Nkx2.2 and CC1, mouse-on-mouse antigen retrieval was performed before immunohistochemistry according to the manufacturer’s instructions (M.O.M.™ kit; Vector
Figure II.4. Model of lysolecithin-mediated focal demyelination of mouse spinal cord. **A**, Experimental time course of focal spinal cord demyelination and analysis at 5, 10, and 20 days post lesion (dpl). **B**, Toludine-Blue staining of ventral spinal cord lesion (black outline). **C-D**, Example immunostaining showing accumulation of Hoechst$^+$ nuclei (**C**, blue) and co-localization with CD11B (**D**, red) in spinal cord lesion. Scale bars represent 100µm. Brightness and contrast adjusted for visualization.
Figure II.5. Markers of stage-specific oligodendrocyte lineage cell differentiation. Oligodendrocyte lineage cells upregulate various proteins as they differentiate from oligodendrocyte progenitor cells (OPCs) to pre-myelinating oligodendrocytes to mature, myelinating oligodendrocytes. Markers used to identify OPCs and myelinating oligodendrocytes throughout this work are magenta.
Laboratories). Oligodendrocyte lineage cells were identified using double labeling of Olig2 (entire oligodendrocyte lineage) and various stage-specific protein markers (Fig. II.5).

J. IMAGING AND CELL COUNTING

For quantification of immunostaining, ImageJ (NIH) cell counter was utilized to manually count cells from low (10x for tissues; 20x for cell cultures) magnification images. For mixed glia cultures, data are represented as proportion of total Olig2+ cells to control for the heterogeneous density and growth of mixed glia cultures. For spinal cord tissues, cells were counted only in the lesioned area. Lesions were identified by abnormally high cell density in the ventral funiculus, as visualized by the accumulation of Hoechst+ nuclei. Cell counts are expressed as percentage (ratio of cells expressing two markers divided by the number of cells expressing a single marker multiplied by 100) or density per mm² (number of cells expressing one or two markers divided by the area in μm² multiplied by 1,000,000). For Nkx2.2, CC1, MBP, and CD11β analysis, a minimum of 2 sections from 3 mice were analyzed and the average proportion or density of cells was determined per mouse. For cleaved caspase-3 analysis, a minimum of 2 sections from 2-3 mice were analyzed. The average and standard error of the mean (SEM) was then calculated for each group using Microsoft Excel.

K. LABELING, IMAGING, AND ANALYSIS OF OLIGODENDROCYTE MITOCHONDRIA

To quantify mitochondrial density, days in vitro (DIV) 10 primary oligodendrocyte cell cultures were treated with DMEM (control), creatine (100μM), or creatine and GPA (100μM) for 24hr, after which mitochondria were labeled with MitoTracker® Red (5nM) for 30mins at 37°C. Fluorescent images were acquired using an EVOS Cell Imaging system. Mature
oligodendrocytes were identified via immunostaining for MBP and ImageJ (NIH) was used to threshold mitochondria outside the cell body for analysis (Fig. II.64,B). To measure mitochondrial membrane potential, purified oligodendrocyte lineage cells plated on a 96-well plate were differentiated until DIV7 and treated with PBS or creatine (100µM) for 24hr. Cells were incubated in 20nM TMRE for 24hr and fluorescent intensity was measured using GloMax®-Multi Detection System. As a control for TMRE sensitivity, cells were next incubated with 20µM FCCP, a known ionophore uncoupler of oxidative phosphorylation, for 20mins to confirm a reduction in TMRE signal.

L. SEAHORSE EXTRACELLULAR FLUX ANALYSIS

O₂ consumption rate (OCR) of primary oligodendrocyte lineage cells was measured using an XF96 Seahorse Extracellular Flux Analyzer according to the manufacturers protocol. Briefly, oligodendrocyte lineage cells were differentiated in defined media and treated on DIV3 with either creatine (100µM) or PBS for 24hr. DIV4 cells were washed in pre-warmed assay media for 1hr in a CO₂-free 37°C incubator. The XF Cell Mito Stress Test Kit (Seahorse Part#103015-100) was then used to quantify ATP production and basal respiration in N=2 wells/condition following sequential injections of oligomycin, FCCP, and rotenone/antimycin A. Oligomycin, a complex IV inhibitor, is injected to differentiate ATP-linked respiration from proton leak. FCCP, an uncoupler of ATP synthesis, is next injected to measure maximal respiratory rate. Finally, rotenone/antimycin A, an inhibitor of complex III, is injected to measure all non-mitochondrial sources of oxygen consumption (Seahorse Bioscience).
Figure II.6. Analysis of mitochondria in oligodendrocyte processes and neuronal axons. 

A, Mitochondria in a mature, MBP+ (green) oligodendrocyte labeled with MitoTracker-Red (magenta). Scale bar represents 50µm. 

B, For analysis, images were imported into ImageJ (NIH) and mitochondria (aqua) located only within oligodendrocyte processes (excluding cell body, black) were thresholded for quantification. 

C-D, Mitochondria in the 20-70µm region of a neuronal axon labeled with MitoTracker-Red (C, magenta) and thresholded (D, black) for analysis. Brightness and contrast adjusted for visualization.
M. NEURONAL MITOCHONDRIA LABELING AND LIVE IMAGING

Neurons were plated in 35mm glass-bottom dishes and maintained in the same co-culture media containing insulin, CNTF, and T3 used for differentiated oligodendrocyte cultures. Mitochondria were labeled on DIV7 using CellLight® Mitochondria-RFP (Life Technologies), a fusion construct of the alpha pyruvate dehydrogenase sequence and TagRFP that provides specific mitochondrial targeting independent of mitochondrial membrane potential. On DIV8, cells were imaged using 3D confocal microscopy (Zeiss). On neurons with 3-5 primary branches, axons were selected for imaging by their characteristic morphology: long, thin, uniform diameter, and sparse branches (Banker and Cowan, 1977). Time-lapse confocal microscopy was performed by collecting z-stacks every 30 seconds for 15 minutes with low laser power to avoid bleaching. Neurons were then treated with OCM obtained from DIV8 primary oligodendrocyte cultures for 2hr at 37°C. Importantly, neuronal cultures were maintained in the same co-culture media as oligodendrocyte cultures prior to adding OCM to ensure that any effects seen could be attributed to oligodendrocyte-secreted factors. Following the 2hr incubation, neurons were reimaged as described above. The 20-70µm region of the axon was selected for analysis and the manual tracking plugin in ImageJ was used to measured mitochondrial density and dynamics (Fig. II.6C,D).

N. OLIGODENDROCYTE MEMBRANE EXPANSION ANALYSIS

Purified oligodendrocyte lineage cells differentiated until DIV10 were treated with PBS (control), creatine (100µM), or creatine and GPA (100µM) for 24hr and immunostained for MBP. Fluorescent images were thresholded in ImageJ (NIH) and fractal dimensions of oligodendrocytes were quantified using the ImageJ Fractal Analysis plugin.
O. TERMINAL DEOXYNUCLEOTIDYL TRANSFER DUTP NICK-END LABELING

Terminal deoxynucleotidyl transfer (TdT) dUTP nick-end labeling (TUNEL) labeling was performed using Click-iT® TUNEL Alexa Fluor® 594 Imaging Assay for microscopy and HCS according to the manufacturers instructions (Molecular Probes™).

P. 5-ETHYNYL-2’-DEOXYURIDINE LABELING

5-ethynyl-2’-deoxyuridine (EdU) labeling was performed using Click-iT® EdU Alexa Fluor® 594 Imaging Kit according to the instructions (Molecular Probes™). EdU was added to cell cultures at a final concentration of 25µM for 24hr beginning at the time of treatment.

Q. PROPIDIDIUM IODIDE LABELING

Cells were incubated with 10µg/mL propidium iodide (PI) for 20min at 37°C, washed once with pre-warmed culture media, and fixed for immunostaining.

R. REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

Reverse transcriptase polymerase chain reaction (RT-PCR) was conducted on complementary DNA (cDNA) from purified oligodendrocyte lineage cultures. The following mouse primer sequences were synthesized by MWG Operon:

- Agat Forward (5’-TCA CGC TTC TTT GAG TAC CG-3’)
- Agat Reverse (5’-TCA GTC GTC ACG AAC TTT CC-3’)
- Gamt Forward (5’-TGG CAC ACT CAC CAG TTC A-3’)
- Gamt Reverse (5’-GAC TGC CGC TAC TAT GCC TT-3’)
- CrT Forward (5’-TCC TGG CAC TCA TCA ACA G-3’)


**CrT Reverse** (5’-ATG AAG CCC TCC ACA CCT AC-3’)

S. MAGNESIUM GREEN LABELING

Mixed glia cultures plated in 35mm glass-bottom dishes were labeled with 1µM cell permanent, Magnesium Green™ AM (Thermo Fisher Scientific, M3735). Following a 20min incubation, cells were rinsed with PBS, and placed in temperature equilibrated live-cell imaging media. Time-lapse confocal microscopy was performed by collecting images every 30 seconds for 15 minutes with low laser power to avoid bleaching.

T. ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY (UPLC)

Ultra performance liquid chromatography (UPLC) experiments were run with Amrita Cheema and Smirithi Menon at the Georgetown University Proteomics and Metabolomics core facility. 25ng/mL internal standard was added to 100µL of sample and processed in MacroSpin Silica C18 Columns (SMM SS18V; Amika). Samples were chromatographed on a 100mm × 2.1mm Acquity BEH 1.7 µm C18 column using an Acquity UPLC system (Waters Corp).

U. STATISTICS

All statistics were performed using Prism. Data are represented as mean ± SEM. Significance was determined using either two-tailed Student's t-tests, or one-way analysis of variance (ANOVA) with Bonferroni test for post-hoc analysis. Statistical significance is reported as *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P<0.0001.
CHAPTER III: CREATINE ENHANCES MITOCHONDRIAL-MEDIATED OLIGODENDROCYTE SURVIVAL FOLLOWING DEMYELINATING INJURY
Oligodendrocytes are glial cells of the central nervous system (CNS) that myelinate axons to promote saltatory conduction and provide neurons with energy metabolites, including lactate (Fünfschilling et al., 2012; Lee et al., 2012). Oligodendrocyte and myelin loss is a hallmark feature of the chronic inflammatory disease multiple sclerosis (MS) (Wolswijk, 1998; Compston and Coles, 2008). While existing MS therapies are able to reduce disease severity, they do not prevent progression into the chronic neurodegenerative phase of the disease (Rice, 2014). Myelinating oligodendrocytes regenerate spontaneously following demyelination in the early phase of MS, owing to the availability of endogenous oligodendrocyte precursor cells (OPCs) in the CNS (Franklin and Gallo, 2014). However, this process becomes increasingly difficult with disease progression and results in progressive axonal loss and the accumulation of clinical disability (Franklin, 2002; Haines et al., 2009; Fitzner and Simons, 2010; Dutta and Trapp, 2011). Why remyelination ultimately fails in MS is not well understood. One possibility is that oligodendrocytes do not survive well under the inflammatory environment in MS. For example, oligodendrocytes are highly vulnerable to hypoxic-ischemic injury (Jablonska et al., 2012; McIver et al., 2010; Ness et al., 2001), a feature of MS lesions (Lassmann, 2016), and readily undergo inflammation-mediated cell death (Akassoglou et al., 1998; Baerwald & Popko, 1998; Vartanian et al., 1995).

Previous studies have demonstrated that oligodendrocyte survival can be achieved in murine autoimmune-mediated models of demyelination by overexpressing the anti-apoptotic protein p53 (Hisahara et al., 2000), enhancing the integrated stress response (Lin et al., 2007; Way et al., 2015), or by deleting FADD (Mc Guire et al., 2010), a receptor adaptor protein involved in apoptotic initiation. Moreover, mice with enhanced oligodendrocyte survival also displayed
reduced disease severity. These studies suggest that enhancing oligodendrocyte survival would be beneficial as a treatment strategy in MS.

One candidate for promoting oligodendrocyte survival is creatine, a cytoprotective organic acid (Matthews et al., 1998, 1999; Klivenyi et al., 1999; Andres et al., 2005) that has been shown to regulate neuronal mitochondrial activity (Lee & Peng, 2008) and protect against oxidative damage (Berti et al., 2012; Hosamani et al., 2010; Saraiva et al., 2012; Sestili et al., 2006). It is hypothesized to function as an intracellular ATP buffer as it is phosphorylated near sites of ATP production by mitochondrial creatine kinase (MtCK) to generate phosphocreatine (PCr), which is then reversibly dephosphorylated by cytoplasmic creatine kinases for rapid ATP regeneration near sites of high utilization (Wyss and Kaddurah-Daouk, 2000). Intriguingly, oligodendrocytes have the highest capacity for the synthesis and utilization of creatine in the CNS (Braissant et al., 2001; Cahoy et al., 2008; Manos et al., 1991; Molloy et al., 1992; Tachikawa et al., 2004; Zhang et al., 2014). In addition, inborn errors of creatine metabolism frequently present with delayed myelination as well as severe mental retardation, autistic-like behavior, motor disorder, and speech delay, suggesting a crucial role for creatine during brain development (Anselm et al., 2006; Barkovich, 2011). Despite its clinical relevance, the precise role of creatine in oligodendrocytes remains poorly understood.

Here, we investigated the role of creatine in oligodendrocyte function. We demonstrate that creatine directly increases oligodendrocyte mitochondrial ATP production and promotes oligodendrocyte survival under inflammatory conditions in vitro and following focal demyelination in vivo in mice. Moreover, we show that creatine does not affect the distribution
of macrophages/microglia in demyelinated lesions, suggesting that the protective effect of creatine occurs independent of inflammatory modulation. These results suggest that therapeutic administration of creatine may promote oligodendrocyte survival in MS.

A. RESULTS
I. Oligodendrocytes Express Components of the Creatine Biosynthesis and Transport Pathway
Creatine synthesis occurs in a two-step reaction; L-arginine:glycine amidinotransferase (AGAT/GATM) first converts arginine and glycine into guanidinoacetate (GAA), which is then converted into creatine by guanidinoacetate-methyltransferase (GAMT). To investigate how oligodendrocyte expression of these enzymes compares to that of other CNS cells, we searched a publicly available RNA-sequencing transcriptome database of the mouse cerebral cortex (http://web.stanford.edu/group/barres_lab/brain_rnaseq.html; Zhang et al., 2014) and graphed the resulting FPKM (fragments per kilobase of transcript sequence per million mapped fragments) values. Astrocytes, neurons, OPCs, newly formed oligodendrocytes, myelinating oligodendrocytes, microglia, and endothelial cells all express AGAT (Fig. III.1A). However, newly formed and myelinating oligodendrocytes express 29X more Gamt than all other CNS cells combined (Fig. III.1B; Zhang et al., 2014), indicating that mature oligodendrocytes have a preferentially high capacity for endogenous creatine synthesis in the CNS. The creatine transporter, known as CrT1 or SLC6A8, is expressed in all CNS cells, with the highest expression in oligodendrocyte lineage cells (Fig. III.1C; Zhang et al., 2014). Reverse transcriptase polymerase chain reaction (RT-PCR) conducted on cDNA isolated from MACS-purified oligodendrocytes lineage cell cultures confirmed the expression of Agat, Gamt, and CrT transcripts (Fig. III.1D). Further, immunohistochemistry demonstrated little co-localization of
Figure III.1. Oligodendrocytes express components of the creatine biosynthesis and transport pathway. A-C, Graphical representation of cell-specific Agat (A), Gamt (B), and SLC6A8 (C) RNA levels obtained from a publicly available RNA-sequencing transcriptome database (http://web.stanford.edu/group/barres_lab/). FPKM represents fragments per kilobase of transcript sequence per million mapped fragments (Zhang et al., 2014). D, MACS-purified oligodendrocytes from n=3 mice (A1-A3) express transcripts for the creatine biosynthetic
enzymes, *Agat* and *Gamt*, and the creatine transporter, CrT, via RT-PCR. *E*, Immunostaining of GAMT (magenta) and GFAP (green) in the corpus callosum of an adult mouse showing minimal co-localization with GFAP⁺ astrocytes. *F*, Immunostaining of GAMT (magenta) and CC1 (green) in the corpus callosum of an adult mouse showing high co-localization with CC1⁺ mature oligodendrocytes. Scale bars represent 50μm.
GAMT with the astrocytic marker glial fibrillary acidic protein (GFAP) in corpus callosum of postnatal day 60 (P60) WT mice (Fig. III.1E). In contrast, nearly all CC1+ mature oligodendrocytes co-localized with GAMT (Fig. III.1F). Together, these data indicate that oligodendrocytes express all components of the creatine biosynthetic and transport pathway as previously reported (Braissant et al., 2010; Tachikawa et al., 2004; Takasaki et al., 2010).

