THE ROLE OF THE GROUP I METABOTROPIC GLUTAMATE RECEPTOR AGONIST, CHPG, IN

OLIGODENDROCYTE REGENERATION AND REPAIR FOLLOWING A CUPRIZONE-INDUCED LESION

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ABSTRACT OF THE DISSERTATION

The Role of the Group I Metabotropic Glutamate Receptor Agonist, CHPG, in

Oligodendrocyte Regeneration and Repair following a Cuprizone-Induced Lesion

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Demyelinating diseases or demyelination in response to toxic agents can be debilitating to patients and can greatly impair the quality of life. Therefore, new therapeutic targets are needed that enhance the regeneration and repair of oligodendrocytes, the myelin-producing cells. In an effort to identify drugs that delay demyelination and promote remyelination, previous work from our lab has been evaluating effects of metabotropic glutamate receptor (mGluR) agonists. The current work assesses the role of one such agonist, 2-chloro-5-hydroxyphenylglycine (CHPG), in the cuprizone model of demyelination in mice. Peripheral injection of CHPG was found to work within the lesion site to influence myelination.

Dedication

This dissertation is dedicated to my Aunt Kathy, who has always been my personal

motivation for doing research related to multiple sclerosis. It is my hope that the

future of research in this field will lead to better treatments for her and others that

are similarly affected by this disease.

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Chapter 1.1, 1.2, 1.3.3, 1.4

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Chapter 1: Introduction

Demyelinating diseases such as Multiple Sclerosis (MS), or demyelination in response to toxic agents, can be debilitating to patients and can greatly impair the quality of life. A 2019 study suggests that the prevalence of MS in 2017 was nearly 1,000,000 people in the United States alone (Wallin et al., 2019), more than twice the estimates of previous studies (Baum and Rothschild, 1981; Anderson et al., 1992; Noonan et al., 2002), making this is a greater problem than originally suggested. Many drug therapies are targeted towards alleviating immune components of these diseases rather than the neurological component, which involves the death of oligodendrocytes, the myelin-producing cells, and the degeneration of axons. Myelin acts as an insulating sheath to neurons to allow for efficient and effective transmission of neuronal signals. When this myelin sheath is lost or damaged, as occurs in MS, this can lead to symptoms such as numbness or tingling of the extremities, pain, fatigue, difficulty walking, and muscle spasms among others. Therefore, it is imperative to find new therapeutic targets for these types of diseases that currently have no cure.

It is now well understood that multiple growth factors impact the response of myelinated axons to demyelination (De Santi et al., 2011; Steinman, 2015). In addition, glial cells (astrocytes, oligodendrocyte lineage cells, and microglia) are recognized to be a source of these growth factors. In particular, our lab has been examining the roles of one of these factors, brain-derived neurotrophic factor (BDNF), on demyelinating lesions and its potential to be affected by stimulating metabotropic glutamate receptors (mGluRs)(Fulmer et al., 2014). Thus, my studies

focus on the role of an mGluR agonist, 2-chloro-5-hydroxyphenylglycine (CHPG), and its ability to increase BDNF and myelin proteins in a demyelinating lesion in mice. Briefly, I found that CHPG acts through astrocytic mGluR5 and astrocyte-derived BDNF to elevate myelin proteins through the BDNF receptor, TrkB, found on oligodendrocytes. To appreciate the work that led to these findings, this introduction will address the following:

1. Astrocyte Heterogeneity

2. Astrocytic Response to Injury

3. Glial mGluR Expression and Function

    a. Roles of mGluRs on oligodendrocytes

    b. Roles of mGluRs on microglia

    c. Roles of mGluRs on astrocytes

4. Astrocytic mGluRs in Demyelinating Disease

5. The Cuprizone Model of Demyelination

6. Glia as a Source of BDNF to Promote Remyelination

1.1 Astrocyte Heterogeneity

Astrocytes are the most abundant glial cell in the central nervous system (CNS) and are specialized cells able to support neuronal activity in both development and adulthood. This includes roles in ion homeostasis, uptake of neurotransmitters, release of growth factors, participation in synaptic transmission, regulation of the blood-brain barrier and involvement in the CNS immune system (Sofroniew and Vinters, 2010). Astrocytes also present a dynamic environment for

axon guidance during development by providing appropriate cell surface receptors and adherent molecules (Powell et al., 1997).

Interestingly, crosstalk between astrocytes and neurons contributes to many astrocytic functions. For example, astrocytes can respond to several factors and neurotransmitters. These include glutamate (Perea and Araque, 2005; Di Castro et al., 2011), adenine triphosphate (ATP) (Bowser and Khakh, 2004; Perea and Araque, 2007), gamma-aminobutyric acid (GABA) (Perea et al., 2016; Mariotti et al., 2018), acetylcholine (Chen et al., 2012) and endocannabinoids (Navarrete and Araque, 2008). When these factors stimulate astrocytes, astrocytes increase levels of intracellular calcium and release gliotransmitters and growth factors that impact neuronal function (Martin, 1992; Nedergaard et al., 2003). Subsequently, astrocytes modulate the function, maintenance, pruning, and remodeling of synapses and they express ion channels, neurotransmitter receptors, and transporters (Kang et al., 1998; Guthrie et al., 1999; Navarrete and Araque, 2008).

Astrocytes are heterogeneous cells that not only differ in morphology and expression of intermediate filament levels, but also in the roles they play (Wilkin et al., 1990). For example, differences in morphology are apparent when subtypes of astrocytes from the human cortex and hippocampus are compared. High levels of glutamine synthetase (GS) and excitatory amino-acid transporters -1 and -2 (EAAT1, EAAT2) are observed in the hippocampus with long-process astrocytes, while low levels of these enzymes and transporters are noted in cortical astrocytes that exhibit reduced numbers of small processes (Sosunov et al., 2014). It is possible that the heterogeneity in morphology may extend to heterogeneity in function. This is

suggested in the observation that astrocytes of different regions release different substances. For example, cultured astrocytes of the substantia nigra are better at maintaining dopaminergic neuron survival than astrocytes of the hippocampus (O'Malley et al., 1992). Such differences are also noted when substantia nigra astrocytes are compared to those of the ventral tegmental area. In this case, astrocytic effects on local dopaminergic neurons suggest that growth and differentiation factor 15 (GDF15), a member of the transforming growth factor beta (TGF-β) superfamily, may be responsible for differences in survival and protection when dopaminergic neurons from the two brain regions are compared (Kostuk et al., 2019).

Astrocytic heterogeneity can also be observed within brain regions. For example, astrocytic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, glutamate transporter 1, and potassium channel Kir4.1 expression are differentially expressed both within and between specific regions of the brain (Poopalasundaram et al., 2000; Emsley and Macklis, 2006; Regan et al., 2007; Hoft et al., 2014). Heterogeneity is also evident with respect to mGluR5 expression as few astrocytes of the spinal cord exhibit this receptor (Silva et al., 1999), while the majority of cortical astrocytes do (Biber et al., 1999). It is interesting to consider what roles these regional differences may play in regards to different disease states.

1.2 Astrocytic Response to Injury

In the case of brain injury, astrocytes become reactive. In this process, astrocytes express a host of new genes resulting in changes in function and

morphology following a variety of lesions (Pekny and Nilsson, 2005; Sofroniew, 2009; Sofroniew and Vinters, 2010; Pekny and Pekna, 2014; Verkhratsky et al., 2014; Pekny and Pekna, 2016). Traditionally, it was thought that these changes are negative. For example, astrocytes can form a physical barrier to axon growth and produce a variety of molecules that serve as an impediment to nerve cell survival (Ohtake and Li, 2015; Adams and Gallo, 2018). Moreover, reactive astrocytes express a wide variety of inflammation-associated molecules and are capable of antigen presentation. These changes have profound pro-inflammatory effects that present an inhibitory environment for glial differentiation and endogenous remyelination (Barnett and Linington, 2013; Hammond et al., 2014; Correale and Farez, 2015; Hammond et al., 2015; Brambilla, 2019). In a more specific example, studies demonstrated that when effects of endothelin-1, a secreted intercellular signaling molecule, were characterized after focal demyelination of the corpus callosum, it acted as a negative regulator of NG2 glial differentiation and functional remyelination (Hammond et al., 2014). Moreover, ablation or inhibition of endothelin receptor-B accelerates oligodendrocyte progenitor differentiation and remyelination (Hammond et al., 2015). Similarly, other proteins, such as bone morphogenetic proteins, have negative effects on oligodendrocytes following spinal cord injury (SCI) (Wang et al., 2011). This has relevance to the response to disease states.

On the other hand, it has become more widely recognized that astrocytes can also have neuroprotective effects and enhance axonal and neuronal regeneration (Faulkner et al., 2004; Anderson et al., 2016). Reactive astrocytes in some cases

suppress immune responses following CNS injury, maintain extracellular homeostasis and produce growth factors (Belanger and Magistretti, 2009). Thus, newly proliferated astrocytes may interact and organize into scars that surround and isolate tissue lesions and protect or enhance regeneration. For example, after SCI (Wanner et al., 2013), signal transducer and activator of transcription 3 (STAT3), expressed by reactive astrocytes, has a key role in regeneration that includes control of inflammation (Zhong et al., 1994; Hong and Song, 2014). Selective deletion of the Stat3 driver after a wound leads to a significant increase of immune cell infiltration and neurodegeneration (Bush et al., 1999; Anderson et al., 2016). Astrocytes also express neurotrophins and growth factors that promote myelination (Eroglu, 2009; Moore et al., 2011; Zamanian et al., 2012; Barnett and Linington, 2013; An et al., 2019). Together, these observations suggest that astrocytes may be critical for the recovery of function and survival at particular times after injury.

To evaluate the effects of injury on astrocyte function and their production of specific molecules, gene transcriptome approaches have been used to characterize subtypes of astroglial cells in response to brain damage. GeneChip analysis of reactive astrocytic populations was evaluated in two brain injury mice models: neuroinflammation induced by a single intraperitoneal injection of lipopolysaccharide and focal ischemic stroke produced by transient middle cerebral artery occlusion (Zamanian et al., 2012). In both models, glial fibrillary acidic protein (GFAP) immunoreactivity is observed after 1 day and persists at least 1 week in combination with increased activated microglia. A core set of genes is

upregulated in reactive astrocytes of both injury models, however, at least 50% of the altered gene expression is specific to a given injury type. These data suggest that there are distinct subtypes of reactive astrocytes, reminiscent of distinct types of quiescent astrocytes, which can have either detrimental or beneficial effects during injury and repair (Zamanian et al., 2012; Liddelow and Barres, 2017; Liddelow et al., 2017).

Neurotoxic astrocytes may have negative effects on surrounding cells in response to inflammation. For example, they may secrete molecules that are inhibitory to neurite outgrowth. In addition, swelling of these astrocytes after injury may result in the release of excessive amounts of glutamate. One study suggests that the activation of these neurotoxic astrocytes may be induced by activated microglia through the secretion of cytokines (Liddelow et al., 2017). After induction, these cells secrete a neurotoxin of uncertain identity that induces rapid death of neurons and oligodendrocytes. Neuroprotective astrocytes, in contrast, are commonly induced by ischemia and their responses to the ischemia are beneficial. This population is geared toward restoring trophic support and synapse repair and to promote the survival and growth of neurons (Hayakawa et al., 2014). These astrocytes express high levels of neurotrophic factors and cytokines, including brain-derived neurotrophic factor (BDNF), cardiotrophin-like cytokine factor 1 (CLCF1), interlukin-6 (IL-6), and GDF15, as well as thrombospondins that promote synapse repair (Eroglu, 2009; Zamanian et al., 2012). Determining the cellular and molecular basis underlying the induction of this astrocytic population remains an issue to address and is also important with respect to disease.

1.3 Glial mGluR Expression and Function

Metabotropic receptors, along with ionotropic receptors, are the two types of receptors that respond to glutamate, a key excitatory neurotransmitter in the CNS. While ionotropic glutamate receptors (iGluRs) are ligand-gated ion channels, metabotropic glutamate receptors (mGluRs) are G protein-coupled receptors consisting of seven transmembrane domains. Moreover, iGluRs are subdivided into N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), and 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainate) receptors, while mGluRs are subdivided into Group I, Group II, and Group III receptors based on their signaling transduction pathways, amino acid sequence homology, and selectivity of agonists and antagonists (Pin and Duvoisin, 1995; Dingledine et al., 1999; D'Antoni et al., 2008; Byrnes et al., 2009a; Spampinato et al., 2018).