II. Exogenous Creatine Addition Increases Mitochondrial Density, Area, and Circularity in Primary Oligodendrocytes

Creatine addition has been shown to increase mitochondrial membrane potential and transport in *Xenopus* spinal neurons (Lee & Peng, 2008) and ATP production in hippocampal neurons (Li et al., 2004) and muscle (Walsh et al., 2001), suggesting that creatine acts directly on mitochondria. To determine if creatine also affects oligodendrocyte mitochondria, MACS-purified oligodendrocyte lineage cells were treated with PBS, creatine (CR), or creatine and the competitive antagonist of CrT, guanidinopropionic acid (GPA), for 24hr. MitoTracker-Red was used to label mitochondria in mature oligodendrocytes, which were identified by expansive MBP staining. We found that creatine significantly increased the density of mitochondria in oligodendrocyte processes (Fig. III.2A-D; N=12 cells/condition; p=0.0002). Moreover, this effect was completely abrogated by co-treatment with GPA (Fig III.2A-D; N=12 cells/condition; p=0.0002). This increase in mitochondrial density was mirrored by an increase in total cellular area represented by mitochondria in creatine-treated oligodendrocytes (Fig. III.2E; N=12 cells/condition; p<0.0001). Interestingly, creatine treatment also altered the morphology of mitochondria, leading to more circular structures in creatine-treated oligodendrocytes (Fig.
Figure III.2. Creatine increases mitochondrial density, area, and circularity in primary oligodendrocytes. A-C, Mitochondria (magenta), labeled with MitoTracker® Red, were analyzed in the processes of primary oligodendrocytes in MACS-purified oligodendrocyte lineage cell cultures expanded until DIV7, differentiated until DIV9, then treated with PBS (A), creatine (B, CR), or CR and guanidinopropionic acid (C, GPA) for 24hr. For all analyses, N=12 cells/condition, one-way ANOVA with Bonferroni post-hoc test. D, Mitochondria per mm² in the processes of MBP⁺ mature oligodendrocytes. E, Average ratio of mitochondrial area:cell area in primary oligodendrocytes. F, Average mitochondrial circularity in primary oligodendrocytes. G, 3D maximum projection of mitochondria (red) in a MBP⁺ (green) oligodendrocyte. H, Example map of mitochondrial center of mass from image H used to reconstruct mitochondrial volume. I, Average mitochondrial volume (µm³) in primary oligodendrocytes. Data are represented as mean ± SEM. Scale bars represent 100µm. Brightness and contrast adjusted for visualization. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
III.2F; N=12 cells/condition; p<0.0001). Despite this morphological shift, mitochondrial volume was not affected by creatine treatment (Fig. III.2G-J).

III. Exogenous Creatine Addition Increases Oligodendrocyte Mitochondrial ATP Production

Since mitochondrial density and morphology are correlated with ATP production (Morris and Hollenbeck, 1993), we next examined the effect of creatine on mitochondrial activity. Oligodendrocyte lineage cell cultures were expanded for 24hr, differentiated until DIV3, and treated with PBS or CR for 24hr. Mitochondria were labeled with tetramethylrhodamine ethyl ester (TMRE; Fig. III.3A). TMRE is a cationic dye that accumulates in mitochondria relative to their membrane potential (ψm), and serves as a proxy for ATP production, such that its fluorescent intensity increases as ATP is produced via oxidative phosphorylation (Perry et al., 2011). As a control for TMRE sensitivity, cultures were treated with FCCP, an ionophore uncoupler of oxidative phosphorylation. Indeed, FCCP significantly reduced the intensity of TMRE signal in both PBS and creatine-treated oligodendrocytes (Fig. III.3B, N=2 wells/condition; p=0.0004 for PBS and p<0.0001 for CR). Removal of FCCP followed by a short TMRE incubation rescued TMRE intensity (Fig. III.3B, N=2 wells/condition; p=0.0005 for PBS and p<0.0001 for CR). Interestingly, oligodendrocyte lineage cells treated with creatine for 24hr had significantly higher TMRE intensity compared to cells treated with PBS (Fig. III.3C; N=2 wells/condition; p=0.0016), suggesting the creatine treatment increases oligodendrocyte mitochondrial activity. We confirmed this finding using Seahorse extracellular flux analysis, which measures oxygen consumption as an indicator of mitochondrial respiration. We found that creatine-treated oligodendrocyte lineage cells displayed significantly increased ATP production.
Figure III.3. Creatine increases oligodendrocyte mitochondrial ATP production. A, Live-cell TMRE (magenta) image showing labeled mitochondria in living oligodendrocyte lineage cells. B-C, Average fluorescent intensity of TMRE signal from oligodendrocyte lineage cell cultures expanded for 24hr, differentiated until DIV3, then treated with PBS or CR for 24hr. TMRE was measured three times following a 24hr TMRE incubation (TMRE), following a 20min incubation with the ionophore uncoupler of oxidative phosphorylation known as FCCP, and following a 20min re-labeling with TMRE (TMRE Rescue). N=2 wells/condition. B indicates statistical results from two-way ANOVA with Bonferroni post-hoc test comparing either FCCP or TMRE Rescue to TMRE for oligodendrocytes treated with PBS (black) and CR (gray) demonstrating that FCCP significantly reduces TMRE intensity regardless of treatment. C indicates statistical results from Student’s T-Test demonstrating that CR-treated oligodendrocytes have significantly higher membrane potential after 24hr compared to PBS-treated oligodendrocytes. D, Average oxygen consumption rate (OCR) in pmoles/min during ATP production in oligodendrocyte lineage cell cultures expanded for 24hr, differentiated until DIV3, then treated with PBS or CR for 24hr. N=2 wells/condition; Student’s T-Test. Data are represented as mean ± SEM. Scale bars represent 50µm. Brightness and contrast adjusted for visualization. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
compared to controls (Fig. III.3D; N=2 wells/condition; p=0.0454). Together, these findings suggest that creatine directly enhances mitochondrial function in primary oligodendrocytes.

IV. Oligodendrocyte Membrane Expansion is Not Affected by Exogenous Creatine Addition or Gamt-Deficiency

Myelination is a highly energy demanding process, requiring an estimated 3.30x10^{23} ATP molecules/gram of myelin synthesized (Harris and Attwell, 2012). To determine if increased ATP production in creatine-treated oligodendrocytes stimulates membrane expansion, purified oligodendrocyte lineage cells were treated with PBS (control), creatine, or creatine and GPA for 24hr. Expansion of MBP^{+} membranes was measured using fractal analysis- a quantitative measurement of morphological complexity that has previously been employed to assess differentiation of oligodendrocyte lineage cells in vitro (Behar, 2001). We found that the fractal dimension of all oligodendrocytes was in the range of those previously reported (Fig. III.4A), however the average fractal dimension of oligodendrocytes was not significantly different across treatment groups (Fig. III.4B). As an additional measure of membrane expansion, ImageJ’s (NIH) concentric circle plugin was utilized to measure average perimeter intensity of MBP^{+} membranes across concentric circles at increasing radii (Fig. III.4C). Again, there were no differences across treatment groups (Fig. III.4D), suggesting that creatine does not affect oligodendrocyte membrane expansion in vitro.

To determine whether creatine loss-of-function is associated with alterations in oligodendrocyte membrane expansion, we measured fractal dimensions and perimeter intensities of MBP^{+} oligodendrocytes in Gamt^{+/+} (GAMT-WT) and Gamt^{-/-} (GAMT-KO) mixed cortical cultures at
Figure III.4. Creatine does not affect oligodendrocyte membrane expansion. 

A, DIV10 MACS cultured primary oligodendrocytes at different stages of membrane expansion and their associated fractal dimension values. 

B, Fractal dimensions of primary oligodendrocytes treated with PBS, creatine (CR), or CR and GPA for 24hr. 

C, Example of concentric circles overlaying a thresholded, MBP+ oligodendrocyte. 

D, Average perimeter intensity of primary oligodendrocytes at DIV10 treated with PBS, creatine (CR), or CR and GPA for 24hr. Data are binned in radii (µm) extending outward from the cell body. $N=10$ oligodendrocytes/condition.
DIV7 (Fig. III.5A). Average fractal dimension was not different between GAMT-WT and GAMT-KO oligodendrocytes (Fig. III.5B). However, analysis of perimeter intensity along concentric circles revealed subtle reductions in morphological complexity of Gamt-deficient oligodendrocytes (Fig. III.5C).

V. Exogenous Creatine Addition Increases Oligodendrocyte Survival Under Basal Conditions

To test whether creatine addition stimulates differentiation of OPCs into oligodendrocytes, proportions of OPCs and mature oligodendrocytes were quantified in primary mixed glia cells cultured in serum-free media with PBS or creatine for 24hr or 48hr (Fig. III.6A). Creatine treatment did not affect the proportions of OPCs (Fig. III.6B) or oligodendrocytes (Fig. III.6C) at either time point, suggesting that creatine does not stimulate differentiation of OPCs into oligodendrocytes. Since no difference was observed in the proportions of oligodendrocyte lineage cells following creatine treatment, we asked if creatine affected the homeostatic turnover of oligodendrocyte lineage cells (Hughes et al., 2013) by assessing the overall proportion of cells undergoing proliferation and cell death. Primary mixed glia were treated with PBS or creatine (CR) for 48hr in the presence of the thymidine analog EdU to label proliferating cells engaged in DNA synthesis. Quantification of cells immunostained for EdU and PDGFRα revealed that creatine treatment resulted in a significant reduction in the proportion of proliferating oligodendrocyte precursor cells (Fig. III.6D,E; N=10 images/condition; p=0.0439). To determine if creatine affects oligodendrocyte lineage cell death, mixed glia treated with PBS or creatine (CR) for 48hr were incubated with propidium iodide (PI), a membrane-impermanent DNA intercalating agent used for the identification of dying cells. We found that creatine treatment significantly reduced the proportion of dying oligodendrocyte lineage cells (Fig. III.6F,G; N=5
Figure III.5. *Gam*-deficient oligodendrocytes exhibit subtle reductions in morphological complexity. 
**A.** Thresholded images of MBP^+^ oligodendrocytes from *Gam^+/−^* (GAMT-WT) and *Gam^−/−^* (GAMT-KO) mixed cortical cultures used for analysis of cellular morphology. **B.** Average fractal dimensions of primary oligodendrocytes in mixed cortical cultures at DIV7. N=5-6 oligodendrocytes/condition; Student’s T-Test. **C.** Average perimeter intensity of primary oligodendrocytes in mixed cortical cultures at DIV7 binned in radii (µm) extending outward from the cell body. N=5-6 oligodendrocytes/condition; Student’s T-Test. Data are represented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
Figure III.6. Creatine increases oligodendrocyte survival. A, Representative image of primary mouse mixed glia culture containing microglia (CD11b⁺; magenta), astrocytes (GFAP⁺; green), and oligodendrocyte lineage cells (Olig2⁺; blue). Scale bar represents 100µm. B-C, Percentage of
OPCs (B, PDGFRα⁺Olig2⁺) and mature oligodendrocytes (C, CC1⁺Olig2⁺) out of total oligodendrocyte lineage cells (Olig2⁺) following 24hr or 48hr treatment with PBS or CR. N=9 images/condition; Student’s T-Test. D-E, Percentage of proliferating OPCs (D, Edu⁺PDGFRα⁺) out of total OPCs (PDGFRα⁺) and representative images (E) following 48hr treatment with PBS or CR. N=10 images/condition; Student’s T-Test. White arrows show Edu⁺PDGFRα⁺ cells. Scale bars represent 50µm. F-G, Percentage of propidium iodide (PI⁺), dying oligodendrocyte lineage cells (F, PI⁺Olig2⁺) out of total Olig2⁺ cells and representative images (G) following 48hr treatment with PBS or CR. N=5 images/condition; Student’s T-Test. White arrows show PI⁺Olig2⁺ cells. Scale bars represent 50µm. H-I, Percentage of dying oligodendrocytes (H, TUNEL⁺CC1⁺) out of total oligodendrocytes (CC1⁺) and representative images (I) following 48hr treatment with PBS or CR. N=12 images/condition; Student’s T-Test. White arrows show TUNEL⁺CC1⁺ cells. Scale bars represent 20µm. J-K, Percentage of propidium iodide (PI) positive, dying oligodendrocytes (J, PI⁺MBP⁺) out of total oligodendrocytes (MBP⁺) and representative images (K) following 24hr treatment of DIV2 purified oligodendrocyte lineage cell cultures with PBS or 100µM creatine (CR). N=5 images/condition; Student’s T-Test. White arrows show PI⁺MBP⁺ cells. Scale bars represent 100µm. Data are represented as mean ± SEM.*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
images/condition; p=0.0002), suggesting that creatine promotes their survival. Further, TUNEL analysis for the detection of fragmented DNA in dying CC1⁺ oligodendrocytes demonstrated that creatine significantly improved survival of mature oligodendrocytes (Fig. III.6J,K; N=12 images/condition; p=0.0070). To determine whether creatine is protective in cultures of purified oligodendrocyte lineage cells, the proportion of PI⁺MBP⁺ dying oligodendrocytes out of total oligodendrocytes (MBP⁺) was measured in DIV2 MACS cultures treated with PBS or creatine for 24hr. We found that creatine treatment significantly reduced the proportion of dying oligodendrocytes (Fig. III.6J,K; N=6 images/condition; p=0.0273), suggesting that creatine acts directly on oligodendrocyte lineage cells to promote their survival.