Group I mGluRs consist of mGluR1 and 5 and function through G_q-proteins, resulting in activation of phospholipase C (PLC), hydrolysis of phosphoinositides, release of calcium, and activation of protein kinase C (PKC). Further downstream signaling pathways include casein kinase 1, cyclin-dependent protein kinase 5, Jun kinase, mitogen-activated protein kinase/extracellular receptor kinase (MAPK/ERK), and mammalian target of rapamycin (mTOR)/p70 S6 kinase (Karim et al., 2001; Hou and Klann, 2004; Warwick et al., 2005; Page et al., 2006; Li et al., 2007; Saugstad and Ingram, 2008). Group II mGluRs, consisting of mGluR2 and 3, and Group III mGluRs, consisting of mGluR4, 6, 7 and 8, are associated with G_i- and

G_0-proteins, and are negatively coupled to adenylate cyclase. Activation of Group II and III mGluRs inhibits voltage-gated calcium entry into the cell and inhibits further glutamate release through a feedback mechanism, with evidence suggesting these receptors could also activate MAPK and phosphatidyl inositol 3-kinase (PI3K) pathways (Pin and Duvoisin, 1995; Iacovelli et al., 2002; D'Antoni et al., 2008; Byrnes et al., 2009a; Niswender and Conn, 2010; Spampinato et al., 2018).

Within the CNS, Group I, Group II, and Group III mGluRs have all been found on neurons, astrocytes, oligodendrocyte lineage cells, and microglia. While the functional roles of mGluRs on neurons have been comprehensively examined (Ferraguti and Shigemoto, 2006; Gerber et al., 2007; Niswender and Conn, 2010; Ribeiro et al., 2017), their role on glia has been minimally described (D'Antoni et al., 2008; Byrnes et al., 2009a; Spampinato et al., 2018). To define roles played by mGluRs on glia, experiments have largely depended on culture work where isolated cells can be manipulated with specific agonists. Critical future work is clearly necessary to extend these studies of function by using new animal models where specific receptors can be deleted from specific cells at particular time points.

1.3.1 Roles of mGluRs on oligodendrocytes: An increasing number of studies are indicating that mGluRs are found on oligodendrocytes. Transcriptome analysis indicates that this expression is distinct based on stage of oligodendrocyte lineage progression. All 8 mGluR subtypes can be found on oligodendrocyte lineage cells (Marques et al., 2016) with most of them highly expressed on early oligodendrocyte progenitors before decreasing in mature cells. mGluR6 expression peaks first, occurring in early oligodendrocyte progenitor stages, with mGluR1, 2, 5 and 8

expression peaking in newly formed but pre-myelinating oligodendrocytes. mGluR4 and 7 then peak during early myelinating stages before mGluR3 expression peaks during the latest stages of mature oligodendrocytes. *In vitro* studies are consistent with the gene arrays. mGluR1, 2, 3, 4 and 5 are highly expressed in young cells (Bagayogo and Dreyfus, 2009) before becoming downregulated in mature MBP+ oligodendrocytes (Luyt et al., 2003; Deng et al., 2004; Luyt et al., 2004; Spampinato et al., 2014; Zhang et al., 2014b).

Analysis in mouse and human tissue indicate that mGluR expression might change on a specific cell at a particular state of development during maturation of the brain. During mouse, as well as human development, mGluR1 and 5 are expressed on O4+ immature oligodendrocytes relatively early in development and then decrease later (Jantzie et al., 2010). In adult humans, immature oligodendrocyte lineage cells when cultured have the potential to express mGluR3 and mGluR5 (Luyt et al., 2004). Other mGluRs have not yet been reported on normal human cells of the oligodendrocyte lineage. It appears then that mGluR agonists may preferentially impact young vs older, more mature oligodendrocytes in normal animals.

In experiments designed to determine what this impact might be, agonists to the receptor have been applied to oligodendrocytes in culture. Group I agonists have been found to reduce oxidative stress and prevent cell death of the oligodendrocyte lineage cells (Deng et al., 2004; Luyt et al., 2006). This agonist can also elicit the release of growth factors such as BDNF from oligodendrocytes (Bagayogo and Dreyfus, 2009). Additionally, Group III agonists, can influence oligodendrocyte

progenitor cell (OPC) maturation and differentiation (Spampinato et al., 2014). Studies regarding the effects of Group II agonists on oligodendrocyte lineage cells thus far are lacking. Collectively, oligodendroglial mGluRs may mediate effects on oligodendrocyte development and ability to influence proximate cells.

1.3.2 Roles of mGluRs on microglia: In microglia cultured from rodents, evidence exists for the expression of all mGluR subtypes with the exception of mGluR7 (Biber et al., 1999; Taylor et al., 2002; Taylor et al., 2003; Taylor et al., 2005; Pinteaux-Jones et al., 2008; Byrnes et al., 2009b). These studies have evaluated effects of the receptors and indicated mixed effects. The application of a Group I mGluR agonist to the BV2 microglial cell line attenuates cell death, oxidative stress, inflammation, and proliferation, yet enhances BDNF expression under toxic conditions (Qiu et al., 2015; Ye et al., 2017; Huang et al., 2018). Treatment with a Group I or III mGluR agonist reverses activation of the microglia (Biber et al., 1999; Taylor et al., 2003; Pinteaux-Jones et al., 2008; Byrnes et al., 2009b). In contrast, in the case of Group II receptor agonists, stimulation of the receptors stimulates the microglia to exhibit a neurotoxic phenotype (Taylor et al., 2003; Pinteaux-Jones et al., 2008). This suggests that microglial mGluRs may be important drug targets, useful in treating neurologic diseases. However, which receptor is involved may determine whether effects will be beneficial or detrimental.

In contrast to culture work, the study of receptors on microglia has generally not been done *in vivo*, limiting at this point what the importance of these receptors is to the function of these cells. The exception is the Group I receptors mGluR1 and 5 that have both been identified on rodent microglia *in vivo* (Jantzie et al., 2010). On

the other hand, mGluRs have not been detected in adult human tissue from patients without neurological disabilities (Geurts et al., 2003; Geurts et al., 2005), suggesting that under normal circumstances the roles of mGluRs on microglia in people may be minimal.

1.3.3 Roles of mGluRs on astrocytes: Of the glial cells, mGluRs are particularly well studied in astrocytes. In general, microarray analysis of hippocampal or cortical astrocytes reveals that while all mGluRs are expressed in adult tissue in mice, the most abundant receptor is mGluR3 followed by mGluR5 (Sun et al., 2013). These two mGluRs are also present in humans under normal conditions, while others are undetectable (Geurts et al., 2003; Geurts et al., 2005; Newcombe et al., 2008; Sun et al., 2013). Importantly, astrocytic mGluR3 expression remains relatively stable at all ages (Sun et al., 2013), while mGluR5 expression is highest during development (Cai et al., 2000; Sun et al., 2013). Specifically, these studies reveal that astrocytic mGluR3 in mice has similar expression levels at 1-, 2-, 3-, and 12-weeks old (Sun et al., 2013). On the other hand, mGluR5 expression peaks at postnatal day 7 before rapidly declining after week 2 and continuously decreasing through adulthood, suggesting that these receptors are downregulated on astrocytes with increasing age (Cai et al., 2000; Sun et al., 2013).

In vitro studies indicate that expression of these receptors can be measured in astrocytes when the cells are removed from developing animals and grown in culture. The culture approach has the advantage of evaluating isolated cells, examining receptors and defining function. Interestingly, culture studies have revealed that the receptors on astrocytes are expressed in a regionally specific

manner. In general, as was the case *in vivo*, mGluR3 and 5 show strong expression when compared to all the other mGluRs (Biber et al., 1999). However, regional differences are apparent. While mGluR3 and 5 are found in astrocytes isolated from thalamus, tegmentum, cortex, hippocampus, and striatum, there are almost undetectable levels of these receptors expressed within the cerebellum. Regional differences are also indicated in studies of mGluR1. It is typically not expressed on cultured astrocytes from hippocampus or cortex (Condorelli et al., 1992; Miller et al., 1995; Balazs et al., 1997; Ciccarelli et al., 1997; Condorelli et al., 1997; Heck et al., 1997; Biber et al., 1999) and has limited expression in astrocytes isolated from thalamus (Biber et al., 1999), but has been found on astrocytes cultured from spinal cord (Silva et al., 1999). Expression of mGluR4, 6, 7 and 8 on astrocytes similarly show regional distribution (Ciccarelli et al., 1997; Biber et al., 1999; Besong et al., 2002). While mGluR4, 6, 7 and 8 are expressed by tegmental astrocytes, mGluR4 and 6 are found on astrocytes isolated from striatum, while mGluR7 and 8 are mostly identified in cerebellum (Biber et al., 1999). It is interesting to consider what these regional differences may signify. Transcriptome analysis has indicated that cultured astrocytes exhibit a phenotype akin to reactive astrocytes of the ischemic brain (Zamanian et al., 2012). These studies suggest that regional differences in astrocyte expression of mGluRs in culture may foretell regional differences that while not evident *in vivo*, will be evident after injury.

Rodent brain slices have been studied to bridge the gap between *in vitro* and *in vivo* studies. In particular, specific agonists of Group I and/or Group II mGluRs induce transient increases in intracellular calcium levels within astrocytes of

hippocampal slices as they do *in vivo* (Pasti et al., 1997; Shelton and McCarthy, 1999; Nett et al., 2002; Zur Nieden and Deitmer, 2006; Copeland et al., 2017). In concordance with *in vivo* studies also is the fact that astrocytic mGluR5 is developmentally regulated in slices with the highest expression occurring in slices isolated from P1-10 rodents before declining into adulthood (Cai and Kimelberg, 1997; Kimelberg et al., 2000).

The stimulation of mGluRs on astrocytes can lead to the release or uptake of neurotransmitters and growth factors. For example, treatment with the Group I/Group II agonist trans-(1S,3R)-1-amino-1,3-cyclopentanedicarboxylic acid (ACPD) leads to the release of neurotransmitters, including glutamate (Miller et al., 1995; Nakahara et al., 1997; Pasti et al., 1997; Biber et al., 1999), as well as BDNF (Jean et al., 2008). Stimulation specifically of Group II mGluRs on astrocytes can elicit the release of other growth factors in addition to BDNF. For example, Group II agonists can enhance BDNF (Durand et al., 2017), glial-derived neurotrophic factor (GDNF) (Battaglia et al., 2015), and TGF-β release (Bruno et al., 1998; Caraci et al., 2011). Astrocytic mGluR activation can also lead to enhanced glutamate uptake through both Group II and Group III receptors (Yao et al., 2005). These data suggest that astrocytes may play positive roles when the brain is damaged.

1.4 Astrocytic mGluRs in Demyelinating Disease

In models of disease and in human disease tissue, levels of astrocytic mGluRs are upregulated in or near lesions. Therefore, the roles of these receptors may be most apparent during development, become downregulated during adulthood, but

emerge to play critical roles during CNS disease. In addition to MS (Fulmer et al., 2014), other disease models in which mGluRs are elevated include Alzheimer's disease (AD) (Shrivastava et al., 2013), amyotrophic lateral sclerosis (ALS) (Vermeiren et al., 2006), epilepsy (Aronica et al., 2000; Ulas et al., 2000; Ferraguti et al., 2001; Umpierre et al., 2016) and spinal cord injury (Nicholson et al., 2012; Kim et al., 2016; Michot et al., 2017).

In at least one rodent model of MS mGluRs are increased in reactive astrocytes. For example, our lab has previously showed that following cuprizone administration in mice, mGluR1 and 5 are substantially upregulated on astrocytes in the midline of the corpus callosum, the site of the demyelinating lesion (Fulmer et al., 2014). On the other hand, these receptors were not found on microglia or CC1+ mature oligodendrocytes using this model. It is less clear whether glial cells are responsive to mGluR agonists in another multiple sclerosis model, the experimental autoimmune encephalomyelitis (EAE) model. Although some studies of tissue samples taken from EAE rodents indicate an increase in mGluR1 and 5 in the whole brain and forebrain, the cells expressing these receptors were not identified (Sulkowski et al., 2009; Sulkowski et al., 2013).