VI. Creatine Promotes Oligodendrocyte Survival Under Chemical and Inflammatory Injury
The pro-survival effect of creatine under basal conditions (48hr) prompted us to test whether creatine also promotes oligodendrocyte survival under acute (24hr) chemical and inflammatory injury. Mixed glia cultures were first treated with 1% dimethyl sulfoxide (DMSO), a solvent known to impair mitochondrial function and reduce cell viability at concentrations as low as 1% (Galvao et al., 2014; Yuan et al., 2014). As expected, DMSO treatment resulted in a significant reduction in the proportion of mature oligodendrocytes (Fig. III.7A,B; N=15 images/condition; p=0.0021), an effect that was abrogated by co-treatment with creatine (Fig. III.7A,B; N=15 images/condition; p=0.003). In contrast to mature oligodendrocytes, DMSO treatment had no effect on OPC proportion (Fig. III.7A,C). Next, we established a robust assay of inflammation-mediated oligodendrocyte cell death using the rodent oligodendroglia cell line, OLN-93 (Richter-Landsberg and Heinrich, 1996). To mimic a pro-inflammatory environment, OLN-93 cells were treated with conditioned media (cm) taken from RAW 264.7 macrophage/monocyte(s) treated
Figure III.7. Creatine promotes oligodendrocyte survival under chemical injury. 

A, Representative images of DIV14 primary mixed glia cultures treated with PBS, 1% dimethyl sulfoxide (DMSO), or DMSO and creatine (CR) for 24hr. Scale bars represent 10µm. 

B, Percentage of mature oligodendrocytes (CC1⁺Olig2⁺) out of total oligodendrocyte lineage cells (Olig2⁺). N=15 images/condition; one-way ANOVA with Bonferroni post-hoc test. 

C, Percentage of OPCs (PDGFRα⁺Olig2⁺) out of total oligodendrocyte lineage cells (Olig2⁺). N=15 images/condition; one-way ANOVA with Bonferroni post-hoc test. Data are represented as mean ± SEM. Brightness and contrast adjusted for visualization. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
with PBS or lipopolysaccharide (LPS). LPS is a potent activator of Toll-like 4 receptor (TLR4) that stimulates macrophages/monocytes to release pro-inflammatory cytokines, and is a known activator of oligodendrocyte cell death in mixed cultures (Li et al., 2005). Cell death analysis by TUNEL assay showed that addition of conditioned media from LPS activated macrophages to OLN-93 cells significantly increased cell death compared to control conditioned media (Fig. III.V8A-D; N=10 images/condition; p<0.0001). Moreover, addition of creatine (100ng/ml) to the LPS conditioned media resulted in a dramatic reduction of OLN-93 cell death compared to PBS addition (Fig. III.8A-B; N=10 images/condition; p=0.0008), suggesting that creatine has a direct pro-survival effect on oligodendrocytes. We next examined the pro-survival effect of creatine on primary oligodendrocytes by treating mixed glia cultures with PBS, LPS, or LPS and creatine followed by co-immunostaining analysis with antibodies against PDGFRα, CC1, and Olig2. Compared to PBS, LPS treatment significantly reduced the proportion of CC1+Olig2+ mature oligodendrocytes (Fig. III.8E, white bars; N=10 images/condition; p=0.0258). This effect was abrogated by co-treatment with 100ng/ml creatine (Fig. III.8E, white bars; N=10 images/condition; p=0.0006). As previously demonstrated (Li et al., 2008; Skripuletz et al., 2011), LPS treatment resulted in a higher proportion of PDGFRα+Olig2+ OPCs compared to PBS (Fig. III.V8E, light gray bars; N=10 images/condition; p=0.019), an effect that was abrogated by co-treatment with creatine (Fig. III.V8E, light gray bars; N=10 images/condition; p=0.017). Interestingly, while LPS increased apoptosis of both OPCs (Fig. III.8F, N=8 images/condition; p=0.016) and oligodendrocytes (Fig. III.8G; N=10 images/condition; p=0.0482), co-treatment with creatine specifically ameliorated apoptosis of oligodendrocytes (Fig. III.8G; N=10 images/condition; p=0.0179). The proportion of apoptotic OPCs in LPS+CR cultures remained elevated compared to PBS (Fig. III.8F; N=10 images/condition; p=0.010). The simultaneous
Figure III.8. Creatine promotes oligodendrocyte cell survival following inflammatory insult. 

**A**, Percentage of dying OLN cells (TUNEL\(^*\)Hoechst\(^*\)) out of total cells (Hoechst\(^*\)) following treatment. OLN-93 cells were treated PBS or CR for 24hr while simultaneously exposed to conditioned media from RAW cells treated with either PBS or LPS the day prior. N=10 images/condition; one-way ANOVA with Bonferroni post-hoc test. 

**B-D**, TUNEL staining (magenta) depicting dying OLN-93 cells (TUNEL\(^*\)Hoechst\(^*\)) in cultures treated with PBS RAW cell conditioned media (cm) + PBS (**B**), LPScm + PBS (**C**), or LPScm + CR (**D**). 

**E**, Percentage of total Olig2\(^+\) cells (dark gray) represented by OPCs (PDGFR\(\alpha\)Olig2\(^+\); light gray) and mature oligodendrocytes (CC1\(^+\)Olig2\(^+\); white) following 24hr treatment with PBS, LPS, or LPS\(^+\)CR. N=10 images/condition; one-way ANOVA with Bonferroni post-hoc test. 

**F**, Percentage of dying OPCs (TUNEL\(^+\)PDGFR\(\alpha\)) out of total OPCs (PDGFR\(\alpha\)) following 24hr treatment with PBS, LPS, or LPS\(^+\)CR. N=10 images/condition; one-way ANOVA with Bonferroni post-hoc test. 

**G**, Percentage of dying oligodendrocytes (TUNEL\(^+\)CC1\(^+\)) out of total oligodendrocytes (CC1\(^+\)) following 24hr treatment with PBS, LPS, or LPS\(^+\)CR. N=10 images/condition; one-way ANOVA with Bonferroni post-hoc test. 

**H-J**, Immunostaining for mature oligodendrocytes (CC1\(^+\)Olig2\(^+\)) and OPCs (PDGFR\(\alpha\)Olig2\(^+\)) in primary mouse mixed glial cultures treated with PBS (**H**), LPS (**I**), or LPS and CR (**J**) for 24hr. Data are represented as mean ± SEM. Scale bars represent 50\(\mu\)m. Brightness and contrast adjusted for visualization. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
increase in OPC proportion and apoptosis is mediated by increased OPC proliferation under LPS treatment (N=10 images/condition; p=0.023; data not shown), suggesting that the OPC response is largely homeostatic. Overall, these results further suggest that creatine promotes oligodendrocyte survival under pro-inflammatory conditions. As numerous studies indicate a critical role for mitochondrial dysfunction in mediating the toxicity of LPS (Bullón et al., 2015; Hunter et al., 2007; Kuwabara & Imajoh-Ohmi, 2004; Mao et al., 2013; Voloboueva et al., 2013), we measured the effect of creatine treatment on ATP production in mixed glia cultures both before and after exposure to LPS. Mixed glia cultures were treated with magnesium-green (Mg-Gr), a cell-permanent compound that binds free Mg+ in solution. As ATP has a much higher affinity for Mg+ than Mg-Gr, lower Mg-Gr intensities indicate higher ATP levels. Live-cell imaging and quantification of average Mg-Gr intensity showed increased ATP levels in mixed glia treated with creatine for 24hr compared to PBS-treated mixed glia (Fig. III.9A; N=4 fields of view/condition). Following addition of LPS, ATP levels in creatine-treated mixed glia remained statistically higher than PBS-treated mixed glia for approximately 20mins (Fig. III.9A; N=4 fields of view/condition). Interestingly, ATP levels gradually increased over time in PBS-treated mixed glia cultures, reaching statistical significance approximately 7.5mins after LPS addition (N=4 fields of view/condition; two-way ANOVA with Bonferroni’s multiple comparisons test). In contrast, ATP levels in creatine-treated cultures remained stable over the entire imaging period.
Figure III.9. Creatine-treated mixed glia exhibit increased ATP levels under basal and inflammatory conditions. A-B, Example images of PBS (A) and CR-treated (B) mixed glia cells labeled with magnesium green (Mg-Gr) before (basal) and after LPS addition (inflammatory response). C, Average MgGr intensity of mixed glia cultures imaged using time-lapse microscopy. N=4 fields of view/condition; one-way ANOVA with Bonferroni post-hoc test. Note: Inverse relationship exists between Mg-Gr Intensity and ATP concentration. Data are represented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
VII. *Gamt*-Deficient Mice Exhibit Reduced Oligodendrocytes in Lesions Following Focal Spinal Cord Demyelination

We next asked if creatine-deficiency *in vivo* impairs oligodendrocyte viability by analyzing mice lacking the creatine-synthesizing enzyme, GAMT (*Gamt<sup>−/−</sup*>). These mice are completely deficient in endogenously synthesized creatine, but do not display the severe learning deficits characteristic of human creatine deficiency (Schmidt et al., 2004). We selected this model to ensure absence of peripherally synthesized creatine in the CNS (Schmidt et al., 2004; Torremans et al., 2005) and because oligodendrocytes are the only CNS cell type expressing high levels of *Gamt* (Zhang et al., 2014; Fig. III.1B). Western blot analysis demonstrates that GAMT protein is undetectable in cortical (Fig. III.10C; N=3 mice/condition; p=0.0136) and cerebellar (Fig. III.10B,C; N=3 mice/condition; p=0.0062) protein lysates of *Gamt<sup>−/−</sup>* mice compared to their WT counterparts. Moreover, we found that *Gamt<sup>−/−</sup>* mice had normal expression of oligodendrocyte lineage-specific proteins (Olig2 and MBP) at postnatal day (P) 30 (Fig. III.10D-E). Interestingly, oligodendrocyte lineage-specific proteins appear to be reduced at P2-3 and P17-20 in *Gamt<sup>−/−</sup>* mice (Fig. III.11A). Further, while there is a significant correlation between GAMT protein level and levels of MBP and Olig2 at P17 (Fig. III.11B), this correlation dissipates by P30 (Fig. III.11C). These results suggest that myelination may be delayed in *Gamt<sup>−/−</sup>* mice, similarly to the hypomyelination phenotype reported in clinical findings (Schulze et al., 1997; Bianchi et al., 2000).

In order to investigate whether creatine plays a role in oligodendrocyte survival during remyelination *in vivo*, we analyzed a previously published remyelination transcriptome from the
Figure III.10. *Gamt*-deficient mice exhibit normal myelin protein levels at P30. 

*A*, Example RT-PCR result demonstrating the identification of *Gamt* wild-type (WT), heterozygous (HET), and knockout (KO) genotypes. 

*B*, Representative immunoblot of cerebellar protein lysates demonstrating lack of GAMT protein in *Gamt*-deficient (KO) mice compared to *Gamt* wild-type (WT) mice. 

*C*, Densitometry analysis for quantification of GAMT protein expression normalized to β-Actin at P30 (n=3 mice/genotype). 

*D*, Densitometry analysis for quantification of MBP protein expression normalized to β-Actin at P30 (n=3 mice/genotype). 

*E*, Densitometry analysis for quantification of Olig2 protein expression normalized to β-Actin at P30 (n=3 mice/genotype). 

Data are represented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
Fig. III.11. *Gamt*-deficiency is associated with delayed expression of oligodendrocyte-specific proteins. **A**, Representative immunoblot showing expression of Olig2, PLP, MBP, and β-Actin in WT and KO mice at postnatal day (P) 3, P17-20, and P30. **C**, Scatter plot demonstrating the high correlation between normalized GAMT protein level and MBP (black) and Olig2 (gray) levels at postnatal day 17. **D**, Scatter plot demonstrating the low correlation between normalized GAMT protein level and MBP (black) and Olig2 (gray) levels at postnatal day 30.
rat CNS (Huang et al., 2011). Similar to other highly expressed oligodendrocyte genes, including g-protein coupled receptor 37 (Gpr37) and myelin basic protein (Mbp), Gamt was significantly differentially upregulated during oligodendrocyte differentiation and remyelination at 14 and 28 days post lesion (dpl), respectively (Fig. III.12A, p=0.0019; Huang et al., 2011). Considering the time course of this upregulation, we hypothesized that Gamt deficient oligodendrocytes would have impaired viability during remyelination. To test this, we utilized a mouse model of focal demyelination in which the toxin lysolecithin is microinjected into the mouse spinal cord white matter. This model was selected due to the known lesion location and well documented time course in which cell death and inflammation, oligodendrocyte differentiation, and remyelination occur at approximately 5, 10, and 20dpl, respectively (Arnett et al., 2004; Bieber et al., 2003; Pavelko et al., 1998). Focal spinal cord lesions were conducted on 9-12 week old Gamt+/+ and Gamt−/− mice and lesions were identified by focal accumulation of Hoechst+ nuclei, thought to represent an influx of inflammatory cells to the injury site. Gamt deficiency did not affect Nkx2.2+Olig2+, CC1+Olig2+, or Olig2+ density in normal appearing white matter (NAWM) adjacent to lesions (Fig. III.13A). While Gamt deficiency had no effect on the recruitment of Nkx2.2+Olig2+ OPCs to the lesion across all three post lesion timepoints examined (Fig. III.13B-D), significantly fewer CC1+Olig2+ oligodendrocytes were present in Gamt−/− lesions at 10dpl (Fig. III.13C,E; N=3 mice/condition; p=0.003) and 20dpl (Fig. III.13D; N=3 mice/condition; p=0.0185), suggesting that Gamt deficiency impaired oligodendrocyte differentiation or reduced survival of newly regenerated oligodendrocytes. Interestingly, co-injection of 25ng creatine with lysolecithin into Gamt−/− animals at the time of surgery (GAMT-KO+CR) led to significantly more CC1+Olig2+ oligodendrocytes in the lesion at 10dpl compared to Gamt−/− animals co-
Figure III.12. *Gamt* is differentially upregulated during oligodendrocyte differentiation and myelination following focal demyelination. A, Relative expression of *Gamt* at 5, 14, and 28dpl following ethidium bromide (EtBR)-mediated experimental demyelination of rat cerebellar peduncle (Huang et al., 2011).
Figure III.13. Reduced oligodendrocyte density in G amt-deficient focal demyelinating lesions can be rescued by creatine injection. A, Quantification of immunostainings for OPCs (Nkx2.2^+Olig2^+), oligodendrocytes (OL; CC1^+Olig2^+), and total oligodendrocyte lineage cells (Lineage; Olig2^+) per mm^2 in normal appearing white matter (NAWM) in G amt^+/− (G AMT-WT) and G amt^-/- (G AMT-KO) mice. B-D, Quantification of immunostainings for OPCs and OLs per mm^2 at 5 (B), 10 (C), and 20dpl (D) in G AMT-WT, G AMT-KO, and G amt^-/- mice co-injected with 25ng creatine (G AMT-KO+CR; 10dpl only). N=3 mice/condition; one-way ANOVA with Bonferroni post-hoc test performed in C, Student’s T-Test performed in D. E, Representative immunostainings of mature oligodendrocytes double positive for Olig2 (magenta) and CC1 (green) at 10dpl. Data are represented as mean ± SEM. Long scale bars represent 100µm. Short scale bars represent 25µm. Brightness and contrast adjusted for visualization. *P<0.05, **P<0.01.
injected with PBS (Fig. III.13C,E; N=3 mice/condition; p=0.008), suggesting that creatine treatment is able to rescue oligodendrocyte density in *Gamt* deficient lesions.