In an attempt to determine roles of mGluR agonists and antagonists in rodent models of MS, these drugs have been injected either locally within the lesion site or systemically. The Group I/Group II mGluR agonist ACPD injected directly into the cuprizone-induced lesion increases synthesis and release of BDNF, an effect that was blocked when BDNF was selectively deleted from astrocytes, suggesting that the mGluRs mediate the increase in this trophic factor in astrocytes (Fulmer et al.,

2014). In the case of the EAE models, effects of the mGluR agonists and antagonists were evaluated on the whole animal making the relative contribution of these receptors on glia compared to other cell types unknown. Of note, systemic administration of an mGluR4 agonist (Fazio et al., 2012; Fazio et al., 2014) or a general Group III agonist (Besong et al., 2002) suppressed clinical signs of the disease. Similarly, enhancing mGluR1 function significantly improved motor coordination in EAE mice (Fazio et al., 2008). On the other hand, mGluR1 and 5 antagonists had no effect on motor function (Fazio et al., 2008), nor did they affect myelin ultrastructure compared to EAE rats receiving vehicle (Sulkowski et al., 2013; Sulkowski et al., 2014; Dabrowska-Bouta et al., 2015). Future studies are needed to identify receptor positive cells to be in position to elucidate the mechanisms of these compounds and determine their potential to act through mGluRs on glia or other cell types.

These observations may be of relevance to human disease. Several studies have shown that many mGluRs are upregulated primarily on astrocytes and microglia in human MS lesions compared to control tissue (Geurts et al., 2003; Geurts et al., 2005; Newcombe et al., 2008). In the case of astrocytes, upregulated mGluR2/3 as well as 5 is reported on virtually all reactive astrocytes within lesion sites, while mGluR1, 4 and 8 are associated only with a subpopulation of those cells (Geurts et al., 2003; Geurts et al., 2005; Newcombe et al., 2008). Microglia also express mGluR1, 2/3 and 8 within active MS lesions (Geurts et al., 2003; Geurts et al., 2005; Newcombe et al., 2008) and cells morphologically resembling young oligodendrocytes have been reported to exhibit mGluR2/3 (Newcombe et al., 2008).

Unlike astrocytes, however, microglia and young oligodendrocytes do not upregulate mGluR5 in human samples, suggesting that astrocytic mGluR5 is particularly relevant to human demyelinating lesions.

1.5 The Cuprizone Model of Demyelination

As indicated above, several animal models of demyelination exist that allow for elucidating mechanisms of demyelination and remyelination. These models also allow for the study of different factors that may influence the demyelination and remyelination processes. Demyelination can be induced by immunizing animals with myelin proteins, as occurs in EAE, or through the use of toxic agents like lysolecithin, ethidium bromide, and cuprizone (bis(cyclohexanone)oxaldihydrazone). While each model has its own advantages and disadvantages, the cuprizone model permits the study of factors that influence remyelination in the absence of a robust immune component, making it less complicated to evaluate effects on the regeneration and repair of oligodendrocytes. Furthermore, cuprizone allow for longer demyelination and remyelination phases compared to other toxicant-induced models of demyelination, which may be more representative of the changes that occur in oligodendrocytes over longer periods of time.

Cuprizone is a copper chelator that induces consistent and reproducible demyelination in the midline of the corpus callosum of mice when milled into their feed and administered for 4-6 weeks. This is advantageous when studying effects on myelin proteins between different treatment groups since the same brain region can

be analyzed for changes in protein levels. This is in contrast to the EAE model in which lesions can occur throughout the CNS, making it difficult to compare changes within a lesion among different treatment groups. Nevertheless, it should be noted that cuprizone-induced demyelination has been reported in other areas of the brain, including the cerebellum (Groebe et al., 2009; Skripuletz et al., 2010) and several gray matter areas (Goldberg et al., 2015), although they do not appear to be as severely or reliably affected compared to the midline of the corpus callosum.

Demyelination in the corpus callosum, cerebellum, and gray matter areas may contribute to the impaired motor and muscle coordination observed after cuprizone treatment. Specifically, cuprizone treatment results in a temporal relationship between motor dysfunction and CNS demyelination (Franco-Pons et al., 2007). For example, rotarod tests that assess motor coordination showed significant decreases in the latency of mice to fall from the rotating rod after 5-6 weeks of cuprizone treatment (Ye et al., 2013; Liu et al., 2015). Moreover, mice fed cuprizone for 5-6 weeks also have motor coordination deficits as assessed by the balance beam assay, in which the number of slips are increased while crossing wooden beams of varying diameters (Ray et al., 2017). Others have used an open-field test, and demonstrated that mice fed cuprizone for 5 weeks had decreased velocity and traveled less distance than control-fed mice (Ye et al., 2013; Vakilzadeh et al., 2015). Additionally, locomotor activity and Y-maze tests have been used to characterize behavioral changes after 12 weeks of cuprizone. These mice exhibited hyperactivity, increased locomotor activity, and impaired spatial working memory compared to controls (Zhang et al., 2012).

Although the exact mechanisms of cuprizone toxicity remain unknown, some possibilities have been hypothesized. One possibility is that cuprizone inhibits copper-dependent enzymes that are either directly or indirectly related to myelin formation. For example, the mitochondrial enzymes cytochrome oxidase and monoamine oxidase in addition to enzymes like copper-zinc superoxide dismutase and ceruloplasmin require copper for proper energy metabolism. When these enzymes are not working properly, this may result in the death of oligodendrocytes and subsequent demyelination (Venturini, 1973; Matsushima and Morell, 2001; Torkildsen et al., 2008). Additionally, cytochrome c oxidase is needed for phospholipid synthesis, a key component of the myelin sheath. If copper deficiency alone was responsible for demyelination, however, then administration of copper to cuprizone-treated mice would be expected to reverse the deficits in myelin. Interestingly, this is not the case (Carlton, 1967), suggesting other mechanisms may be responsible for cuprizone-induced demyelination.

Another hypothesized mechanism of cuprizone-induced demyelination is that systemic copper deficiency may disrupt the homeostasis of other transition metals, including manganese, iron, and zinc (Moldovan et al., 2015). One study shows that 6 weeks of 0.2% cuprizone treatment does not change copper levels in the brain despite decreases in the blood, but notes decreased manganese levels in the cerebellum and striatum (Moldovan et al., 2015). Others have reported demyelination in human patients associated with copper deficiency and hyperzincemia (Prodan et al., 2002), supporting a possible link between copper deficiency and altered transition metal homeostasis. Outside of the CNS, cuprizone

decreases copper and manganese levels in the liver but increases iron in this organ (Moldovan et al., 2015). These changes in the liver are interesting to consider since the formation of megamitochondria have been reported in this organ following cuprizone treatment (Suzuki, 1969; Suzuki and Kikkawa, 1969). Taken together, while the mechanisms of cuprizone toxicity remain unknown, mitochondrial dysfunction in the brain as well as other organs has been implicated to play a role in this model.

Within the corpus callosum, 0.2% cuprizone causes the apoptotic death of oligodendrocytes between week 2 and 4 and peak demyelination occurs between week 4 and 5 (Matsushima and Morell, 2001). Upon removal of cuprizone after 6 weeks of treatment, spontaneous remyelination occurs in which approximately 90% of the axons remyelinate in the first 4 weeks of the recovery period (Matsushima and Morell, 2001). Some spontaneous remyelination also occurs prior to the removal of cuprizone at 6 weeks. Of note, numbers of OPCs, microglia, and astrocytes peak after 4-5 weeks of cuprizone, corresponding with the peak demyelination period. While astrocytes undergo many changes following a demyelinating lesion, our lab has previously found that cuprizone induces upregulated expression of mGluR1 and mGluR5 on these cells (Fulmer et al., 2014). Furthermore, our lab and others have reported upregulated expression of growth factors and neurotrophins in astrocytes after cuprizone, including BDNF (Fulmer et al., 2014; An et al., 2019), ciliary neurotrophic factor (CNTF), and insulin-like growth factor II (IGF-II) (An et al., 2019). Thus, astrocytes may be optimally positioned to enhance myelination after a lesion.

1.6 Glia as a Source of BDNF to Promote Remyelination

In addition to astrocytes, BDNF has also been reported in oligodendrocytes (Dougherty et al., 2000; Dai et al., 2003; Bagayogo and Dreyfus, 2009) and microglia (Dougherty et al., 2000; Trang et al., 2009; Trang et al., 2011; Zhang et al., 2014a). BDNF itself is also capable of acting on oligodendrocytes (Du et al., 2003; Du et al., 2006a; Du et al., 2006b; Xiao et al., 2010; Wong et al., 2013; Fletcher et al., 2018; Nguyen et al., 2019) and microglia (Mizoguchi et al., 2009; Spencer-Segal et al., 2011; Zhang et al., 2014a), raising the possibility that a positive autocrine feedback loop may occur in these cells.

To determine the possible roles of BDNF related to myelination, several studies have investigated the role of this neurotrophin on oligodendrocytes, both *in vitro* and *in vivo*. Cell culture studies reveal that BDNF enhances the differentiation and total numbers of oligodendrocytes of the basal forebrain (Du et al., 2003; Du et al., 2006b). *In vivo*, BDNF deficient mice (BDNF +/- mice) have decreases in myelin proteins during development in the spinal cord, optic nerve, and basal forebrain (VonDran et al., 2010; Xiao et al., 2010). While myelin protein levels in the spinal cord and optic nerve recover into adulthood (Xiao et al., 2010), myelin deficits in the basal forebrain persist throughout life (VonDran et al., 2010).

Animal models of demyelination further demonstrate the importance of BDNF on myelin proteins. In the cuprizone model, BDNF deficient mice have an exacerbated loss of myelin proteins in the lesioned corpus callosum compared to their wildtype counterparts (VonDran et al., 2011). These deficits correlate with

decreases in myelin proteins during the demyelination and remyelination phases of the model.

Because BDNF acts on TrkB receptors, studies using mice with deleted TrkB receptors have been beneficial in examining the role of BDNF signaling. These reports indicate that the conditional knockout of TrkB from MBP+ mature oligodendrocytes results in decreased myelin proteins in the spinal cord and corpus callosum both during development and in adulthood (Wong et al., 2013). Likewise, in the cuprizone model, conditional deletion of TrkB from CNPase+ oligodendrocytes worsens demyelination (Fletcher et al., 2018; Nguyen et al., 2019). On the other hand, enhancing endogenous BDNF in the lesion site (Fulmer et al., 2014) or delivering a BDNF mimetic or TrkB agonist via intracerebroventricular administration (Fletcher et al., 2018; Nguyen et al., 2019) increases myelin proteins, suggesting that BDNF may aid in the repair of a demyelinating lesion.

Furthermore, the role of BDNF in demyelinating diseases may also have human relevance. For example, evidence suggests that levels of BDNF are decreased in patients with MS (Sarchielli et al., 2002; Azoulay et al., 2005; Caggiula et al., 2005; Azoulay et al., 2008; Mehrpour et al., 2015; Vacaras et al., 2017). In addition, some therapeutic agents used for MS elevate levels of BDNF in patients and this typically corresponds to a decrease in the severity of the disease (Azoulay et al., 2005; Mehrpour et al., 2015; Vacaras et al., 2017). Thus, enhancing BDNF levels in patients with these types of diseases may be beneficial.

In considering how to take advantage of these observations, one approach is to administer BDNF or otherwise stimulate TrkB receptors. However, application of

exogenous BDNF to enhance oligodendrocyte repair may prove difficult since the molecule does not readily cross the blood-brain-barrier (Poduslo and Curran, 1996). Therefore, previous work in our lab considered the feasibility of examining small molecules that enhance endogenous sources of BDNF. It was found that endogenous BDNF could be synthesized and released from cultured astrocytes when stimulated with the Group I/II mGluR agonist ACPD (Jean et al., 2008). ACPD also elevates BDNF, and subsequently myelin proteins, *in vivo* when injected directly into a cuprizone-induced lesion (Fulmer et al., 2014). Astrocytes were the only cells within the lesion to exhibit both BDNF and mGluRs, particularly Group I mGluRs, suggesting that they are the only cells within the lesion that have the ability to respond to ACPD and regulate BDNF levels. Supportive of this claim, inducing the deletion of BDNF from GFAP+ astrocytes blocked the increases in myelin proteins elicited by ACPD. These data suggest that Group I mGluRs on astrocytes may be a potential target to reverse deficits associated with demyelination through astrocyte-derived BDNF.

In the present work, I have examined whether a more specific mGluR agonist that binds only to Group I receptors, CHPG, can mimic effects of ACPD in cuprizone-treated mice. Moreover, in considering the therapeutic potential of an mGluR agonist, I examined effects of CHPG when it is injected through the more clinically relevant route of a peripheral injection. I hypothesize that CHPG will act through Group I mGluRs on astrocytes to release astrocytic BDNF that then binds to TrkB on oligodendrocytes to increase myelin proteins. Furthermore, I hypothesize that CHPG will promotes the differentiation of oligodendrocyte lineage cells, enhance

myelination itself, and reverse behavioral deficits associated with a cuprizone-induced lesion. These studies may suggest that Group I mGluRs on astrocytes could be a potential therapeutic target to promote myelination in the diseased brain by stimulating the production and release of BDNF and that manipulation of astrocytes may be a clinically important direction worth pursuing.

Chapter 2: Materials and Methods

2.1 Experimental animals

Mice were managed by the Rutgers Robert Wood Johnson Animal Facility. Animal maintenance, husbandry, housing, transportation, and use were in compliance with the Laboratory Animal Welfare Act (PL 89-544; PL-91-579) and with NIH guidelines (NIH Manual Chapter 4206). Mice were housed in a temperature- and humidity-controlled environment with a 12-hour light-dark cycle and maintained on standard mouse chow with water *ad libitum* prior to cuprizone treatment.

Wild-type mice and inducible conditional knockout mice in which BDNF or mGluR5 was deleted from GFAP+ cells were on a C57BL/6 background. Inducible conditional knockout mice in which TrkB was deleted from PLP+ cells were on a C57/Balbc/129 background. For experiments in which BDNF was conditionally deleted from GFAP+ cells, hGFAP-CreERT2-BDNF$^{fl/fl}$-eGFP mice or BDNF$^{fl/fl}$-eGFP mice lacking cre expression (used as controls) were utilized. hGFAP-CreERT2-BDNF$^{fl/fl}$-eGFP mice were generated by crossing hGFAP-CreERT2 mice (MMRRC) with BDNF$^{fl/fl}$ mice (The Jackson Laboratory). We have previously reported successful deletion of BDNF from GFAP+ cells in these mice (Fulmer et al., 2014). Similarly, hGFAP-CreERT2-mGluR5$^{fl/fl}$-eGFP mice or mGluR5$^{fl/fl}$-eGFP mice lacking cre expression (used as controls) were used for experiments in which mGluR5 was conditionally deleted from GFAP+ cells. hGFAP-CreERT2-mGluR5$^{fl/fl}$-eGFP mice were generated by crossing hGFAP-CreERT2 mice (MMRRC) with mGluR5$^{fl/fl}$ mice (a gift from Anis Contractor, Northwestern University, Chicago, Illinois). These mice

exhibited 51% deletion of mGluR5+GFAP+/total GFAP+ cells compared to controls. Finally, PLP-CreERT2-TrkB$^{fl/fl}$-lacZ mice or TrkB$^{fl/fl}$-lacZ mice lacking cre expression (used as controls) were used for experiments in which TrkB was conditionally deleted from PLP+ cells. PLP-CreERT2-TrkB$^{fl/fl}$-lacZ mice were generated by crossing PLP-CreERT2 mice (a gift from Ueli Suter, ETH Zürich, Zürich, Switzerland) with TrkB$^{fl/fl}$ mice (a gift from Luis Parada, University of Texas Southwestern, Dallas, Texas). These mice exhibited 69% deletion of TrkB+CC1+/total CC1+ cells compared to controls. To induce the deletion of BDNF or mGluR5 from astrocytes, or TrkB from oligodendrocytes, 1 mg tamoxifen dissolved in 10% ethanol in sunflower seed oil was injected intraperitoneally twice daily for 5 days the week before being placed on cuprizone feed. Both Cre- and Cre+ mice were treated with tamoxifen.

2.2 Cuprizone treatment

Demyelination was induced by feeding 8-10-week-old male mice 0.2% cuprizone (Sigma-Aldrich) milled into mouse feed (Envigo Teklad) for 4-6 weeks. The control feed was identically processed, but without cuprizone supplementation (Envigo Teklad). Cuprizone and control feed were changed every 2 to 3 days and animal cages were changed every 1-2 weeks.

2.3 Intraperitoneal and stereotaxic injections

Intraperitoneal injections of CHPG (Tocris Bioscience) in saline vehicle were administered to mice at doses of 20 or 40 mg/kg and given as 20 μL/g. Injections were given either 24 hours before or 6 and 24 hours before euthanasia.

Alternatively, CHPG was injected every other day for 2 weeks. Controls received 0.9% saline injections at similar times. In some experiments, 100 μg 5-ethynyl-2'-deoxyuridine (EdU) in saline vehicle was injected intraperitoneally 15 minutes and 22 hours after the first CHPG injection.

Stereotaxic injections were made into the corpus callosum of mice at coordinates from bregma -1.0 mm and 0.5 mm lateral to the sagittal sinus. A Hamilton syringe was lowered to a depth of 1.875 mm to target the midcaudal corpus callosum over the fimbria-fornix. Stereotaxic injections were given as 2 μL infusions at a concentration of 5 μM for CHPG (Tocris Bioscience) or 150 μM for 2-methyl-6-(phenylethynyl)pyridine (MPEP) (Tocris Bioscience) in saline vehicle. MPEP was given one hour before intraperitoneal injection of CHPG (40 mg/kg) or vehicle. 2 μL of 0.9% saline vehicle was infused in control animals.

2.4 Western blot

The midline of the corpus callosum overlying the fimbria-fornix and rostral hippocampus, from bregma -0.06 to -2.06, was dissected using a mouse brain matrix that allows 1 mm coronal cuts along the rostral-caudal axis. Tissue was immediately frozen at -80°C before being lysed by triturating tissue in 60 μL of buffer (pH 7.5) containing 50 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 2 mM EGTA, 1% CHAPS, 0.5% NP-40, 1% Triton X-100, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 20 μg/mL soybean trypsin inhibitor, 50 mM NaF, 1 mM PMSF, 0.5 μM microcystin, and 1 mM ortho-vanadate. Protein concentrations were determined using a BCA protein assay kit (Pierce).

Antibodies for Western blots include rabbit polyclonal antibodies to BDNF (1:200, sc-546, Santa Cruz Biotechnology), NG2 (1:750, AB5320, Millipore), and PDGFRα (1:750, sc-338, Santa Cruz Biotechnology), a mouse monoclonal antibody to MBP (1:400, MCA184S, Serotec), and a goat polyclonal antibody to PLP (1:1,000, sc-18529, Santa Cruz Biotechnology). Mouse monoclonal antibodies to GAPDH (1:1,000,000, H86504M, Meridian Life Science) and beta-tubulin (1:200,000, T4026, Sigma-Aldrich) were used as loading controls.

For the analysis of BDNF, MBP, and PLP, 10 μg protein was run on 12% Bis-Tris gels (Invitrogen). For the analysis of NG2 and PDGFRα, 20 μg protein was run on 3-8% Tris-Acetate gels (Invitrogen). Protein was then transferred to a PVDF membrane (Millipore) and membranes were exposed to HRP-linked anti-rabbit (NA934, GE Healthcare), anti-mouse (NA931, GE Healthcare) or anti-goat (705-035-003, Jackson ImmunoResearch) antibodies as appropriate. All Western blots were visualized with a chemiluminescence system (GE Healthcare) and data were analyzed using Quantity One V 4.2.1 software (Bio-Rad).

2.5 Immunohistochemistry

Brains from mice perfused with 4% paraformaldehyde (PFA) were post-fixed in PFA for 2 hours followed by 30% sucrose/PBS for 48 hours. Brains were embedded in OCT (Tissue Tek) and frozen at -80°C before 20 μm serial sections were taken in the coronal plane from the basal forebrain to the caudal hippocampus using a Leica cryostat.

Antibodies for immunohistochemistry include mouse monoclonal antibodies

to CC1 (1:800, OP80, Calbiochem), GFAP (1:500, sc-33673, Santa Cruz Biotechnology), and PCNA (1:1,000, sc-25280, Santa Cruz Biotechnology), rabbit polyclonal antibodies to Olig2 (1:250, 18953, Immuno-Biological Laboratories), mGluR1 (1:500, 07-617, Millipore), mGluR5 (1:500, AB5675, Millipore), and cleaved Caspase-3 (1:400, D175, Cell Signaling Technology), and goat polyclonal antibodies to PDGFRα (1:50, AF-307-NA, R&D Systems) and SOX10 (1:50, AF2864, R&D Systems). Secondary antibodies used include anti-mouse AlexaFluor 488 (Invitrogen), anti-mouse AlexaFluor 594 (Invitrogen), anti-rabbit Cy3 (Invitrogen), and anti-goat AlexaFluor 488 (Invitrogen). EdU was stained according to the manufacturer's instructions (Click-iT Edu Imaging Kit, C10339, Invitrogen).

A Leica fluorescent microscope equipped with a Lumenera Infinity3S-1UR CCD camera was used for characterizing co-localization and Infinity Analyze software was used for image capture. Images of 20 μm serial sections separated by 80 μm were obtained with a 40× objective lens from the midline corpus callosum (a width of 240 μm) overlying the fimbria-fornix and rostral hippocampus, from bregma -0.06 to -2.06. Double counting was corrected for based on cell body diameter and section thickness allowing for an estimation of cell number (Abercrombie, 1946). An equal number of matched sections were analyzed for each treatment group. Cells were counted using the ImageJ Cell Counter plug-in and analysis was done blinded to treatment groups.

2.6 Pathology studies

150 mg/kg CHPG was injected intraperitoneally in five 12 week old male C57BL/6 mice. Two matched controls received 0.9% saline vehicle. Mice were examined daily for signs of morbidity. At study end, mice were perfused with saline followed by 4% PFA. At necropsy, gross findings were recorded and all tissues were collected in 10% neutral buffered formalin. Five levels of brain, lung, liver, kidney, heart and spleen were processed to hematoxylin and eosin stained microscope slides and examined by Dr. Michael Goedken, a board certified toxicological pathologist.

2.7 Transmission electron microscopy

Following cardiac perfusion with 4% paraformaldehyde/2.5% glutaraldehyde, the brain was removed and sectioned sagitally in 150 μm sections using a Leica vibratome. Sections of the midline of the corpus callosum were post-fixed in 1% osmium tetroxide, stained with uranyl acetate, dehydrated with graded ethanols, and embedded in Poly/Bed812 resin (Polysciences, Inc.). Thin sections (1 μm) were stained with toluidine blue for orientation and ultrathin sections (0.13 μm) were subsequently collected on copper grids. Images of the axons cut in cross section were obtained using a transmission electron microscope (Philips CM12) at 5000X magnification. For each mouse, images were taken in the corpus callosum immediately caudal to its junction with the fornix. All axons in 10 random fields were analyzed to calculate the percentage of myelinated fibers. ImageJ software was used to calculate the axonal diameter, myelin thickness, and g-ratios of 3 animals; 150 axons per mouse were randomly selected for evaluation using the ImageJ G

Ratio plug-in. The g-ratio represents the ratio of the diameter of the axon to the diameter of the axon plus myelin. Analysis was done blinded to treatment groups.

2.8 Balance beam test

A round plexiglass rod, 1.3 cm in diameter and 70 cm in length, was placed 17 cm above soft bedding and used to test motor coordination. This test took place over 2 consecutive days: 1 day of training and 1 day of testing. A black box was placed at the end of the beam as the finish point. Nesting materials from home cages were placed in the black box to attract the mouse to the finish point. On training days, each mouse crossed the beam 2 times. On the test day, times to cross the beam and the number of paw slips were recorded with a video camera. Video analysis was done blinded to treatment groups. 12 mice per treatment group were used for analysis.

2.9 Wire hang test

A round metal wire, 2 mm in diameter and 43 cm in length, was used to test grip strength. The wire was attached to two poles at a height of 43 cm above a cage with soft bedding to break the fall. Mice began the test with the forelimbs attached to the wire and the time to fall was recorded with a video camera. Video analysis was done blinded to treatment groups. 8 mice per treatment group were used for analysis.

2.10 Data analysis

For each experiment, a control- and cuprizone-fed mouse injected with saline or CHPG were compared. In some cases, cuprizone-fed mice injected with saline or CHPG were also injected with MPEP or vehicle and compared with one another. In other cases, tamoxifen-injected mice were fed cuprizone and then injected with saline or CHPG. For the CC1+EdU+ cell counts, cuprizone-treated mice were injected with saline or CHPG and compared with one another. Each experiment was repeated ≥ 3 times and the data are presented as ± SEM. Statistical differences were determined using ANOVA followed by Fisher's protected least significant *post hoc* test or Student's *t* test as appropriate. For analysis of g-ratio vs. axon diameter, linear regression analysis was used to compare differences in slopes between cuprizone-fed mice ± CHPG. Conditions were considered significant at p < 0.05.