The robust pro-survival effect of creatine *in vitro* led us to hypothesize that this reduction may be the result of impaired oligodendrocyte viability. To quantify oligodendrocyte cell death in lesions, co-immunostaining analysis for CC1 and cleaved caspase-3 (Clv-Csp3), a critical executioner protein involved in oligodendrocyte cell death (Casaccia-Bonnefil, 2000), was performed. While the number of Clv-Csp3⁺CC1⁺ oligodendrocytes was not different between groups at 5dpl (data not shown), it was significantly increased in *Gamt⁻/⁻* lesions at 10dpl (Fig. III.14A-C; N=3 mice/condition; p=0.001). Compared to co-injection with PBS, co-injection of creatine reduced the number of Clv-Csp3⁺CC1⁺ oligodendrocytes in *Gamt⁻/⁻* lesions by 47% (Fig. III.14A,D; N=3 mice/condition; p=0.004). Thus, despite regenerating from OPCs, oligodendrocytes within *Gamt* deficient lesions are unable to survive in the absence of creatine.

To investigate whether changes in inflammation contributed to altered oligodendrocyte viability in lesions, we quantified the density of CD11b⁺ macrophages/microglial cells at 5dpl and 10dpl. We found no differences between WT and *Gamt⁻/⁻* mice at 5 or 10dpl (Fig. III.14E-G). It is possible to alter the balance of pro-inflammatory and anti-inflammatory macrophages/microglia without affecting the overall number of CD11b⁺ cells. Therefore, we quantified the densities of YM1⁺CD11b⁺ and iNOS⁺CD11b⁺ cells, generally considered to represent pro-inflammatory/classically-activated and anti-inflammatory/alternatively-activated macrophages/microglia, respectively, although these cells can acquire a diverse range of activation programs depending on the external stimuli received (Katsumoto et al., 2014). We observed no differences in the YM1⁺:iNOS⁺ ratio (Fig. III.15A), density of YM1⁺ cells
Figure III.14. *Gamt*-deficient oligodendrocytes exhibit reduced survival following focal spinal cord demyelination. 

**A**, Quantification of immunostaining showing the proportion of oligodendrocytes positive for cleaved caspase-3 (Clv-Csp3⁺CC1⁺) out of total oligodendrocytes (CC1⁺) at 10dpl in *Gamt*⁺/⁺ (WT), *Gamt*⁻/⁻ (KO), and *Gamt*⁻/⁻ mice treated with 25ng creatine (KO+CR). N=3 mice/condition; one-way ANOVA with Bonferroni post-hoc test. 

**B-D**, Representative immunostainings of dying oligodendrocytes in GAMT-WT (**B**), GAMT-KO (**C**), and GAMT-KO+CR (**D**) lesions at 10dpl. White arrows indicate cells double positive for Clv-Csp3 and CC1. Scale bars represent 25µm. 

**E**, Quantification of immunostaining for CD11b⁺ cells/mm² in GAMT-WT and GAMT-KO lesions at 5dpl and 10dpl. N=3 mice/condition. 

**F-G**, Representative immunostainings of macrophages/microglia (CD11b⁺; magenta) in WT (**F**) and KO (**G**) lesions at 5dpl. Scale bars represent 50µm. Data are represented as mean ± SEM. Brightness and contrast adjusted for visualization. *P<0.05, **P<0.01.
Figure III.15. *Gamt*-deficient mice exhibit normal pro- and anti-inflammatory macrophage/microglia densities following focal spinal cord demyelination. **A**, Ratio of anti-inflammatory (YM1⁺) to pro-inflammatory (iNOS⁺) macrophages/microglia in lesions of *Gamt*⁺/⁺ (WT) and *Gamt*⁻/⁻ (KO) mice at 5dpl. N=2 mice/condition. **B-C**, Quantification of immunostainings for YM1⁺CD11B⁺ anti-inflammatory macrophages/microglia per mm² (B) and iNOS⁺CD11B⁺ pro-inflammatory macrophages/microglia per mm² (C) at in WT and KO mice 5dpl. N=2 mice/condition. **D-E**, Representative immunostainings of YM1⁺CD11B⁺ (D) and iNOS⁺CD11B⁺ (E) macrophages/microglia at 5dpl in WT and KO lesions. Scale bars represent 100µm. Brightness and contrast adjusted for visualization. Data are represented as mean ± SEM.
(Fig. III.15B,D), or density of iNOS+ cells (Fig. III.15C,E) between WT and Gamt−/− mice at 5dpl, suggesting that GAMT does not affect macrophage/microglia number in lesions and is required specifically for oligodendrocyte viability. To determine whether increased oligodendrocyte death in Gamt−/− mice was associated with changes in mitochondria, we conducted immunostaining for the mitochondrial-associated proteins, Tom20 and VDAC (voltage-dependent anion channel). We observed no changes in the average fluorescent intensity of Tom20 (Fig. III.16A,B) or VDAC (Fig. III.16C,D) in Gamt−/− lesions at 10dpl. Unfortunately, the resolution of these mitochondrial markers is not sufficient for intracellular quantification, thus it remains unknown whether oligodendrocyte mitochondria are specifically affected in Gamt−/− mice.

VIII. Creatine Administration Increases Oligodendrocyte Density Following Focal Spinal Cord Demyelination

Creatine has proven safe and well tolerated in clinical trials (Malin et al., 2008; Rosenfeld et al., 2008), however its effect on oligodendrocyte survival in MS remains unknown. To determine if creatine administration promotes oligodendrocyte survival in CNS lesions, spinal cords of WT mice were co-injected with lysolecithin and either PBS or creatine (25ng). We found that creatine had no effect on the number of Nxk2.2+Olig2+ OPCs at any of the post lesion timepoints examined (Fig. III.17A; N=3 mice/condition), but significantly increased the density of CC1+Olig2+ oligodendrocytes at both 5 and 10dpl (Fig. III.17B,C; N=3 mice/condition; p=0.0254, p=0.0022). The observation that creatine-treated mice exhibited increased oligodendrocyte density in lesions by 5dpl (Fig. III.17C), prior to the expected time of oligodendrocyte differentiation, suggests that creatine may promote survival of existing...
Fig. III.16. Expression of Tom20 and VDAC in Gamt-deficient lesions. A, Representative immunostainings of Tom20 in Gamt+/- (WT) and Gamt-- (KO) lesions at 10dpl. B, Quantification of average Tom20 intensity within the lesion. N=2 mice/condition. C, Representative immunostainings of VDAC in Gamt+/- (WT) and Gamt-- (KO) lesions at 10dpl. D, Quantification of average VDAC intensity within the lesion. N=2 mice/condition. Data are represented as mean ± SEM. Scale bars represent 100µm. Brightness and contrast adjusted for visualization.
Figure III.17. Creatine administration enhances oligodendrocyte restoration following focal spinal cord demyelination. \( A-B \), Quantification of immunostaining for Nkx2.2\(^+\)Olig2\(^+\) OPCS per mm\(^2\) (\( A \)) and CC1\(^+\)Olig2\(^+\) oligodendrocytes per mm\(^2\) (\( B \)) at 5, 10, and 20dpl in PBS-treated (PBS) and creatine-treated (CR) mice. N=3 mice/condition; Student’s T-Test. \( C \), Representative immunostaining of mature oligodendrocytes double-positive for Olig2 (magenta) and CC1 (green) in PBS and CR lesions at 5dpl. Data are represented as mean ± SEM. Scale bars represent 100µm. Brightness and contrast adjusted for visualization. N=3 mice/condition; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
oligodendrocytes within the lesion. Indeed, we observed that creatine-treated mice had significantly fewer Clv-Csp3+ oligodendrocytes at 5dpl compared to PBS-treated mice (Fig. III.18A,B; N=3 mice/condition; p=0.0051), demonstrating a role for creatine in the inhibition of caspase-dependent oligodendrocyte apoptosis. Moreover, we detected increased MBP staining in lesions at 20dpl in creatine treated mice compared to control, suggesting that creatine-mediated oligodendrocyte survival enhanced CNS remyelination (Fig. III.18C,D; N=3 mice/condition; p=0.0019). Creatine did not affect the density of inflammatory macrophages/microglia at 5dpl or 10dpl (Fig. III.18E,F). In addition, there were no differences in the YM1+:iNOS+ ratio (Fig. III.19A), density of YM1+ cells (Fig. III.19B,D), or density of iNOS+ cells (Fig. III.19C,E) between PBS and CR-treated mice at 5dpl. Therefore, changes in inflammation are unlikely to mediate the beneficial effect of creatine treatment. This result suggests that creatine administration can directly enhance oligodendrocyte survival in the CNS. To determine whether creatine promotes survival of newly generated oligodendrocytes in CNS lesions, PBS and CR-treated mice were injected twice daily from 1-4dpl with 25mg/kg EdU (Fig. III.20A), a nucleoside analog of thymidine that becomes incorporated into DNA during active DNA synthesis. The co-localization of EdU with CC1, a marker for mature oligodendrocytes, indicates a newly generated oligodendrocyte. Interestingly, we observed a significant increase in the density of EdU+CC1+ cells in CR-treated lesions at 5dpl (Fig. III.20B,C; N=2 mice/condition; p=0.0365), suggesting that creatine promotes survival of newly generated oligodendrocytes at the peak of inflammation.
Figure III.18. Creatine administration enhances oligodendrocyte survival following focal spinal cord demyelination. 

A, Representative immunostainings of dying oligodendrocytes in PBS-treated (PBS) and creatine-treated (CR) lesions at 5dpl. White arrows indicate cells double-positive for Clv-Casp3 and CC1. Scale bars represent 50µm. 

B, Proportion of oligodendrocytes positive for cleaved caspase-3 (Clv-Casp3⁺/CC1⁺) out of total oligodendrocytes (CC1⁺) in PBS and CR lesions at 5dpl. N=2 mice/condition; Student’s T-Test.

C, Representative immunostainings of myelin basic protein (MBP) in PBS-treated (PBS) and creatine-treated (CR) lesions at 20dpl. Scale bars represent 100µm. 

D, Average MBP intensity per µm² in PBS and CR lesions at 20dpl. N=3 mice/condition; Student’s T-Test.

E, Quantification of immunostaining for CD11b⁺ cells/mm² in PBS and CR lesions at 5dpl and 10dpl. N=3 mice/condition; Student’s T-Test.

F, Representative immunostainings of macrophages/microglia (CD11b⁺; magenta) in PBS and CR lesions at 5dpl. Data are represented as mean ± SEM. Scale bars represent 50µm. Brightness and contrast adjusted for visualization. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
Figure III.19. Creatine-treated mice exhibit normal pro- and anti-inflammatory macrophage/microglia densities following focal spinal cord demyelination. A, Ratio of anti-inflammatory (YM1⁺) to pro-inflammatory (iNOS⁺) macrophages/microglia in lesions of PBS and creatine (CR)-treated mice at 5dpl. N=2-3 mice/condition. B-C, Quantification of immunostainings for YM1⁺CD11B⁺ anti-inflammatory macrophages/microglia per mm² (B) and iNOS⁺CD11B⁺ pro-inflammatory macrophages/microglia per mm² (C) at 5dpl in PBS and CR-treated mice. N=2-3 mice/condition. D-E, Representative immunostainings of YM1⁺CD11B⁺ (D) and iNOS⁺CD11B⁺ (E) macrophages/microglia at 5dpl in PBS and CR-treated mice. Scale bars represent 100µm. Brightness and contrast adjusted for visualization. Data are represented as mean ± SEM.
Figure III.20. Creatine administration enhances survival of newly generated oligodendrocytes following focal spinal cord demyelination. 

A, Experimental paradigm for detection of newly generated oligodendrocytes in mice treated with PBS or creatine (CR). Mice were given interperitoneal (IP) injections of 25mg/kg EdU at 11am and 4pm daily from 1-4dpl.

B, Representative immunostainings of newly generated oligodendrocytes (Edu⁺CC1⁺) in lesions at 5dpl. Scale bars represent 50µm.

C, Proportion of oligodendrocytes positive for EdU (EdU⁺CC1⁺) out of total oligodendrocytes (CC1⁺) in PBS and CR-treated lesions at 5dpl. N=2 mice/condition; Student’s T-Test. Data are represented as mean ± SEM. Brightness and contrast adjusted for visualization. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
B. DISCUSSION

The CNS utilizes 20% of the body’s energy (Mergenthaler et al., 2013), with the human cortex alone requiring \( \sim 3 \times 10^{23} \text{ ATP/s/m}^3 \) (Howarth et al., 2012). Creatine is thought to play a critical role in meeting this energy demand by allowing for rapid ATP regeneration in the cytoplasm (Wyss and Kaddurah-Daouk, 2000). Within the CNS, oligodendrocytes have a preferentially high capacity for creatine synthesis (Braissant et al., 2001; Cahoy et al., 2008; Tachikawa et al., 2004; Zhang et al., 2014), suggesting that they may be a major source of creatine in the brain. Therefore, we investigated the role of creatine in oligodendrocytes using both \textit{in vitro} and \textit{in vivo} approaches.

In purified oligodendrocyte lineage cells, creatine directly increased oligodendrocyte mitochondrial density, membrane potential, and ATP production. Surprisingly, enhanced mitochondrial function in creatine-treated oligodendrocytes did not increase membrane expansion or differentiation, but rather promoted oligodendrocyte survival. Creatine treatment of primary mixed glia cultures for 48hr significantly reduced oligodendrocyte cell death. Interestingly, OPC proliferation was also reduced in creatine-treated cultures. Despite these underlying changes, no differences were observed in the overall proportions of OPCs and oligodendrocytes at either 24 or 48hr treatment. As we did not directly quantify O4\(^+\)MBP\(^-\) pre-oligodendrocytes, we cannot rule out the possibility that creatine may have affected the proportion of this intermediate population. However, at the experimental endpoint, O4\(^+\) highly overlaps with MBP\(^+\) in our cultures, making robust quantification of this population difficult. Inclusion of additional time points in the 24 to 48hr treatment window could address this question in future studies. The simultaneous changes in oligodendrocyte death and OPC
proliferation suggest an active mechanism for maintaining homeostatic density of oligodendrocytes reminiscent to that recently described *in vivo* (Hughes et al., 2013).

By contrast, acute (24hr) LPS-mediated inflammatory injury significantly altered the proportions of oligodendrocyte lineage cells. LPS treatment reduced the proportion of oligodendrocytes and concomitantly increased oligodendrocyte apoptosis, both of which were returned to control levels by co-treatment with creatine. As previously reported, we found that OPCs exhibit both increased apoptosis and proliferation in response to LPS treatment (Li et al., 2008; Skripuletz et al., 2011). However, creatine did not significantly affect either of these parameters, indicating that changes in OPC proportion are a reflection of oligodendrocyte loss rather than a direct effect of creatine on OPCs. It is interesting that creatine did not reduce the level of oligodendrocyte apoptosis below PBS in this experiment. This effect may be due to the acute timing of LPS treatment (24hr) rather than different underlying mechanisms of inflammatory and non-inflammatory cell death *in vitro*, as previous work suggests that oligodendrocytes undergo apoptosis, both as a normal turnover response, and as a result of cytokine or glutamate-induced excitotoxicity in the presence of activated microglia (Aktas et al., 2006; Barres et al., 1992; Matute et al., 2006).