<center>**Chapter 3: Results**</center>

I hypothesize that CHPG will act through Group I mGluRs on astrocytes to release astrocytic BDNF that then binds to TrkB on oligodendrocytes to increase myelin proteins. Furthermore, I hypothesize that CHPG will promote the differentiation of oligodendrocyte lineage cells, enhance myelination itself, and reverse behavioral deficits associated with a cuprizone-induced lesion. Three Specific Aims have been developed to test this by cellular and behavioral changes.

1. Define the effects of CHPG on oligodendrocyte lineage cells following a cuprizone-induced lesion.
2. Investigate the effects of CHPG on myelination and behavior.
3. Determine the roles of Group I mGluRs on astrocytes, astrocyte-derived BDNF, and oligodendroglial TrkB on CHPG-mediated effects.

3.1 Define the effects of CHPG on oligodendrocyte lineage cells following a cuprizone-induced lesion.

3.1.1 CHPG increases MBP when injected directly into the lesioned corpus callosum at 6 hours

In previous studies, injection of the mGluR Group I/II agonist ACPD directly into the lesioned corpus callosum increases BDNF and myelin proteins (Fulmer et al., 2014). Since ACPD binds to both Group I and Group II mGluRs, preliminary studies in our lab injected a Group I agonist, CHPG, or a Group II agonist, DCGIV, directly into the corpus callosum of mice fed cuprizone for 4 weeks to further elucidate the receptors responsible for the effects of ACPD. Only injections of the

Group I agonist CHPG, but not the Group II agonist DCGIV, enhanced myelin proteins in the lesion site after 6 hours, consistent with the previous observation that astrocytes contain both Group I mGluRs and BDNF (Fulmer et al., 2014). My initial experiments repeated these preliminary studies with CHPG. 5 μM CHPG was injected stereotaxically into the corpus callosum of control-fed and cuprizone-fed mice 6 hours prior to dissection. Tissue from the midline of the corpus callosum was then collected and processed for Western blot analysis. As was shown with ACPD, 4 weeks of cuprizone treatment results in significant decreases in MBP. Following injection with CHPG, however, a significant increase in MBP was observed in cuprizone-fed mice (Fig. 1), suggesting that Group I mGluRs are responsible for the increases in myelin proteins following injection with ACPD or CHPG.

3.1.2 Intraperitoneal injection of CHPG increases BDNF and myelin proteins in the demyelinated corpus callosum at 24 hours

To determine if CHPG can mimic the effects of ACPD even when injected through the more clinically relevant route of a peripheral injection, CHPG was injected intraperitoneally at 20 or 40 mg/kg 24 hours and 6 hours prior to dissection. While cuprizone treatment results in significant decreases in BDNF, MBP, and PLP protein levels in saline vehicle-injected mice, both 20 mg/kg (Fig. 2A) and 40 mg/kg (Fig. 2B) doses of CHPG significantly increase BDNF, MBP, and PLP when compared to their own cuprizone-fed controls (Fig. 2A-B, insets). 40 mg/kg doses elicit a greater increase (> 4-fold) in these proteins than does the 20 mg/kg dose (>

2-fold) when cuprizone treated samples are compared. Interestingly, CHPG has no effect in control-fed mice.

3.1.3 CHPG does not affect oligodendrocyte cell numbers at 24 hours

Changes in myelin proteins could be due to an increase in myelin proteins per cell or due to an increase in numbers of mature myelin-producing cells. To address this question, immunofluorescent staining was performed using Olig2 as a marker for all oligodendrocyte lineage cells and CC1 as a marker for mature oligodendrocytes (Fig. 3A; experiments performed with Lauren Lercher). Cuprizone treatment causes a decrease in Olig2+CC1+ cell numbers in saline-injected mice, but CHPG has no effect on these cells in either control-fed or cuprizone-fed mice at 20 or 40 mg/kg (Fig. 3B). This observation suggests that CHPG increases myelin proteins per cell at 24 hours. Furthermore, the total numbers of Olig2+ cells, indicative of total oligodendrocyte lineage cells, remain relatively unchanged due to cuprizone or CHPG treatment (Fig. 3C). This suggests that oligodendrocyte progenitor cells (OPCs) increase as CC1+ mature oligodendrocytes decrease.

3.1.4 CHPG decreases OPC marker protein levels at 24 hours

To determine if CHPG affects OPCs, Western blots were performed for PDGFRα and NG2. Cuprizone treatment for 4 weeks significantly increases these markers, consistent with previous work (VonDran et al., 2011). While CHPG does not affect PDGFRα and NG2 protein levels at 20 mg/kg, 40 mg/kg significantly reduces these markers (Fig. 4). These data suggest that CHPG could be promoting

the differentiation of these cells into more mature oligodendrocytes. Alternatively, CHPG may be decreasing OPC proliferation or increasing death of OPCs.

3.1.5 CHPG does not affect proliferation or cell death of OPCs

Since decreases in OPC markers do not necessarily indicate decreases in numbers of OPCs, immunofluorescent staining for PDGFRα was performed. Consistent with previous results, cuprizone increases the numbers of PDGFRα+ cells (Fig. 5; experiments performed with Lauren Lercher). Following intraperitoneal injections of 40 mg/kg CHPG, however, numbers of PDGFRα+ cells decrease, consistent with the Western blot findings.

Since changes in OPC proliferation or cell death could contribute to the decreases in PDGFRα cell numbers, co-staining of PDGFRα with PCNA or cleaved caspase-3 was performed, respectively. As shown in Fig. 6A (experiments performed with Lauren Lercher), numbers of PDGFRα+PCNA+ relative to total numbers of PDGFRα+ cells are unchanged after CHPG treatment. Furthermore, numbers of PDGFRα+Caspase+ relative to total numbers of PDGFRα+ cells are also unaltered following CHPG (Fig. 6B; experiments performed with Danielle Sainato), suggesting that CHPG does not affect the proliferation or survival of OPCs at 24 hours. This is consistent with the possibility that CHPG may enhance the differentiation of these cells. Although CHPG did not increase the numbers of mature CC1+ oligodendrocytes at 24 hours (Fig. 3A-B), longer time points may be needed to observe changes in differentiation.

3.1.6 Effects of CHPG last up to 3 days

To investigate if CHPG-elicited increases in BDNF and myelin proteins are maintained beyond 24 hours, 2 injections of 40 mg/kg CHPG were administered as described earlier. Mice were then dissected at 1 day, 3 days, or 7 days after injection. Interestingly, BDNF, MBP, and PLP remain elevated up to 3 days following CHPG administration and this effect is reduced at 7 days (Fig. 7).

3.1.7 CHPG does not induce toxicity in major organs

To examine if CHPG elicits any toxic effects at the gross or microscopic level in major organs, control-fed mice were injected with saline vehicle or 150 mg/kg CHPG, approximately 5-fold the therapeutic dose, and perfused after 3 days. Histomorphological surveillance of hematoxylin and eosin stained sections was performed by Dr. Michael Goedken, a board certified toxicological pathologist, blinded to treatment groups. There were no noteworthy pathological findings in the brain, liver, lung, heart, kidney, or spleen in any of the 5 mice that received 150 mg/kg CHPG compared to 2 matched control mice that received saline (Table 1). The results suggest that there are no gross or microscopic compound-related findings for CHPG at doses up to 5 times the observed therapeutic dose.
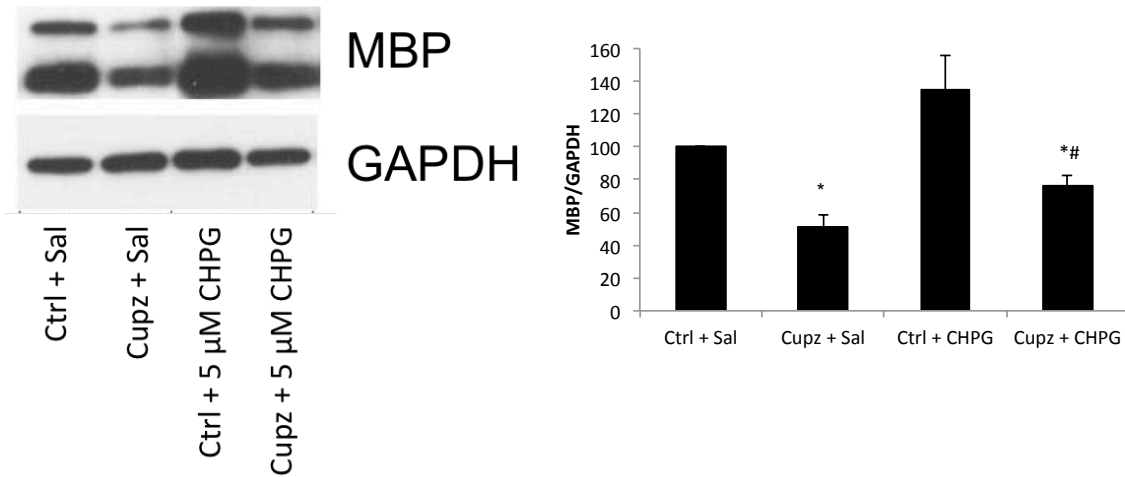
Figure 1. CHPG increases MBP in cuprizone-fed mice 6 hours after stereotaxic injection directly into the lesioned corpus callosum. Western blots demonstrate MBP protein levels in the corpus callosum of mice subjected to a 4 week cuprizone lesion and injected stereotaxically with 5 μM CHPG or 0.9% saline vehicle 6 hours prior to dissection. Graph represents densitometric analysis of Western blots normalized to GAPDH and presented as percent saline-injected control-fed mice, ANOVA. *Sig. diff. from saline-injected control-fed mice, $*p<0.05$, #Sig. diff. from saline-injected cuprizone-fed mice, $\#p<0.05$. Each experiment consists of one mouse per treatment group and each experiment was repeated four times.

Figure 2. Cuprizone-lesioned mice exhibit increases in BDNF and myelin proteins 24 hours after intraperitoneal injection of CHPG. Western blots demonstrate BDNF, MBP, and PLP protein levels in the corpus callosum of mice subjected to a 4 week cuprizone lesion and injected ip with 20 mg/kg **(A)** or 40

mg/kg **(B)** CHPG or 0.9% saline vehicle 6 and 24 hours prior to dissection. Graph represents densitometric analysis of Western blots normalized to GAPDH and presented as percent saline-injected control-fed mice, ANOVA. Insets represent densitometric analysis of Western blots presented as percent saline-injected cuprizone-fed mice, t-test. *Sig. diff. from saline-injected control-fed mice, *p<0.05, #Sig. diff. from saline-injected cuprizone-fed mice, #p<0.05. Each experiment consists of one mouse per treatment group and each experiment was repeated four times.

A.

B.