To determine whether creatine also affected oligodendrocyte survival *in vivo*, we conducted loss of function experiments by performing focal spinal cord demyelination on *Gamt* deficient mice and quantifying the process of spontaneous oligodendrocyte regeneration. Lesions of *Gamt* deficient mice contained fewer total oligodendrocytes and a higher proportion of cleaved caspase-3-positive oligodendrocytes compared to controls. Injection of creatine at the time of
surgery rescued the detrimental effect of *Gamt* deficiency on oligodendrocyte apoptosis, suggesting that creatine is necessary for survival of newly generated oligodendrocytes. Oligodendrocyte lineage cell numbers were not different in the NAWM of *Gamt* deficient mice, suggesting that survival of the oligodendrocyte lineage is either not affected during development, or that compensatory mechanisms allow for normalization of these densities over time.

Further supporting a role for creatine in the survival of newly generated oligodendrocytes, lesions treated with exogenous creatine at the time of injury contained more oligodendrocytes at 5 and 10dpl due to a reduction in caspase-mediated oligodendrocyte cell death. Interestingly, by 20dpl, PBS-treated lesions contain similar numbers of oligodendrocytes as those treated with creatine. Thus, it appears that creatine serves to promote oligodendrocyte survival early in the injury time course when inflammation remains high. Creatine treatment was also associated with elevated MBP expression, suggesting that creatine may enhance myelin synthesis or speed up the spontaneous process of myelin regeneration *in vivo*. Addition of exogenous creatine, or loss of *Gamt* did not affect the recruitment of inflammatory cells, suggesting that creatine directly promotes oligodendrocyte viability.

Despite the high expression of creatine-synthesizing enzymes in oligodendrocytes (Braissant et al., 2010; Tachikawa et al., 2004; Takasaki et al., 2010; Zhang et al., 2014), its physiological importance in these cells had not been investigated. Our results suggest a model in which creatine promotes the viability of newly generated oligodendrocytes by enhancing mitochondrial function (Fig. III.21). Several mechanisms of creatine-mediated protection have been proposed. Creatine can serve as a direct antioxidant (Lawler et al., 2002), and reduces markers of oxidative
Figure III.21. Hypothesized model for creatine-mediated protection of oligodendrocytes. Creatine treatment promotes oligodendrocyte restoration following demyelinating injury by inhibiting caspase-dependent oligodendrocyte death. Although the precise mechanism remains to be elucidated, it is hypothesized that creatine activation of mitochondrial creatine kinase (MtCK) may directly inhibit apoptotic initiation.
stress in rodent models of neurological insult (Hosamani et al., 2010; Saraiva et al., 2012; Cunha et al., 2013; Rambo et al., 2013). Creatine also inhibits loss of mitochondrial membrane potential (Rambo et al., 2013), which has been shown to precede initiation of cellular apoptosis (Kroemer et al., 2007). Additionally, creatine activation of MtCK directly inhibits opening of the mitochondrial permeability transition pore (mPTP), an early apoptotic event concomitant with cytochrome-c (cyt-c) release (Beutner et al., 1998; Beutner et al., 1996; O’Gorman et al., 1997; Schlattner et al., 2006; Vyssokikh & Brdiczka, 2003). Our loss and gain-of-function studies yielded complementary results demonstrating that creatine increases oligodendrocyte density during CNS regeneration by inhibiting caspase-dependent cell death of mature oligodendrocytes. Although caspase-3 can be activated by both intrinsic (mitochondrial) and extrinsic (death receptor) pathways, our finding that creatine directly increases mitochondrial membrane potential and ATP production in oligodendrocyte lineage cells suggests that creatine protection is mediated by a mitochondrial-dependent mechanism. Future studies would be necessary to address whether creatine activation of MtCK promotes oligodendrocyte viability.

Oligodendrocyte death plays a crucial role in the pathology of MS (Matute et al., 2006; Macchi et al., 2015). In demyelinated lesions, oligodendrocytes death may be achieved with, or without complement activation (Barnett & Prineas, 2004; Lucchinetti et al., 2000). Moreover, oligodendrocytes can undergo either apoptosis or necrosis, depending on how mitochondrial function is affected (Casaccia-Bonnefil, 2000). Interestingly, many oligodendrocytes appear to survive demyelination in chronic-stage MS, but are lost from lesioned areas gradually over time (Wolswijk, 2000), suggesting that their survival may be compromised under chronic inflammation. Preventing oligodendrocyte cell death in MS is particularly important considering
that current therapies do not prevent transition into secondary-progressive disease (Huang and Franklin, 2012), in which chronic demyelination is thought to contribute to neurodegeneration (Jeffery and Blakemore, 1997; Kornek et al., 2000; Irvine and Blakemore, 2008). In addition, the past few decades have demonstrated novel roles for oligodendrocytes in maintaining neuronal health and integrity (Fünfschilling et al., 2012; Lee et al., 2012; Nave & Trapp, 2008), underscoring the importance of maintaining the oligodendrocyte-axon connection. Our work demonstrates that creatine promotes survival of oligodendrocytes under inflammatory conditions both in vitro and in vivo. It is therefore exciting to consider creatine as a potential treatment strategy for protecting oligodendrocytes in patients with MS. Dietary creatine supplementation has proven safe and well tolerated in clinical trials (Kahler and Fahey, 2003). It has also been demonstrated to improve brain performance (Rae et al., 2003) and protect neurons during oxygen deprivation (Turner et al., 2015). Thus, future work would be needed to elucidate whether dietary creatine supplementation, or administration of creatine analogs can protect oligodendrocytes in both the lab and clinic.

In summary, we have found a novel role for creatine in promoting oligodendrocyte viability. Our results suggest that creatine may be a potentially relevant therapeutic agent for promoting oligodendrocyte survival in MS.
CHAPTER IV: CREATINE MODULATES AXONAL MITOCHONDRIAL DYNAMICS
Individual neurons acquire complex morphologies as they transform from immature, neuronal spheres to mature, polarized neurons. This process is characterized by sequential sprouting, elongation, and branching of neurites, cylindrical processes with growth cones at the tips, which later become identified as dendrites or axons (Da Silva and Dotti, 2002). While morphological maturation is highly energetically demanding, maintenance of the mature neuronal structure also requires significant energy supplies to power action potentials, axonal transport, maintenance of the ionic gradient and synaptic compartments (Schwarz, 2013). In fact, even at rest, cortical neurons require an estimated 4.7 billion ATP molecules per second (Zhu et al., 2012).

A crucial component in meeting this energy demand as well as buffering local Ca$^{2+}$ concentrations are mitochondria, intracellular organelles which generate large amounts of ATP via oxidative phosphorylation. Due to the unique morphology of neurons, which can extend axons up to a meter long in humans, neuronal mitochondria must be properly trafficked and placed within these compartmentalized structures in order to meet local energy demand (Schwarz, 2013). Neurons have evolved intricate mechanisms for orchestrating this finely tuned distribution of mitochondria in dendritic and axonal regions (Cai et al., 2011).

Mitochondria are attached to motor proteins that travel long ranges via microtubule trafficking through a complex of mitochondrial-specific linker proteins known as Milton and Miro (Schwarz, 2013). Molecular motors are broken up into two families- kinesin and dynein proteins. Kinesins are (+)-oriented molecular motors, meaning they move from the (-)- to (+)- ends of microtubules. As axonal microtubules are organized in linear arrays with the (+)-ends directing away from the cell body (Baas et al., 1988), kinesin-dependent movement occurs in the
anterograde direction. Dyneins are the (-)-oriented molecular motors that carry organelles towards the cell body, referred to as retrograde movement (Schwarz, 2013).

Interestingly, in both axons and dendrites, approximately 70-90% of mitochondria are held stationary for an extended period of time while only ~10-30% are motile (Cai et al., 2005; Hollenbeck and Saxton, 2005; Saxton and Hollenbeck, 2012). Stationary mitochondria appear to allow for local ATP generation at regions of high energetic demand. However, the balance between stationary and motile phases is critical for maintaining the overall health of the mitochondrial population (Schwarz, 2013; Sheng, 2014) and aberrant accumulation of stationary mitochondria is detrimental to neuronal health in diseases such as MS (Campbell et al., 2012; Ohno et al., 2014). How an individual mitochondrion switches its behavior from motile to stationary and vice versa is not well understood. Recently, an outer mitochondrial membrane protein known as syntaphilin (SNPH) was identified and discovered to have a microtubule binding domain that anchors mitochondria to the microtubules, thereby holding them stationary (Kang et al., 2008).

To alleviate stress on the mitochondrial system, neuronal cells utilize a form of ATP buffering known as creatine-phosphocreatine (PCr) shuttling (Ellington, 2001). Creatine is an organic acid that becomes phosphorylated to form high-energy PCr, which can rapidly donate its phosphate group to ADP for local regeneration of ATP (Wyss and Kaddurah-Daouk, 2000). The activity of creatine kinase increases nearly 5X from P0-P40 (Manos et al., 1991), suggesting that this system is crucial for neuronal development. Further, inborn errors of creatine metabolism are
characterized by severe neurological deficits (Kahler and Fahey, 2003; Anselm et al., 2006; Gordon, 2010; Barkovich, 2011).

Exogenous creatine is protective in models of neurodegeneration (Matthews et al., 1998, 1999; Klivenyi et al., 1999; Andres et al., 2005; Ducray et al., 2007; Juravleva et al., 2011), possibly due to its role in mitigating oxidative damage and mitochondrial dysfunction (Hosamani et al., 2010; Berti et al., 2012; Saraiva et al., 2012; Cunha et al., 2013; Rambo et al., 2013). However, precisely how creatine deficiencies stress the mitochondrial system in neurons remains unknown. Here, we utilized mice lacking the creatine-synthesizing enzyme, Gamt (Gamt<sup>−/−</sup>), to investigate how creatine affects neuronal mitochondrial dynamics. We demonstrate the Gamt deficiency impairs neuronal respiration and axonal mitochondrial dynamics, leading to reduced morphological complexity of neurons. Further, we suggest that oligodendrocyte-derived creatine is necessary for reducing the energetic demand placed on axonal mitochondria. Overall, this work implicates the creatine-PCr system as a potentially useful therapeutic target for normalizing mitochondrial dynamics in neurological diseases, including MS and other disorders of oligodendrocyte dysfunction.

A. RESULTS
I. Gamt-Deficient Neurons Exhibit Reduced Bioenergetic Capacity

As creatine plays an integral role in buffering intracellular ATP levels, we reasoned that creatine deficiency might lead to mitochondrial dysfunction in developing neurons. To quantify the effect of Gamt deficiency on neuronal mitochondrial function, we conducted Seahorse analysis on Gamt<sup>+/+</sup> and Gamt<sup>−/−</sup> cortical neurons early in development (DIV3) using the Seahorse XF Cell
Mito Stress Test. Compared to controls, Gamt-deficient neurons exhibited significantly reduced basal respiration (Fig. IV.1A; N=2 wells/condition; p=0.0221), however there was no significant difference in ATP production between Gamt<sup>+/+</sup> and Gamt<sup>/−</sup> neurons (Fig. IV.1B). Maximal respiration (Fig. IV.1C; N=2 wells/condition; p=0.0162) and non-mitochondrial respiration (Fig. IV.1D; N=2 wells/condition; p=0.0153) were both reduced in Gamt<sup>/−</sup> neurons compared to Gamt<sup>+/+</sup> neurons. Average spare respiratory capacity was reduced by 71% in Gamt<sup>/−</sup> neurons (Fig. IV.1D; N=2 wells/condition; p=0.0242). These findings suggest that mitochondrial respiration is deficient in Gamt<sup>/−</sup> neurons. Treatment with 100µM creatine was unable to restore respiration in Gamt<sup>/−</sup> neurons. Rather, Gamt<sup>/−</sup> neurons receiving creatine exhibited respiration levels in between those of control and Gamt<sup>/−</sup> neurons receiving PBS (Fig. IV.1A-C,E). Intriguingly, the only parameter that remained significantly different in Gamt<sup>/−</sup> neurons receiving creatine was non-mitochondrial respiration, which was reduced by ~80% compared to Gamt<sup>+/+</sup> neurons (Fig. IV.1D; N=2 wells/condition; p=0.0193).

II. Gamt-Deficiency Induces Movement of Axonal Mitochondria

To determine whether bioenergetic deficits associated with Gamt-deficiency resulted in long-lasting changes in mitochondrial dynamics, cortical neurons were cultured from Gamt<sup>+/+</sup> and Gamt<sup>/−</sup> mice. At DIV13, neurons were treated with PBS or 100µM creatine for 24hr. Mitochondria were simultaneously labeled with CellLight® Mitochondria-RFP (Life Technologies), a fusion construct of the alpha pyruvate dehydrogenase sequence and TagRFP that provides specific mitochondrial targeting independent of mitochondrial membrane potential. On DIV14, neurons with 3-5 primary branches were imaged by sequential acquisition of z-stacks every 30sec for 15min. Mitochondrial dynamics were analyzed solely in the axon, identified as
Figure IV.1. *Gamt*-deficient neurons exhibit reduced bioenergetic capacity. *A*, Results of Seahorse analysis using the Seahorse XF Cell Mito Stress Test to measure respiration in *Gamt*<sup>+/+</sup> (GAMT-WT) and *Gamt*<sup>−/−</sup> (GAMT-KO) cortical neurons treated with PBS or *Gamt*<sup>−/−</sup> neurons treated with creatine (CR) for 24hr at DIV3. Following measurement of oxygen consumption rate (pmol/min) during basal respiration (*A*), cells received sequential injections of oligomycin, FCCP, and Antimycin/Rotenone. Oligomycin inhibits ATP synthase and allows for measurement of mitochondrial ATP production (*B*). FCCP collapses the mitochondrial membrane gradient, which allows for complex IV to utilize O2 at its maximal rate, known as maximal mitochondrial respiration (*C*). Antimycin/Rotenone shut down complexes I and III, which allows for the measurement of non-mitochondrial respiration (*D*). Spare respiratory capacity (*E*) is the
difference between basal and maximal respiration and reflects the ability for mitochondria to meet increased energy demand. N=2 wells/condition; one-way ANOVA with Bonferroni post-hoc test. Data are represented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 in comparison to WT+PBS.
the longest neurite with a thin, uniform diameter and sparse branching pattern (Banker and Cowan, 1977). The 20-70µm region of the axon was selected for analysis (Fig. IV.2A). Kymographs of mitochondrial movement over time and space were generated in ImageJ (NIH) and used to quantify mitochondrial density and dynamics (Fig. IV.2B). There was no effect of Gamt-deficiency on axonal mitochondrial density (Fig. IV.2C). However, there were significantly more moving mitochondria, identified as any mitochondria moving more than 5µm (Fig. IV.2D,F; N=4-5 neurons/condition; p=0.0835 for D and p=0.0063 for F), and significantly fewer stationary mitochondria (Fig. IV.2E,G; N=4-5 neurons/condition; p=0.0062 for E and p=0.0026 for G) in Gamt+/− (KO) neurons compared to Gamt++ (WT). The effect of Gamt-deficiency on mitochondrial dynamics was rescued by treatment with 100uM creatine for 24hr (KO+CR), which returned numbers of moving mitochondria (Fig. IV.2F; N=4-5 neurons/condition; KO+PBS vs. KO+CR, p=0.0131) and stationary mitochondria (Fig. IV.2E,G; N=4-5 neurons/condition; KO+PBS vs. KO+CR, p=0.0026 for E and p=0.0131 for G) to control levels.

III. TNFα Induces Movement of Axonal Mitochondria

To determine whether increased mitochondrial movement, as observed in Gamt+/− neurons, is a generalized response to pathological conditions characterized by low-respiring mitochondria, we analyzed mitochondrial dynamics in wild-type neurons treated with tumor necrosis factor alpha (TNFα). TNFα is a signaling cytokine released by pro-inflammatory/classically-activated macrophages/microglia that can exert pathophysiological effects in the CNS (Olmos and Lladó, 2014). Concentrations as low as 10-1000pg of TNFα induce neuronal mitochondrial dysfunction by 1.5hr, as measured by reduced basal respiration during Seahorse XF analysis (Doll et al.,
Figure IV.2. *Gamt*-deficiency induces movement of axonal mitochondria. **A**, Representative images of mitochondria in the 20-70µm axonal region of DIV14 *Gamt*<sup>WT</sup> (GAMT-WT) and *Gamt*<sup>KO</sup> (GAMT-KO) cortical neurons treated with PBS or *Gamt*<sup>KO</sup> neurons treated with creatine (CR) for 24hr. **B**, Corresponding kymographs of mitochondrial movement for axons shown in **A**. Scale bar represents 4µm. **C-G**, Average axonal mitochondrial density (**C**), percentage of moving mitochondria (**D**), percentage of stationary mitochondria (**E**), number of moving mitochondria per 50µm axon (**F**), and number of stationary mitochondria per 50µm axon (**G**). N=5 axons/condition; Student’s T-Test. Data are represented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
To quantify the effect of mitochondrial dysfunction resulting from TNFα exposure on axonal mitochondrial movement, we treated DIV8 cortical neurons with PBS or 1000pg/mL TNFα for 2hr and measured mitochondrial dynamics as described above. TNFα-treated axons exhibited significantly more moving mitochondria (Fig. IV.3D,F; N=5 neurons/condition; p=0.0275 for D and p=0.0486 for F) and significantly fewer stationary mitochondria (Fig. IV.3E,G; N=5 neurons/condition; p=0.0362 for E and p=0.0508 for G) compared to PBS-treated controls. Total mitochondrial density was not affected by TNFα treatment (Fig. IV.3C). These results suggest that proportional increases in motile mitochondria without changes in the overall density may be a compensatory response to low-respiring mitochondria in the axon.

IV. Gamt-Deficient Neurons Exhibit Reduced Morphological Complexity

Although human creatine deficiencies are associated with severe neurological problems (Kahler and Fahey, 2003; Anselm et al., 2006; Gordon, 2010; Barkovich, 2011), neuronal morphology has not been quantified in existing animal models. As the process of neuritogenesis and branching is energy demanding and requires regional immobilization of mitochondria (Ruthel and Hollenbeck, 2003; Courchet et al., 2013), we hypothesized that the combined bioenergetic deficit and decreased proportion of immobilized mitochondria observed in Gamt-deficient neurons might impair neuritogenesis. Cortical neurons were cultured from Gamt+/+ and Gamt−/− mice and NeuronJ, an ImageJ (NIH) plugin designed for tracing and quantifying elongated neurites (Meijering et al., 2004), was used to quantify their morphological complexity at DIV14. We found that compared to wild-type neurons, Gamt−/− neurons had significantly reduced morphological complexity (Fig. IV.4). Gamt−/− neurons had fewer neurites overall (Fig. IV.4A,B; N=10 neurons/condition; p=0.0087). This reduction primarily reflected fewer secondary (Fig.
Figure IV.3. TNFα induces movement of axonal mitochondria. A, Representative images of mitochondria in the 20-70µm axonal region of DIV8 cortical neurons treated with PBS or tumor necrosis factor alpha (TNFα). B, Corresponding kymographs of mitochondrial movement for axons shown in A. Scale bar represents 4µm. C-G, Average axonal mitochondrial density (C), percentage of moving mitochondria (D), percentage of stationary mitochondria (E), number of moving mitochondria per 50µm axon (F), and number of stationary mitochondria per 50µm axon (G). N=5 axons/condition; Student’s T-Test. Data are represented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
Figure IV.4. *Gamt*-deficient neurons exhibit reduced morphological complexity. 

A. Traces of representative neurons from *Gamt*+/+ (GAMT-WT) and *Gamt*−/− (GAMT-KO) mixed cortical cultures at DIV14. 

B. Average number of neurites of WT and KO neurons; N=10 neurons/condition; Student’s T-Test. 

C-E. Average number of primary (C), secondary (D), and tertiary (E) neurites of WT and KO neurons; N=10 neurons/condition; Student’s T-Test. 

F. Example image of concentric circles overlaying a neuronal trace used to quantify neuronal morphology. 

G. Average number of intersections of WT and KO neurons at binned radii (µm) from the cell body; N=10 neurons/condition; Student’s T-Test. Data are represented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
IV. Creatine Reduces the Density of Axonal Mitochondria

It has previously been demonstrated that creatine increases mitochondrial membrane potential and alters mitochondrial movement in *Xenopus* spinal neurons (Lee and Peng, 2008). Therefore, we investigated the effect of creatine on axonal mitochondrial dynamics in wild-type cortical neurons. Mitochondria were labeled with CellLight Mitochondria-RFP™ on DIV7 for 24hr. On DIV8, cells were treated with PBS for 2hr and imaged every 30sec for 30 cycles (PBS). Cells were then treated with 500uM creatine for 2hr, after which the media was replaced and the same neurons were reimaged as described above (CR). Kymographs from the 20-70µm region of the axon were analyzed for mitochondrial movement (Fig. IV.5A,B). Creatine-treated neurons exhibited significantly reduced axonal mitochondrial density (Fig. IV.5C; N=5 neurons/condition; p=0.0021). Interestingly, moving mitochondria were not significantly different in PBS and creatine-treated neurons (Fig. IV.5D,F). Rather, creatine only significantly reduced the number of stationary mitochondria (Fig. IV.5E,G; N=5 neurons/condition; p=0.0135 for G). Considering that the overall proportions of moving (Fig. IV.5D) and stationary (Fig.
Figure IV.5. Creatine reduces the density of stationary axonal mitochondria.  

A, Representative images of mitochondria in the 20-70µm axonal region of DIV8 cortical neurons imaged first after a 2hr PBS treatment (PBS) and again following 2hr treatment with creatine (CR).  

B, Corresponding kymographs of mitochondrial movement for axons shown in A. Scale bar represents 4µm.  

C-G, Average axonal mitochondrial density (C), percentage of moving mitochondria (D), percentage of stationary mitochondria (E), number of moving mitochondria per 50µm axon (F), and number of stationary mitochondria per 50µm axon (G). N=5 axons/condition; Paired Student’s T-Test. Data are represented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
IV.5E) mitochondria were not different between PBS and creatine-treated neurons, it is likely that longer imaging periods and/or regions of axonal analysis are necessary in order to detect differences within the small fraction of moving mitochondria. Overall, these results indicate that creatine reduces axonal mitochondria density.

VI. Oligodendrocyte-Derived Creatine May Be Transported to Neurons

The above work illustrates that creatine deficiency is associated with impaired bioenergetics, abnormal mitochondrial dynamics, and immature neuronal morphology, clearly indicating an important role for creatine in regulating neuronal energetics within the CNS. However, neurons exhibit very low levels of the creatine biosynthetic enzyme *Gamt* (Fig. III.1; Zhang et al., 2014), which prompts the hypothesis that creatine must be transported into neurons. Transport of creatine through the BBB is minimal (Ohtsuki et al., 2002; Perasso et al., 2003), therefore it is likely that neurons import creatine secreted from locally synthesizing cells (Braissant et al., 2001; Tachikawa et al., 2004). As oligodendrocytes have a preferentially high capacity for creatine synthesis within the CNS (Braissant et al., 2001; Cahoy et al., 2008; Tachikawa et al., 2004; Zhang et al., 2014), it is possible that oligodendrocyte-derived creatine is transported into neurons for utilization. RNA-sequencing has demonstrated that neurons express transcripts for creatine transporter (*CrT*), also know as *SLC6A8* (Fig. IV.6A; Zhang et al., 2014). Further, neurons express high levels of mitochondrial creatine kinase 1 (*MTCK1*; Fig. IV.6B; Tachikawa et al., 2004; Zhang et al., 2014) and exhibit increased CK activity across early development (Manos et al., 1991), suggesting that neurons utilize intracellular creatine. Immunostaining of cortical neurons at DIV7 reveals expression of CrT protein throughout the dendritic and axonal compartments (Fig. IV.6C). Thus, neurons are poised to import creatine from the extracellular
Figure IV.6. Oligodendrocyte-derived creatine may be transported to neurons. 

*A-B*, Graphical representation of cell-specific *Slc6a8* (A) and *Mtck1* (B) RNA levels obtained from a publicly available RNA-sequencing transcriptome database (http://web.stanford.edu/group/barres_lab/). FPKM represents fragments per kilobase of transcript sequence per million mapped fragments (Zhang et al., 2014). 

*C*, Representative image of DIV7 cortical neurons immunostained for SLC6A8 (magenta) and β-Tubulin (green). Scale bar represents 50µm. 

*D*, Low magnification scan image of a DIV7 mixed cortical culture containing a small proportion of mature, myelinating MBP⁺ (magenta) oligodendrocytes. Scale bar represents 500µm. 

*E*, Higher magnification image of a DIV7 mixed cortical culture showing MBP⁺ (magenta) oligodendrocytes and NeuN⁺ cortical neurons. Scale bar represents 100µm. 

*F*,
DIV7 mixed cortical cultures (N#1, N#2) express transcripts for oligodendrocyte-specific transcripts, including MBP and CNP via RT-PCR. As a positive control, cDNA from MACS-purified oligodendrocyte lineage cells (O4#1, O4#2) is included. G, Average concentration of creatine (ng/mL) in conditioned media harvested from DIV14 mixed glia cells cultured from Gamt+/+ (GAMT-WT) and Gamt–/– (GAMT-KO) mice; N=2 wells/condition; Student’s T-Test. H, Concentration of creatine (ng/mL) in conditioned media harvested from OLN-93 cells spiked with PBS or 100µM creatine; N=1 well/condition. Data are represented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
environment. Immunostaining also reveals a small proportion of MBP+ oligodendrocytes in addition to NeuN+ neuronal cells (Fig. IV.6D,E). Further, RT-PCR analysis demonstrates oligodendrocyte-specific transcripts (PLP, MBP) in cortical cultures (Fig. IV.6F), indicating that neurons could receive oligodendrocyte-derived factors in our in vitro system. We reasoned that if creatine was released from oligodendrocytes, it should be detectable in conditioned media from cultured cells. We first measured creatine in conditioned media harvested from high-density mixed glial cultures to ensure sufficient levels for detection and optimization. Ultra performance liquid chromatography (UPLC) was utilized to measure creatine concentration in conditioned media collected from Gamt+/+ and Gamt−/− mixed glia cultures. Indeed, creatine was detectable in conditioned media (Fig. IV.6G), suggesting that glia-derived creatine is released into the extracellular environment. In addition, the concentration of creatine was significantly reduced in Gamt−/− conditioned media compared to control (Fig. IV.6G; N=2 wells/condition; p=0.029). That some creatine signal is still present in the Gamt−/− sample likely reflects interference of a non-specific component. As mixed glia cultures contain numerous cell types aside from oligodendrocytes, we next measured creatine concentration in conditioned media taken from the rodent oligodendroglia cell line, OLN-93 (Richter-Landsberg and Heinrich, 1996). Creatine was detectable in OLN-93 conditioned media (Fig. IV.6H), suggesting that creatine is also secreted by immortalized oligodendrocytes. Spiking the media with 100µM creatine for 24hr increased creatine concentration by ~8X (Fig. IV.6H). Together, these data suggest that oligodendrocyte-derived creatine is released into the extracellular space.
VII. Oligodendrocyte-Derived Factors Reduce the Density of Axonal Mitochondria

If neurons utilize oligodendrocyte-derived creatine, then axonal mitochondria exposed to oligodendrocyte-conditioned media (OCM) would be expected to exhibit similar dynamic patterns to those observed in creatine-treated axons. To test this hypothesis, mitochondria in wild-type cortical neurons were labeled with CellLight Mitochondria-RFP™ on DIV7 for 24hr. On DIV8, cells were treated with PBS for 2hr and imaged every 30sec for 30 cycles (PBS). Cells were then treated with OCM obtained from DIV8 primary oligodendrocyte cultures for 2hr at 37°C. Importantly, neuronal cultures were maintained in the same co-culture media as oligodendrocyte cultures prior to adding OCM to ensure that any effects seen could be attributed to oligodendrocyte-secreted factors. Following the 2hr incubation, the media was replaced and the same neurons were reimaged as described above (OCM). Kymographs from the 20-70μm region of the axon were analyzed for mitochondrial movement (Fig. IV.7A,B). Similar to creatine treatment, OCM-treated neurons exhibited significantly reduced axonal mitochondrial density (Fig. IV.7C; N=5 neurons/condition; p=0.0002) and reductions in the number of stationary axonal mitochondria (Fig. IV.7E,G; N=5 neurons/condition; p=0.0231 for G). Moving mitochondria were not different in PBS and OCM-treated neurons (Fig. IV.7D,F).

B. DISCUSSION

Genetic mutations in the biosynthetic and transport pathway for the intracellular ATP buffer, creatine, lead to devastating neurological effects including mental retardation (Anselm et al., 2006; Barkovich, 2011). However, very little is known about how neuronal cells are affected by creatine deficiency mechanistically. Here, we investigated the impact of Gamt-deficiency on
Figure IV.7. Oligodendrocyte-derived factors reduce the density of stationary axonal mitochondria. A, Representative images of mitochondria in the 20-70µm axonal region of DIV8 cortical neurons imaged first after a 2hr PBS treatment (PBS) and again following 2hr treatment with oligodendrocyte conditioned media (OCM). B, Corresponding kymographs of mitochondrial movement for axons shown in A. Scale bar represents 4µm. C-G, Average axonal mitochondrial density (C), percentage of moving mitochondria (D), percentage of stationary mitochondria (E), number of moving mitochondria per 50µm axon (F), and number of stationary mitochondria per 50µm axon (G). N=5 axons/condition; Paired Student’s T-Test. Data are represented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
neuronal respiration, axonal mitochondrial dynamics, and neuronal morphology. Our results demonstrate that Gamt-deficient neurons exhibit severely reduced respiration. Changes in respiration were associated with abnormal mitochondrial dynamics and reduced morphological complexity in Gamt-deficient neurons compared to their wild-type counterparts, indicating that bioenergetic deficit leads to impaired neuritogenesis and branching.

Using Seahorse XF analysis, we observed that Gamt-deficient neurons exhibit significantly reduced basal respiration. In addition, the mitochondrial stress test revealed impaired maximal respiration, non-mitochondrial respiration, and spare respiratory capacity of Gamt-deficient neurons compared to controls. Interestingly, treating Gamt-deficient neurons with 100uM creatine produced an intermediate respiratory phenotype, such that basal respiration, maximal respiration, and spare respiratory capacity were not significantly different from either controls or Gamt-deficient neurons treated with PBS. These results suggest that creatine concentration or treatment timing was insufficient to return respiration to control levels. Although we treated neurons relatively young at DIV3, it is possible that earlier intervention may be necessary to restore the bioenergetic capacity of Gamt-deficient neurons.