C.
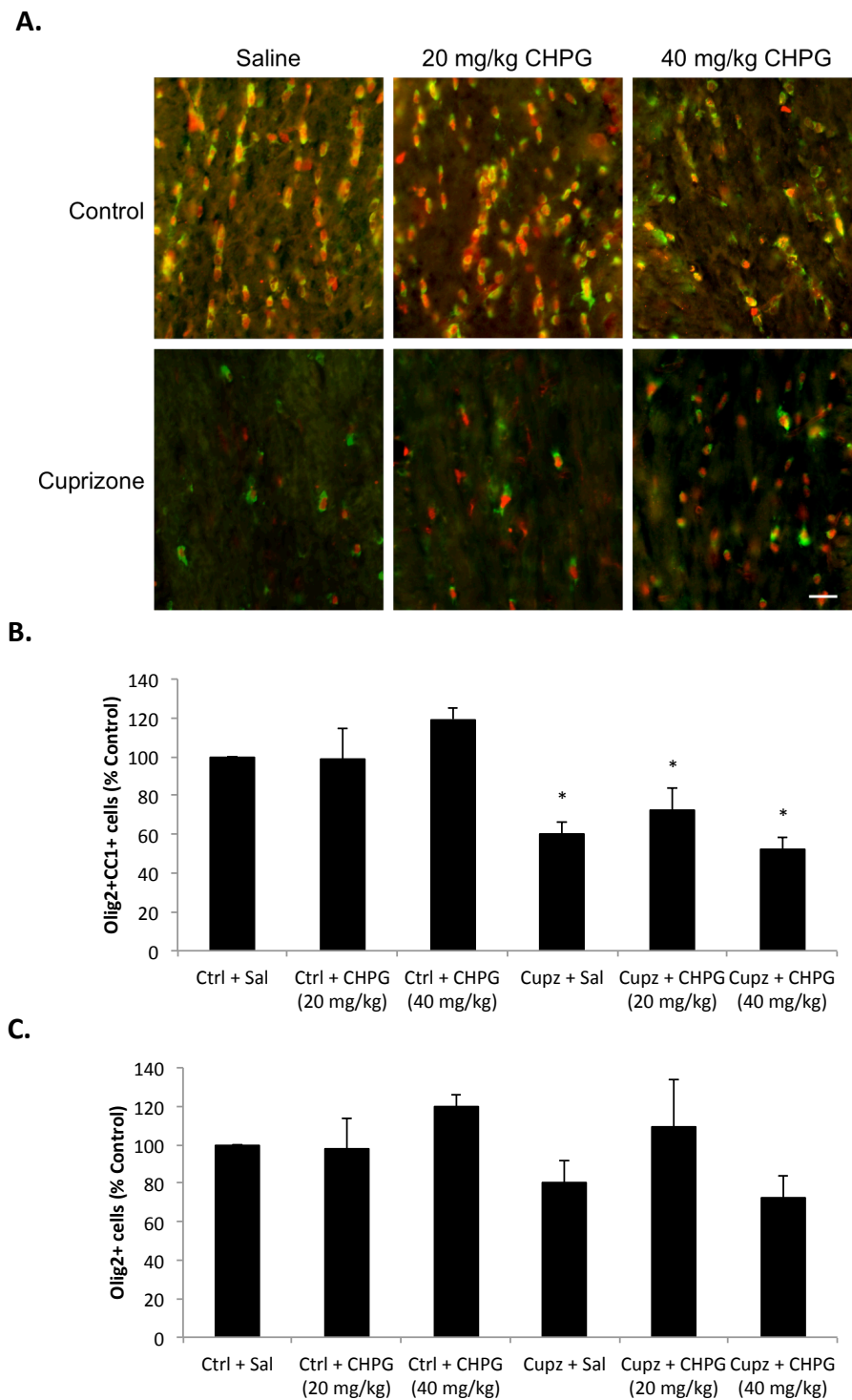
Figure 3. CHPG does not affect oligodendrocyte cell numbers at 24 hours, suggesting that CHPG increases myelin proteins per cell. (A) Olig2+CC1+ staining in the corpus callosum of mice subjected to a 4 week cuprizone lesion and

injected ip with 20 or 40 mg/kg CHPG or 0.9% saline vehicle 6 and 24 hours prior to perfusion. Green, CC1; Red, Olig2. **(B)** Quantification of co-localized Olig2+CC1+ cells. *Sig. diff. from saline-injected control-fed mice, *$p<0.05$, ANOVA. **(C)** Quantification of total Olig2+ cells. 30 matched 20 μm sections taken from the corpus callosum were counted per mouse (Experiments performed with Lauren Lercher). Each experiment consists of one mouse per treatment group and each experiment was repeated three times. Scale bar, 20 μm.
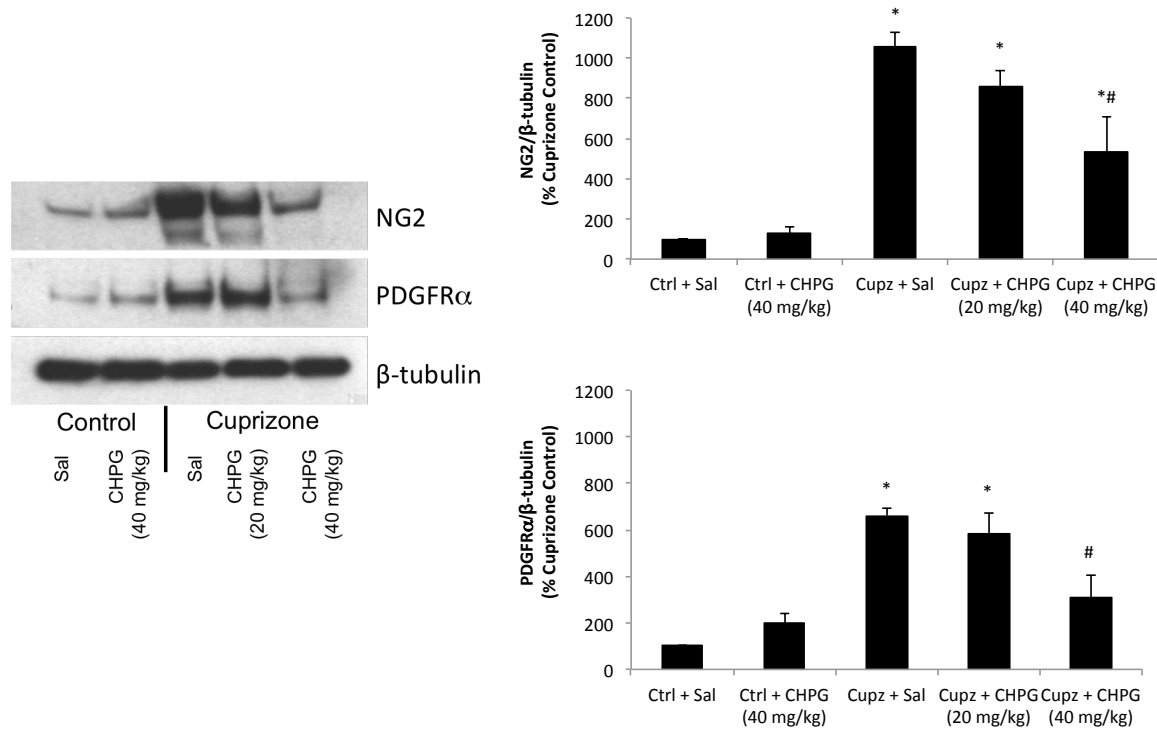
Figure 4. Cuprizone-lesioned mice exhibit decreases in OPC markers 24 hours after intraperitoneal injection of 40 mg/kg CHPG. Western blots demonstrate NG2 and PDGFRα protein levels in the corpus callosum of mice subjected to a 4 week cuprizone lesion and injected ip with 20 mg/kg or 40 mg/kg CHPG or 0.9% saline vehicle 6 and 24 hours prior to dissection. Graph represents densitometric analysis of Western blots normalized to β-tubulin and presented as percent saline-injected control-fed mice, ANOVA. *Sig. diff. from saline-injected control-fed mice, *p<0.05, #Sig. diff. from saline-injected cuprizone-fed mice, #p<0.05. Each experiment consists of one mouse per treatment group and each experiment was repeated four times.
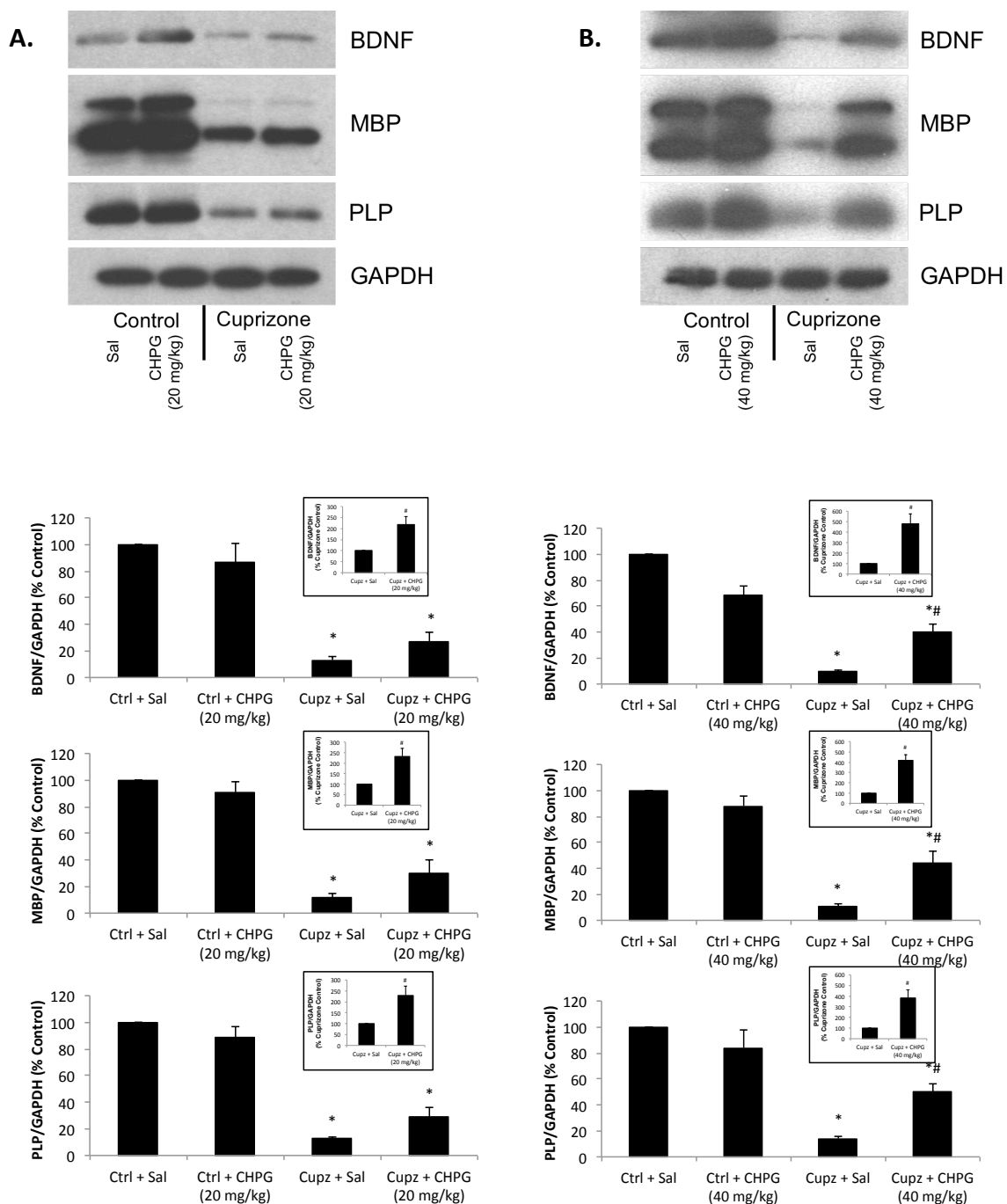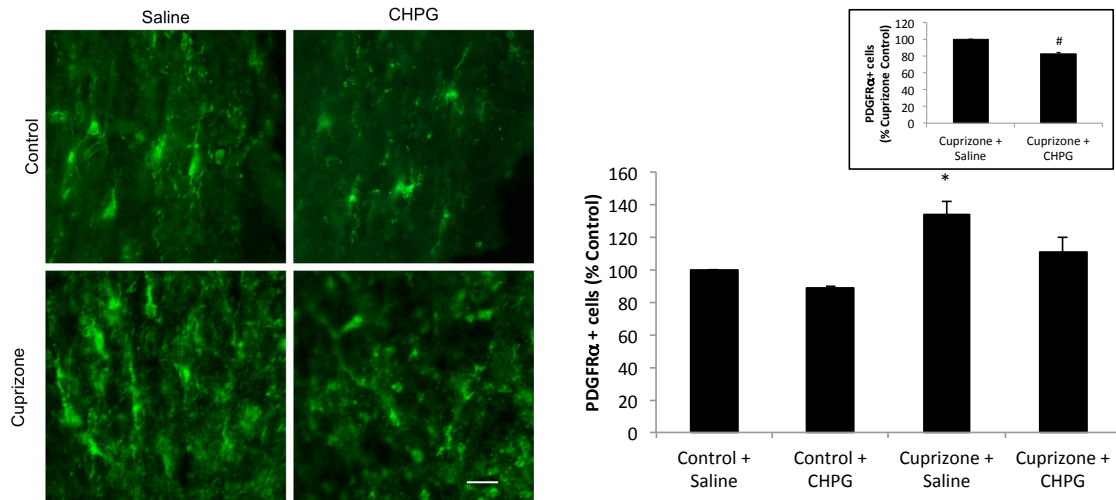
Figure 5. Cuprizone-lesioned mice exhibit decreases in PDGFRα cell numbers 24 hours after intraperitoneal injection of 40 mg/kg CHPG. PDGFRα+ staining in the corpus callosum of mice subjected to a 4 week cuprizone lesion and injected ip with 40 mg/kg CHPG or 0.9% saline vehicle 6 and 24 hours prior to perfusion. Data presented as percent saline-injected control-fed mice, ANOVA. Inset represents data presented as percent saline-injected cuprizone-fed mice, t-test. *Sig. diff. from saline-injected control-fed mice, *p<0.05, #Sig. diff. from saline-injected cuprizone-fed mice, #p<0.05. 18 matched 20 μm sections taken from the corpus callosum were counted per mouse (Experiments performed with Lauren Lercher). Each experiment consists of one mouse per treatment group and each experiment was repeated three times. Scale bar, 20 μm.
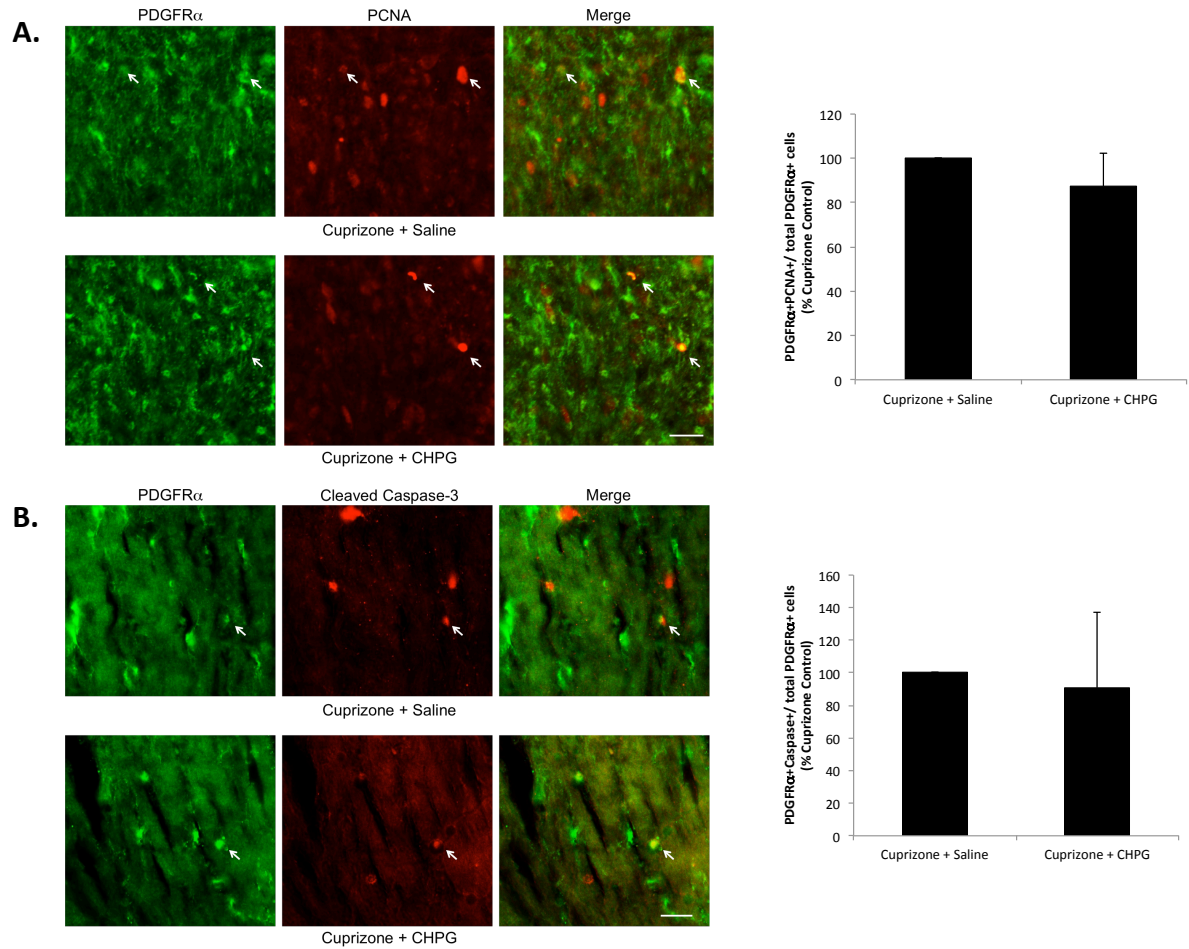
Figure 6. 40 mg/kg CHPG does not affect proliferation or cell death of OPCs 24 hours after intraperitoneal injection. PDGFRα+PCNA+ **(A)** or PDGFRα+cleaved caspase-3+ **(B)** staining in the corpus callosum of mice subjected to a 4 week cuprizone lesion and injected ip with 40 mg/kg CHPG or 0.9% saline vehicle 6 and 24 hours prior to perfusion. Data presented as percent saline-injected cuprizone-fed mice, t-test. 18 matched 20 μm sections taken from the corpus callosum were counted per mouse (PDGFRα+PCNA+ experiments performed with Lauren Lercher; PDGFRα+cleaved caspase-3+ experiments performed with Danielle Sainato). Arrows indicate co-localized cells. Each experiment consists of one mouse per treatment group and each experiment was repeated three times. Scale bar, 20 μm.
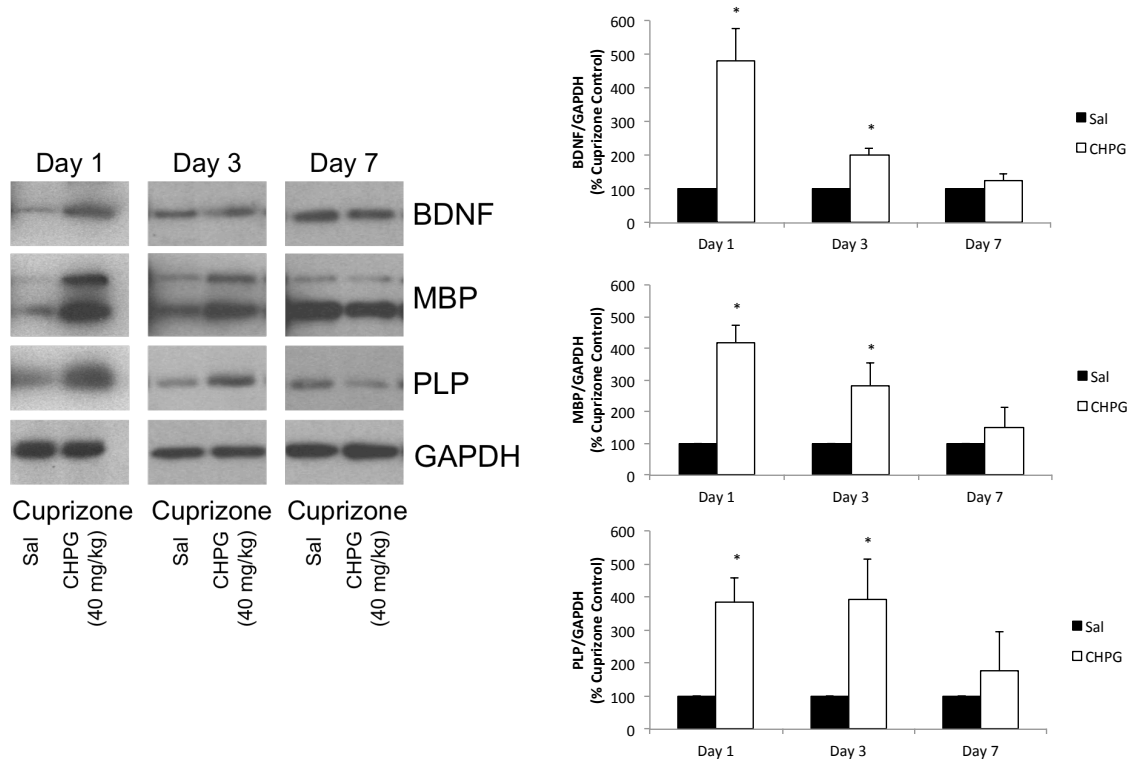
Figure 7. CHPG-elicited effects last up to 3 days. Western blots demonstrate BDNF, MBP, and PLP protein levels in the corpus callosum of mice subjected to a 4 week cuprizone lesion that received 2 ip injections of 40 mg/kg CHPG or 0.9% saline vehicle within the first 18 hours and dissected at Day 1, Day 3, or Day 7. Graph represents densitometric analysis of Western blots normalized to GAPDH and presented as percent saline-injected cuprizone-fed mice, t-test. *Sig. diff. from saline-injected cuprizone control, *$p<0.05$. Each experiment consists of one mouse per treatment group and each experiment for Day 1 was repeated four times; each experiment for Day 3 was repeated three times; each experiment for Day 7 was repeated two times.

Mouse #	Treatment	Slide #	Tissue	Findings
1	Saline	1	brain	NNF (no noteworthy findings)
1	Saline	2	lung	NNF
1	Saline	3	liver	NNF
1	Saline	3	kidney	NNF
1	Saline	3	heart	NNF
1	Saline	3	spleen	NNF
2	Saline	4	brain	NNF
2	Saline	5	lung	NNF
2	Saline	6	liver	NNF
2	Saline	6	kidney	NNF
2	Saline	6	heart	NNF
2	Saline	6	spleen	NNF
3	CHPG	7	brain	NNF
3	CHPG	8	lung	NNF
3	CHPG	9	liver	NNF
3	CHPG	9	kidney	NNF
3	CHPG	9	heart	NNF
3	CHPG	9	spleen	NNF
4	CHPG	10	brain	NNF
4	CHPG	11	lung	NNF
4	CHPG	12	liver	NNF
4	CHPG	12	kidney	NNF
4	CHPG	12	heart	NNF
4	CHPG	12	spleen	NNF
5	CHPG	13	brain	NNF
5	CHPG	14	lung	NNF
5	CHPG	15	liver	NNF
5	CHPG	15	kidney	NNF
5	CHPG	15	heart	NNF
5	CHPG	15	spleen	NNF
6	CHPG	16	brain	NNF
6	CHPG	17	lung	NNF
6	CHPG	18	liver	NNF
6	CHPG	18	kidney	NNF
6	CHPG	18	heart	NNF
6	CHPG	18	spleen	NNF
7	CHPG	19	brain	NNF
7	CHPG	20	lung	NNF
7	CHPG	21	liver	NNF
7	CHPG	21	kidney	NNF
7	CHPG	21	heart	NNF
7	CHPG	21	spleen	NNF

Table 1. No pathological findings observed 3 days after intraperitoneal injection of 150 mg/kg CHPG. Control-fed mice were injected with saline vehicle

or 150 mg/kg CHPG and perfused after 3 days. Histomorphological surveillance of hematoxylin and eosin stained sections was performed by Dr. Michael Goedken, a board certified toxicological pathologist, blinded to treatment groups. N = 2 for saline-injected mice; N = 5 for CHPG-injected mice.

3.2 Investigate the effects of CHPG on myelination and behavior.

3.2.1 CHPG enhances BDNF and myelin proteins in cuprizone-fed mice when injected every other day for 2 weeks

To begin to examine effects on myelin itself, continual injections of 20 mg/kg CHPG began after 4 weeks of cuprizone treatment and continued to be administered every other day for an additional 2 weeks while remaining on cuprizone feed. To determine if elevations in BDNF and myelin proteins are maintained over this time period, Western blots were performed. The data show that BDNF, MBP, and PLP are significantly increased with this dosing regimen of CHPG (Fig. 8), suggesting that CHPG's effects can be prolonged over time. While deficits in BDNF and MBP were not fully reversed after 2 weeks of CHPG treatment, PLP levels in cuprizone-fed mice receiving CHPG were not significantly different than control-fed mice, suggesting that CHPG is able to completely reverse decreases in PLP. Again, no CHPG effects were observed in control-fed mice, suggesting that CHPG is only effective in the presence of a lesion.