In addition to respiratory changes, Gamt-deficient neurons exhibited abnormal patterns of axonal mitochondrial movement. While the overall density of mitochondria was not altered, Gamt-deficient axons contained a higher proportion of motile mitochondria compared to controls. It is possible that increased mitochondrial movement may represent increased turnover events, in which low energy-producing or damaged mitochondria may be returned to the soma or have the opportunity to fuse with newer mitochondria for delivery or exchange of mitochondrial
components (Schwarz, 2013). In agreement, it has been demonstrated that mitochondria with low membrane potential tend to move retrogradely (Miller and Sheetz, 2004). Therefore, we suspect that increased motility of mitochondria in Gamt-deficient axons may represent an effort to remove or repair low-respiring mitochondria. In support of this hypothesis, axons treated with TNFα, a pro-inflammatory cytokine known to lower the basal respiration of mitochondria in immortalized hippocampal neurons (Doll et al., 2015), exhibited a nearly identical pattern of increased motile mitochondria with no effect on overall density.

We next demonstrated that Gamt-deficient neurons have significantly reduced morphological complexity, with fewer secondary and tertiary neurites. It has previously been demonstrated that adding exogenous creatine to striatal neural progenitor cells increases the morphological complexity of GABA-ergic neurons in vitro (Andres et al., 2016). In addition, dendritic mitochondria are known to act synergistically with creatine kinase in maintaining ATP levels to allow for proper dendritic outgrowth of cerebellar Purkinje neurons (Fukumitsu et al., 2015). Mitochondria, and specifically stationary mitochondria, are also necessary to supply ATP for axonal branching in cortical neurons in vivo (Courchet et al., 2013). Thus, it is possible that the morphological deficits of Gamt−/− neurons may be the result of insufficient local ATP concentrations caused by the increased proportion of mitochondria in the motile phase.

As dendritic branches serve as the primary location for synaptic input, elaboration and branching of neurites is crucial for the formation of complex neural networks. Reduced branching in Gamt-deficient neurites suggests fewer synaptic contacts with neighboring neurons and possibly reduced network complexity overall. If neuronal connectivity is reduced in Gamt-deficient mice,
behavioral abnormalities would be expected. Surprisingly, a battery of behavioral tasks designed to test cognitive performance, including morris water maze (MWM), passive avoidance, activity, open field exploration, social exploration, dark-light transition, and rotarod, revealed only subtle changes in Gamt-deficient mice, which exhibited impaired retrieval of learned information during MWM compared to controls (Torremans et al., 2005). These results suggest that Gamt-deficient mice are only mildly cognitively impaired, at least at the age tested (6 months). The first possible explanation for these findings is that reduced morphological complexity of Gamt-deficient neurons is transient. It may simply take a longer period of time for neuritogenesis and branching to occur due to low energy supply. To address this possibility, morphological complexity of Gamt-deficient neurons should be measured in vivo across early postnatal development. The second possible explanation is that despite impaired neuronal complexity, developmental or functional compensatory mechanisms exist to normalize cognition in Gamt-deficient mice.

One puzzling aspect of this work is why Gamt-deficient neurons exhibit such severe bioenergetic and morphological deficits when Gamt is primarily expressed in pre-myelinating and myelinating oligodendrocytes in the CNS (Braissant et al., 2001; Cahoy et al., 2008; Tachikawa et al., 2004; Zhang et al., 2014). The high expression of MtCK and CrT in neurons suggests that these cells may import and utilize oligodendrocyte-derived creatine from the extracellular environment. Indeed, oligodendrocytes are present in our cortical cultures. Further, our preliminary findings demonstrate that creatine can be detected in conditioned media harvested from mixed glia and immortalized oligodendrocytes, suggesting that oligodendrocyte-derived creatine is released into the extracellular environment. Similar to the proposed model of creatine
transport, previous work has demonstrated that axons rely on oligodendrocyte-derived energy metabolites, including lactate and pyruvate (Fünfschilling et al., 2012; Lee et al., 2012). In fact, disruption of this transport via downregulation of monocarboxylate transporter 1 (MCT1) induces neuronal degeneration (Lee et al., 2012).

Exogenous creatine treatment increases mitochondrial membrane potential in Xenopus spinal neurons (Lee and Peng, 2008) and in the cerebral cortex when given systemically (Rambo et al., 2013). We hypothesized that increased mitochondrial function in creatine-treated neurons would be associated with altered mitochondrial dynamics. We found significantly reduced mitochondrial density and stationary mitochondria in cortical axons after creatine treatment compared to before. Under basal conditions, it is hypothesized that stationary mitochondria help mitigate the low diffusion capacity of ATP (Hubley et al., 1996). By a poorly understood mechanism, it is believed that mitochondria become recruited to sites of high energetic demand in response to low ATP levels (Mironov, 2007; Sheng, 2014). The creatine-phosphocreatine shuttle buffers ATP concentrations by allowing for rapid regeneration of ATP without the need for de novo oxidative phosphorylation (Wyss and Kaddurah-Daouk, 2000), therefore it is possible that creatine-treated axons require fewer mitochondria to meet local energy demand.

Mitochondrial density was also reduced due in cortical axons after treatment with oligodendrocyte-conditioned media (OCM) compared to before. Intriguingly, the opposite phenotype of abnormally accumulated axonal mitochondria is a common hallmark of MS pathology (Griffiths et al., 1998; Garbern et al., 2002; Lappe-Siefke et al., 2003; Edgar et al., 2004; Ferreirinha et al., 2004; Andrews et al., 2006; Hogan et al., 2009; Mahad et al., 2009;
Witte et al., 2009; Kiryu-Seo et al., 2010; Zambonin et al., 2011). Disruption of mitochondrial dynamics, suggested by overabundant expression of the mitochondrial anchoring protein SNPH in chronic MS (Mahad et al., 2009), could cause a buildup of dysfunctional mitochondria along the axon. Indeed, deletion of SNPH in the dysmyelinating Shiverer mouse prolonged survival and was associated with reduced oxidative stress and increased mitochondrial health (Joshi et al., 2015), suggesting that mitochondrial anchoring may be a viable target for disorders associated with oligodendrocyte dysfunction. The striking resemblance in the patterns of axonal mitochondrial movement in creatine-treated neurons and OCM-treated neurons suggests that the effects of OCM on axonal mitochondria may be mediated by the transport of oligodendrocyte-derived creatine into neurons. More work utilizing OCM derived from Gamt<sup>+/+</sup> and Gamt<sup>−/−</sup> oligodendrocytes is needed to investigate this possibility.

Overall, this work reinforces the notion that neuronal mitochondria respond adaptively to various energetic states and highlights the importance of creatine in modulating neuronal energy production and mitochondrial dynamics. It further implicates oligodendrocytes, independent of myelin sheaths, in supporting neurons by providing secreted factors that modulate axonal mitochondrial dynamics. Our results suggest that therapeutic administration of creatine may be beneficial for alleviating neuronal mitochondrial dysfunction in neurodegenerative diseases and possibly disorders of primary oligodendrocyte dysfunction.
CHAPTER V: DISCUSSION
The overarching goal of this dissertation was to investigate the cell-specific roles of creatine in the CNS. Creatine came of interest to our lab when its synthesizing enzyme, GAMT, emerged as a highly differentially expressed gene during oligodendrocyte differentiation and remyelination. While many publications have emphasized the preferentially high expression of GAMT in oligodendrocytes, an explanation of its function in these cells had not been previously reported. Even more intriguing was the understanding that neurons are heavily reliant on creatine despite their inability to synthesize it. Due to the known role of creatine in energy buffering, my dissertation research focused on elucidating how creatine affects mitochondrial function and cellular integrity of both oligodendrocytes and neurons.

I. CREATINE PROMOTES OLIGODENDROCYTE SURVIVAL FOLLOWING DEMYELINATING INJURY

My results demonstrate that creatine enhances mitochondrial ATP production in oligodendrocytes and serves to protect them from cell death under both basal and inflammatory conditions in vitro. Importantly, creatine-mediated oligodendrocyte protection was also observed under more physiologically relevant injury conditions in vivo. Following focal spinal cord demyelination, Gamt-deficient mice exhibited increased apoptosis of regenerated oligodendrocytes in CNS lesions. Co-injecting Gamt-deficient mice with creatine at the time of surgery rescued oligodendrocyte density in the lesions and returned levels of apoptotic oligodendrocytes to control. In addition, exogenous creatine treatment reduced oligodendrocyte apoptosis in demyelinated lesions and promoted survival of newly generated oligodendrocytes. Overall, these findings demonstrate a novel function for creatine in promoting oligodendrocyte viability during CNS remyelination.
I. If Creatine is Important for Oligodendrocyte Survival, Then Why Are Oligodendrocytes Normal in Gamt-Deficient Mice?

Quantification of oligodendrocyte lineage cells in uninjured spinal cord demonstrates that OPC and oligodendrocyte densities are not different in Gamt-deficient adult animals. Myelin proteins are also not different from controls by P30. However, my preliminary findings suggest that myelin proteins are reduced in Gamt-deficient mice at early postnatal time points, indicating delayed myelination. Human patients with inborn errors in creatine metabolism frequently exhibit delayed myelination (Schulze et al., 1997; Bianchi et al., 2000), supporting the notion that Gamt may be important for timely oligodendrocyte differentiation and myelin production. Our finding that Gamt-deficient mice exhibit decreased oligodendrocyte survival during remyelination strongly suggests that delayed myelination may be the result of increased oligodendrocyte turnover during early development. Creatine did not strongly affect oligodendrocyte membrane expansion in vitro, however our system does not accurately recapitulate myelination of neuronal axons in the CNS. Therefore, it is also possible that Gamt-deficient oligodendrocytes survive during early development but do not have the energetic resources for myelin protein production and assembly. One additional possibility to consider is that delayed myelination may be a combination of direct and indirect effects on oligodendrocytes. Our results demonstrate that Gamt-deficient neurons are morphologically less complex than wild-type neurons. As myelination is at least in part, stimulated by neuronal activity, it may be that Gamt-deficient axons are not as amenable to myelination.
II. Why Are Behavioral Measures Normal in Gamt-Deficient Mice?

One related question is why Gamt-deficient mice are cognitively normal (Torremans et al., 2005) whilst Gamt-deficiency in humans is associated with severe cognitive disability. While there are many possible explanations, we hypothesize that the detrimental effects of Gamt-deficiency might be exacerbated in humans due to the vastly increased amount of white matter in humans compared to mice. It is also possible that compensatory mechanisms in mice combat the detrimental effects of creatine deficiency. Lastly, it is possible that different Gamt expression patterns and timing produce distinctive phenotypes in humans and mice. Additional studies comparing Gamt expression in brain cells across various developmental time points and brain regions are needed to address this hypothesis.

III. Could Creatine Be Beneficial For the Treatment of Multiple Sclerosis?

Clinical trials have demonstrated that high doses (30g/day) of oral creatine, necessary to permeate the BBB, are safe and well tolerated (Rosas et al., 2014). Only two studies have evaluated creatine in the context of MS. They report that creatine ingestion of 20g/day for 5 days (Lambert et al., 2003) or ingestion of 5-20g/day for 14 days (Malin et al., 2008) had no effect on muscle capacity in MS patients. As both studies only measured outcomes related to muscle and exercise capacity, how creatine ingestion affects the CNS in MS patients is completely unknown. Generally, my results illustrate that creatine significantly impacts function and survival of CNS cells, an intuitive finding based on the severe phenotype of creatine deficiencies. More importantly, the specific pro-survival effect of creatine on oligodendrocytes in the demyelinated CNS suggests that clinical trials aimed at measuring cognitive function over longer durations of high-dose creatine in MS patients is a worth while consideration.
Whether creatine is capable of promoting oligodendrocyte survival under direct, immune-mediated attacks remains to be determined. Assuming that existing oligodendrocytes still undergo cell death during bouts of auto-immunity, my results suggest that creatine supplementation could serve to promote the survival of newly generated oligodendrocytes in what remains a highly inflammatory environment for some time. Continued survival of newly generated oligodendrocytes may be particularly important in SPMS, as previous reports suggest that many oligodendrocytes actually do survive initial demyelination, but disappear from the lesion slowly over time (Wolswijk, 2000). It is likely that oligodendrocytes succumb to persistent mitochondrial dysfunction and oxidative stress under chronic inflammation (Casaccia-Bonnefil, 2000), therefore my observation that creatine directly bolsters oligodendrocyte mitochondrial function makes it an ideal candidate for oligodendrocyte protection under these conditions.

B. CREATINE ENHANCES NEURONAL RESPIRATION AND MODULATES AXONAL MITOCHONDRIAL DYNAMICS IN CORTICAL NEURONS

My results indicate that creatine also plays an important role in neuronal mitochondrial homeostasis. Gamt-deficient neurons had reduced aerobic respiration, indicating that creatine-phosphocreatine buffering is a crucial component of neuronal energy production. Reduced respiratory capacity was associated with aberrantly motile axonal mitochondria in Gamt-deficient neurons. This shift in dynamics is likely a homeostatic response to repair low-respiring mitochondria by returning them to the cell body or promoting fusion events. However, the alternative scenario, in which altered mitochondrial dynamics causes the overall population of mitochondria to be low respiring, remains possible. Considering that the overall density of
mitochondria remains the same in Gamt-deficient axons, it is difficult to consider how this might occur. One possibility is that fusion requires one motile mitochondrion to fuse with one stationary mitochondrion. If this were true, the increased proportion of motile mitochondria might discourage the number of fusion events, generating a population of low-respiring mitochondria.

Addition of exogenous creatine treatment was previously shown to enhance mitochondrial respiration in wild-type neurons. Interestingly, creatine-treated neurons exhibited significantly fewer axonal mitochondria compared to PBS-treated neurons. The fact that overall mitochondrial density is not altered in Gamt-deficient axons, but is lowered in creatine-treated axons is a subtle, but important distinction. Fewer mitochondria in creatine-treated axons suggest a reduction in their overall energetic demand. Future studies are needed to determine whether the reduction in axonal mitochondrial density in creatine-treated neurons is due to reduced biogenesis or altered transport/docking dynamics. Irrespective of the mechanism, these findings suggest that creatine may useful for normalizing mitochondrial density in pathological conditions.

C. OCM REDUCES THE DENSITY OF AXONAL MITOCHONDRIA

The demonstration that transport of oligodendrocyte-derived lactate to axons is important for maintaining neuronal integrity (Fünfschilling et al., 2012; Lee et al., 2012), suggests that oligodendrocyte-derived secreted factors play an important role in regulating axonal metabolic homeostasis. Utilizing an in vitro approach, I have demonstrated that conditioned media taken from purified oligodendrocyte lineage cell cultures directly affects axonal mitochondrial dynamics. Axons treated with OCM exhibited fewer mitochondria overall, due to a specific
reduction in the number of immobilized mitochondria. The striking resemblance in the patterns of axonal mitochondrial movement in creatine-treated neurons and OCM-treated neurons suggests that the effects of OCM on axonal mitochondria may be mediated by the transport of oligodendrocyte-derived creatine into neurons.

I. How Might Oligodendrocyte-Derived Creatine Be Transported Into Neurons?
My preliminary findings indicate that oligodendrocyte-derived creatine is released into the extracellular space. At present, it is unknown how creatine moves extracellularly. While it has long been established that creatine is exported from hepatocytes in the periphery (daSilva et al., 2009), the transporter responsible for this export remains a mystery. The only transporter identified for creatine, SLC6A8, is a Na+/Cl- symporter, which drives creatine into cells by actively utilizing the sodium gradient generated via Na+/K+-ATPase (Brosnan and Brosnan, 2007). This direction of transport does not make sense for oligodendrocytes given that they, like hepatocytes, are expected to have high intracellular creatine levels. Interestingly, it has previously been demonstrated that creatine transporter reversal is thermodynamically possible in cardiac tissue with very high intracellular creatine concentrations (Hove et al., 2008). Additional work is needed to determine whether creatine transporter reversal occurs in oligodendrocytes or whether creatine is exported through a yet unidentified mechanism.

II. Disruption of Axonal Mitochondria in Multiple Sclerosis
A common hallmark of MS pathology is the accumulation of abnormally activated mitochondria in demyelinated axons (Griffiths et al., 1998; Garbern et al., 2002; Lappe-Siefke et al., 2003; Edgar et al., 2004; Ferreirinha et al., 2004; Andrews et al., 2006; Hogan et al., 2009; Mahad et
al., 2009; Witte et al., 2009; Kiryu-Seo et al., 2010; Zambonin et al., 2011). Evidence from rodent MS models suggests that axonal mitochondria activity and morphology are altered prior to demyelination (Nikić et al., 2011; Acs et al., 2013), suggesting that mitochondrial changes may be an early pathological event. As mitochondria play crucial homeostatic roles in the axonal and synaptic microenvironments (Nave, 2010), it is not surprising that their dysfunction leads to ATP depletion, Ca2+ dyshomeostasis, and the accumulation of reactive oxygen species (ROS), all of which can lead to axonal degeneration (Court and Coleman, 2012). Disruption of mitochondrial dynamics, such as overabundant expression of the mitochondrial anchoring protein SNPH in chronic MS (Mahad et al., 2009), could cause a buildup of dysfunctional mitochondria along the axon. Indeed, deletion of SNPH in the dysmyelinating Shiverer mouse prolonged survival and was associated with reduced oxidative stress and increased mitochondrial health (Joshi et al., 2015). Our observation that OCM reduces the density of axonal mitochondria strongly suggests that changes in mitochondria in MS may be due to loss of oligodendrocyte-derived support as a whole (Nave, 2010).

III. Contribution of Bioenergetic Homeostasis to the Oligodendrocyte:Axon Unit

Axonal pathology is commonly observed in models of oligodendrocyte dysfunction in which myelin is preserved (Edgar et al., 2009, 2010; Traka et al., 2010; Oluich et al., 2012). Even more surprising is the demonstration that many axons exhibit distinct, intermittent stretches of myelinated and unmyelinated axon (Tomassy et al., 2014). Together, these observations suggest that oligodendrocytes perform alternative functions completely independent of their role in promoting saltatory conduction and highlight that we have much to learn about the complexities of oligodendrocytes.
While more work is needed to prove that oligodendrocyte-derived secreted factors modulate axonal mitochondrial dynamics \textit{in vivo}, it is interesting to consider how this process might proceed. On one hand, it is possible that oligodendrocytes continuously release metabolic factors to alleviate stress on the axonal mitochondrial system. Alternatively, oligodendrocytes might actively “listen” to axonal activity and modulate the amount of metabolic support based on axonal demand. Implied in this hypothesis is that oligodendrocytes have a mechanism for listening to neuronal activity. Where there is no evidence for direct electrical coupling of oligodendrocytes and neurons, oligodendrocytes are extensively coupled to astrocytes via gap junctions (Bennett and Zünken, 2004). Interestingly, these junctions have been shown to mediate action-potential evoked K$^+$ inward rectifying currents in satellite oligodendrocytes (Battefeld et al., 2016), which reside near neuronal somas. As a single oligodendrocyte can contact up to 40 different neurons, this electrical syncytium could theoretically allow for modulation of axonal energy metabolism on a network level.

Herein, I have presented evidence demonstrating that creatine promotes mitochondrial-dependent oligodendrocyte survival following demyelinating injury. In addition, creatine plays a crucial role in regulating neuronal mitochondrial respiration and dynamics within the axon. While applicable to all neurological diseases including primary creatine deficiencies, these findings have particularly interesting implications for MS, a disease in which both oligodendrocyte and neuronal damage contribute to severe disability.
CHAPTER VI: FUTURE DIRECTIONS
My work demonstrates that creatine enhances oligodendrocyte mitochondrial function and promotes oligodendrocyte survival by inhibiting cleavage of caspase-3. It remains to be seen whether creatine binding to MtCK directly inhibits opening of the mitochondrial permeability transition pore (mPTP), as has been demonstrated in vitro (Beutner et al., 1996; O’Gorman et al., 1997; Beutner et al., 1998; Vyssokikh et al., 2003; Schlattner et al., 2006). Focal demyelination should be performed in MtCK and cytosolic CK-knockout mice to determine which, if either, kinase is necessary for creatine-mediated protection. Development of genetic tools to study the intracellular patterns and timing of creatine kinase expression in oligodendrocytes would be useful to further this line of investigation. Creatine can also serve as a direct antioxidant (Lawler et al., 2002), therefore it would be interesting to investigate whether creatine-mediated reductions in ROS generation indirectly promote oligodendrocyte survival in lesioned spinal cords.

Due to the reduced viability of Gamt-deficient mice, it will be necessary to generate conditional, oligodendrocyte-specific Gamt-deficient mice for future experiments. As the global Gamt-deficient mouse utilized in this study does not exhibit overt cognitive deficits, behavioral experiments should be repeated in conditional Gamt knockouts. In addition, our preliminary experiments measuring myelin proteins in early postnatal development suggest that Gamt-deficiency is associated with delayed myelination. Future work should elaborate on these experiments by measuring myelin proteins at additional time points during early postnatal development. The mechanism for myelin protein delay, whether increased oligodendrocyte turnover or delayed myelin protein synthesis, should be elucidated.
In vivo EdU labeling of lesions suggests that creatine promotes survival of newly generated oligodendrocytes. This should be confirmed by combining fluorescent immunostaining of EdU\(^+\)CC1\(^+\) with colorimetric detection of cleaved caspase-3 to determine whether the reduction in dying oligodendrocytes at 5 and 10dpl in creatine-treated mice is due primarily to enhanced survival of newly generated oligodendrocytes rather than pre-existing oligodendrocytes. Further, these experiments should be conducted at 10 and 20dpl for PB vs. creatine-treated mice as well as all post-lesion time points for Gamt-deficient mice and control littermates. My data thus far suggests creatine is primarily protective during time periods of high inflammation (5 & 10dpl), thus it will be interesting to correlate the timing of creatine-mediated protection of newly generated oligodendrocytes with inflammation. In addition, it would be informative to perform focal demyelination in mice harboring an inducible caspase (iCP9) sequence and reporter under an OPC-specific promoter. With a diminished OPC population, these mice would be expected to have very few new oligodendrocytes, allowing investigators to assess the contribution of survival of pre-existing oligodendrocytes in the presence of creatine.

My work demonstrates that co-injection of creatine at the time-of-injury promotes oligodendrocyte regeneration in focally demyelinated lesions. It will be critically important to conduct studies aimed at evaluating the efficacy of creatine supplied at different times and through different methods. Experiments in which mice are fed creatine prior to surgery could determine whether prophylactic creatine treatment is a viable approach to enhancing oligodendrocyte survival in demyelinating lesions. While it is expected that creatine could access lesions with active BBB damage, alternative methods for getting creatine through the BBB would likely be necessary to treat chronic lesions. Two possibilities worth exploring are
intranasal delivery of creatine or administration of cyclocreatine, a creatine analog with enhanced BBB permeability.

In this study, I utilized the lysolecithin-mediated model of focal spinal cord demyelination to investigate the effect of creatine on oligodendrocyte injury. The strength of this model comes from the precise timing of injury, allowing tightly controlled analysis of the demyelination and remyelination processes. However, repetition of this work in other models of demyelination, primarily EAE, is urgently needed. Oligodendrocyte demyelination in response to lysolecithin toxicity is not immune-mediated, thus this model does not accurately recapitulate the inflammatory component of MS. In contrast, subcutaneous injection of adjuvant and myelin-derived proteins in EAE induce activation and expansion of peripheral T-cells, which invade the CNS to induce oligodendrocyte death and demyelination (Denic et al., 2011). It will be very interesting to test whether creatine also promotes oligodendrocyte survival in the EAE model. It is possible that creatine may promote survival of oligodendrocytes during the initial, direct immune-mediated attack. Perhaps more likely is a scenario in which creatine promotes survival of newly generated oligodendrocytes born into a highly inflammatory environment in which cytokines released from neighboring, activated macrophages/microglia stress oligodendrocyte mitochondria (Mahad et al., 2015b).

My finding that creatine protects newly generated oligodendrocytes suggests that increasing creatine levels could be beneficial for MS patients with active lesions. Quantitative measurements of Agat, Gamt, and CrT expression levels, as well as activity patterns, in healthy and MS populations would be very informative. In addition, it will be crucially important to
understand how aging affects these parameters in both healthy and MS populations in order to parse apart disease-specific versus normal aging processes.

My preliminary work investigating the effect of creatine deficiency on neuronal bioenergetics demonstrates impaired respiration in Gamt-deficient neurons. Future work should attempt to rescue these deficits with higher concentrations of exogenous creatine. It is also possible that earlier intervention is necessary. Neuronal cultures could be maintained in media spiked with creatine from the time of plating to test this hypothesis. Alternatively, pregnant mothers could be fed a creatine-enriched diet (2%) prior to culturing pups at P3-5. In addition to impaired respiration, I also found that Gamt-deficient neurons exhibited reduced morphological complexity in vitro. It will therefore be important to examine neuronal morphology in Gamt-deficient mice using golgi staining in future experiments.

Gamt-deficient neurons, as well as neurons treated with TNFα, exhibit higher proportions of motile axonal mitochondria despite having similar densities compared to controls. Previous work indicates that low-respiring mitochondria are more likely to be in motion (Miller and Sheetz, 2004), possibly to return damaged mitochondria to the cell body for degradation or to facilitate fusion events for the exchange of newly synthesized mitochondrial components (Amiri and Hollenbeck, 2008; Schwarz, 2013). It will be necessary to examine whether moving mitochondria in Gamt-deficient axons are moving anterogradely or retrogradely, as the latter would suggest return to the soma for degradation. Additionally, experiments utilizing two different-colored mitochondrial dyes would be ideal for assessing the impact of Gamt-deficiency on mitochondrial fusion and fission. Lastly, although the Seahorse analysis indicates low
respiring mitochondria in *Gamt*-deficient neurons as a whole, it would be useful to correlate mitochondrial activity with direction of movement in the axon to confirm previous reports of low-respiring mitochondria moving towards the soma.

Based on the preferential expression of GAMT in oligodendrocytes, we hypothesize that GAMT-KO neurons are pathologically affected by lack of oligodendrocyte-derived creatine in the extracellular space. Although numerous studies have identified oligodendrocytes as the primary GAMT-expressing cells in the brain, it remains possible that GAMT expression patterns vary significantly across developmental time points. If this is true, the detrimental effect of *Gamt*-deficiency on oligodendrocytes could theoretically be a secondary effect of neuronal mitochondrial alterations. Future work should focus on measuring cell-specific GAMT expression patterns at various developmental time points and correlating these patterns with aberrant cellular phenotypes.

I present preliminary evidence to suggest that oligodendrocyte-derived creatine is detected in the extracellular space. However, future experiments should elaborate on these findings in order to confirm the origin of creatine. Ideally, stable isotopic labeling by amino acid (SILAC) would be conducted in cultures of MACS-purified oligodendrocyte lineage cells by addition of labeled arginine and glycine, the amino acid building blocks of creatine. Conditioned media from these cultures on subsequent days *in vitro* could then assessed for concentration of labeled creatine via mass spectrometry. More generally, this method would be useful in constructing a more complete picture of the various oligodendrocyte-derived compounds released into the extracellular milieu.
The mechanism by which creatine is released into the extracellular environment remains unknown. It has been reported that creatine transporter reversal is thermodynamically possible in cardiac tissue with very high intracellular creatine levels (Hove et al., 2008). The preferentially high expression of \textit{Gamt} in oligodendrocytes suggests that they too would contain very high intracellular creatine levels. Therefore, it would be very interesting to study the effect of creatine transporter antagonism in this context. Creatine concentrations should be measured in conditioned media derived from MACS-purified oligodendrocyte cultures treated with PBS or guanidinopropionic acid (GPA), the competitive antagonist of CrT.

Mechanistic studies investigating how oligodendrocyte-secreted factors influence axonal integrity are sparse. My finding that axonal mitochondrial dynamics is altered in neurons treated with OCM suggests that neurons actively adapt to extracellular cues from oligodendrocytes. We currently know very little about which factors are secreted by oligodendrocytes and in what concentrations or when. Therefore, it is not technically feasible to test for the effect of oligodendrocyte-derived factors \textit{in vivo} without significant confounding variables. Controlled \textit{in vitro} experimentation using MACS-purified populations of oligodendrocyte lineage cells should be utilized to investigate how neuronal morphology and signaling is affected by OCM.

I have demonstrated that cortical axons treated with both creatine and OCM exhibit fewer axonal mitochondria. As mitochondria appear to be recruited to sites of high energetic demand (Schwarz, 2013), we hypothesize that the ATP buffering capacity of creatine relieves the mitochondrial system of some of this demand, thereby reducing the need for mitochondria. In order to test this hypothesis, ATP concentration should be measured in neuronal axons via
magnesium green imaging and changes in ATP concentration should be correlated with alterations in the number of stationary mitochondria. Further, the TMRE and fusion/fission experiments proposed above for Gamt-deficient neurons should be conducted in creatine- and OCM-treated neurons to determine how the behavior of an individual mitochondrion is affected by its activity under these treatment paradigms.

It would also be interesting to determine whether the reduction in stationary mitochondria in creatine- and OCM-treated axons affects overall levels or binding activity of the mitochondrial docking protein SNPH (Kang et al., 2008; Chen and Sheng, 2013). As creatine directly interacts with MtCK located in the outer mitochondrial membrane protein, it is possible that conformational changes in MtCK during creatine binding might interfere with syntaphilin attachment to mitochondria. Alternatively, SNPH binding activity may be regulated by mitochondrial membrane potential and therefore indirectly affected by creatine and/or OCM.

In summary, my work demonstrates novel roles for creatine in promoting the survival of oligodendrocytes and regulating mitochondrial function in both oligodendrocytes and neurons. The severe neurological disabilities caused by creatine deficiency in the human brain illustrate the importance of elucidating how creatine functions in the CNS on a mechanistic level. Further, while creatine treatment has been studied as a therapeutic agent in the context of numerous neurological disorders, additional work is needed to determine the value of creatine treatment in the context of primary oligodendrocyte disorders, such as MS and leukodystrophy.
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