3.2.2 CHPG enhances myelination in cuprizone-fed mice when injected every other day for 2 weeks

Transmission electron microscopy revealed that changes in myelin proteins correspond with enhanced myelin structure (Fig. 9A; experiments performed with Dr. W. Geoff McAuliffe). Cuprizone-fed mice receiving CHPG had significantly more myelinated fibers and decreased g-ratio, indicative of increased myelin thickness, compared to saline-injected cuprizone-treated mice (Fig. 9B-C; analyzed with Ashish

Patel). As was the case with myelin protein levels, CHPG had no effect on myelin structure in control-fed mice. Furthermore, linear regression analysis of g-ratio against axon diameter indicated that CHPG treatment resulted in a significant increase in slope compared to vehicle-treated animals (Fig. 9D). These data suggest that CHPG may be acting preferentially on smaller diameter axons (Fig. 9D-E) and that CHPG is enhancing remyelination of the small diameter axons (Fletcher et al., 2018; Nguyen et al., 2019). This is consistent with the actions of the small molecule TrkB agonist, LM22A-4, in the cuprizone model (Nguyen et al., 2019).

3.2.3 CHPG reverses behavioral deficits of cuprizone-fed mice when injected every other day for 2 weeks

To determine if improvements in myelination have a functional consequence, behavioral tests were performed. Cuprizone-induced demyelination leads to impaired motor and muscle coordination with a temporal relationship between motor dysfunction and CNS demyelination (Franco-Pons et al., 2007). In addition to the corpus callosum, other brain regions affected by cuprizone have also been identified including the cerebellum (Groebe et al., 2009; Skripuletz et al., 2010) and several gray matter areas (Goldberg et al., 2015), all of which may contribute to the behavioral deficits observed in this model.

The balance beam test was used to measure balance and motor coordination, while the wire hang test was used as an indicator of grip strength and muscle coordination. Cuprizone-fed mice performed worse on these tests compared to their control-fed counterparts, as they had more paw slips (Fig. 10A), required more time

to cross the balance beam (Fig. 10B), and exhibited less grip strength (Fig. 10C). Excitingly, CHPG reversed all of these endpoints in cuprizone-fed mice, suggesting that CHPG attenuates both the myelination and behavioral deficits associated with cuprizone treatment.

3.2.4 CHPG increases the differentiation of oligodendrocyte lineage cells when injected every other day for 2 weeks

To assess whether improvements in myelination and behavior are due to an increase in oligodendrocyte cell numbers or due to an increase in myelin proteins per oligodendrocyte after 2 weeks of treatment, numbers of CC1+ mature oligodendrocytes were counted using immunofluorescence. Remarkably, CHPG induces a small but significant increase in CC1+ cells over 2 weeks. To explore if this is due to enhanced differentiation of oligodendrocyte lineage cells, EdU (100 μg, ip) was injected 15 minutes and 22 hours after the first CHPG injection and the numbers of EdU+CC1+ cells were counted at the end of the 2-week CHPG treatment (Fig. 11; experiments performed with Lauren Lercher). CHPG treatment results in an increase in the number of EdU+CC1+ cells relative to total CC1+ cells. Since EdU is only incorporated into actively dividing cells at the time of its administration, any cell that is co-labeled for EdU+CC1+ after 2 weeks of CHPG treatment is indicative of a recently differentiated oligodendrocyte. These results are consistent with the previous observation that BDNF can enhance differentiation of OPCs in culture (Du et al., 2003; Du et al., 2006b; Xiao et al., 2010) and *in vivo* (VonDran et al., 2010; Xiao et al., 2010). These findings may also explain why OPC markers decreased 24 hours

following CHPG administration (Fig. 4 and Fig. 5) without changes in proliferation or death of these cells (Fig. 6), supporting the notion that OPCs may have just begun the differentiation process at 24 hours, which is not evident until after 2 weeks of CHPG treatment.
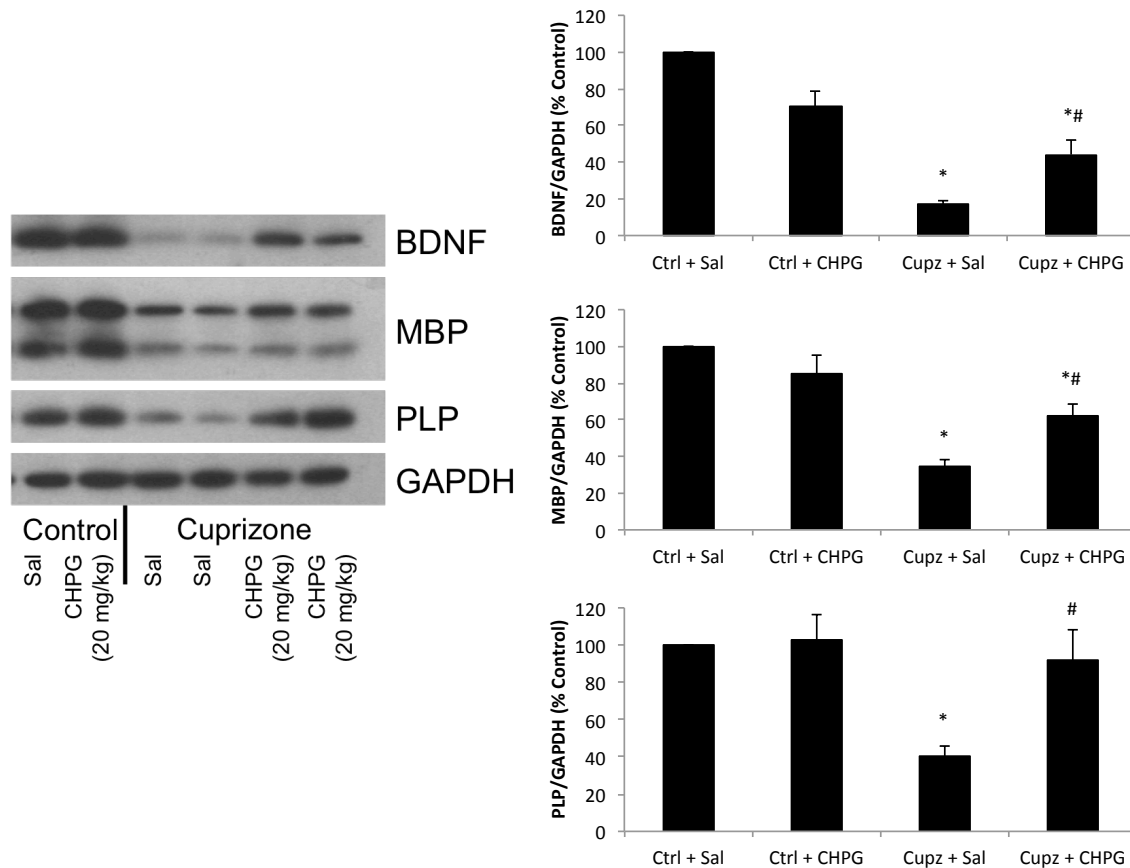
Figure 8. After 2 weeks, BDNF and myelin proteins remain elevated after multiple intraperitoneal injections of CHPG. Western blots demonstrate BDNF, MBP, and PLP protein levels in the corpus callosum of mice subjected to a 4 week cuprizone lesion and injected ip with 20 mg/kg CHPG or 0.9% saline vehicle every other day for an additional 2 weeks while remaining on cuprizone feed. Graph represents densitometric analysis of Western blots normalized to GAPDH and presented as percent saline-injected control-fed mice. *Sig. diff. from saline-injected control-fed mice, *p<0.05, #Sig. diff. from saline-injected cuprizone-fed mice, #p<0.05, ANOVA. Each experiment consists of one mouse per treatment group and each experiment was repeated ten times. Western blot results of two cuprizone experiments are shown, while densitometric analysis includes all ten experiments.
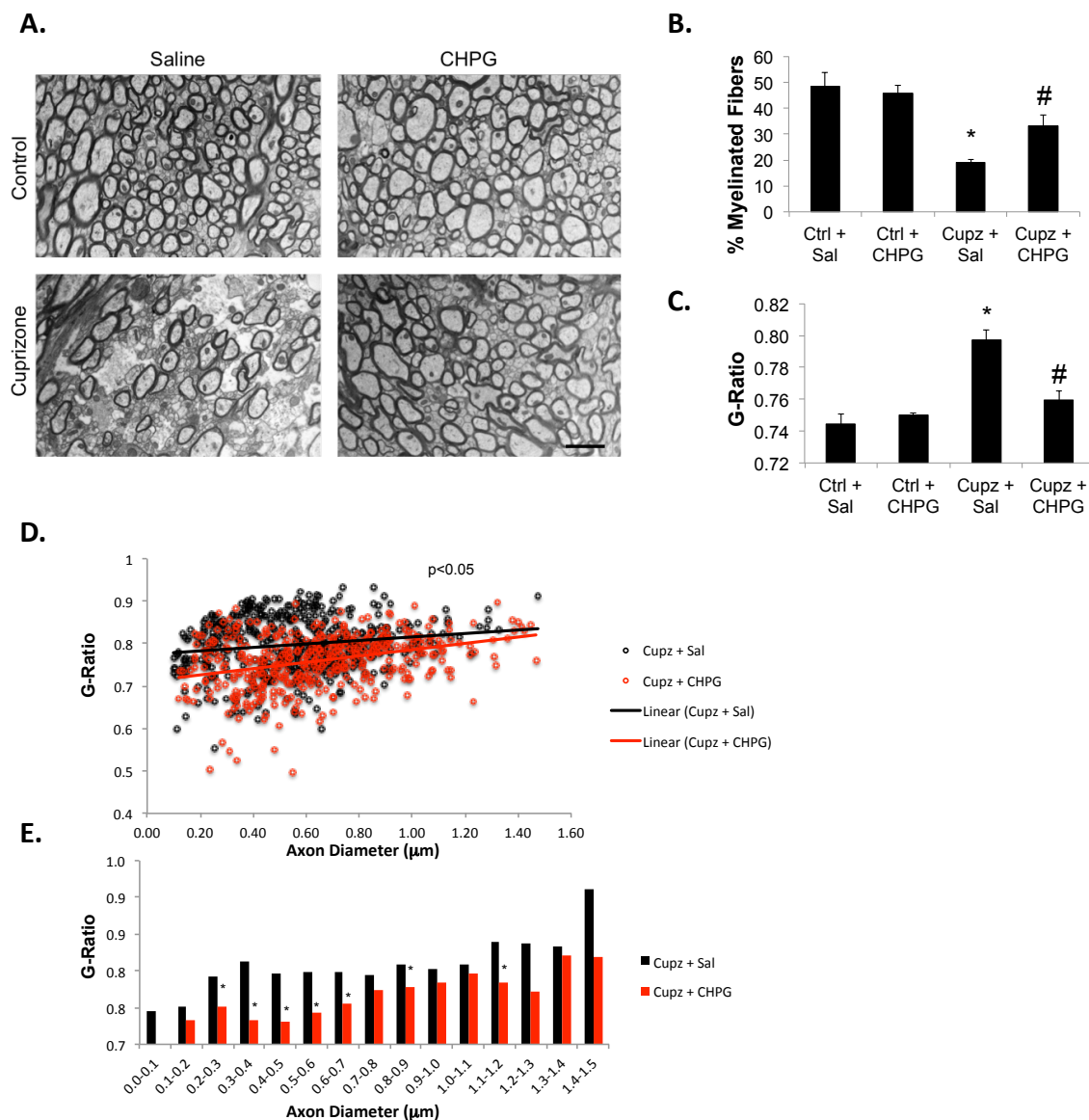
Figure 9. Multiple intraperitoneal injections of CHPG over 2 weeks increases myelin thickness and the percentage of myelinated fibers. (A) Transmission electron microscopy (performed with Dr. W. Geoff McAuliffe) at 5000X magnification of the corpus callosum of mice subjected to a 4 week cuprizone lesion and injected ip with 20 mg/kg CHPG or 0.9% saline vehicle every other day for an additional 2 weeks while remaining on cuprizone feed. Scale bar, 2 μm. Graphs represent the

mean percentage of myelinated fibers **(B)** or mean g-ratio **(C)** among treatment groups. Analysis was done blinded to treatment groups. 150 axons analyzed per mouse for g-ratios; all axons analyzed in 10 different fields per mouse for percent myelinated fibers (Analyzed with Ashish Patel). *Sig. diff. from saline-injected control-fed mice, *$p<0.05$, #Sig. diff. from saline-injected cuprizone-fed mice, #$p<0.05$, ANOVA. Each experiment consists of one mouse per treatment group and each experiment was repeated three times. **(D)** Scatter-plot of g-ratio against axon diameter of cuprizone-fed mice ± CHPG. Linear regression analysis revealed a significant difference in slopes between saline-injected and CHPG-injected mice at $p<0.05$. **(E)** G-ratio against axon diameters grouped by size intervals. *Sig. diff. from saline-injected cuprizone-fed mice, *$p<0.05$, t-test performed at each size interval.
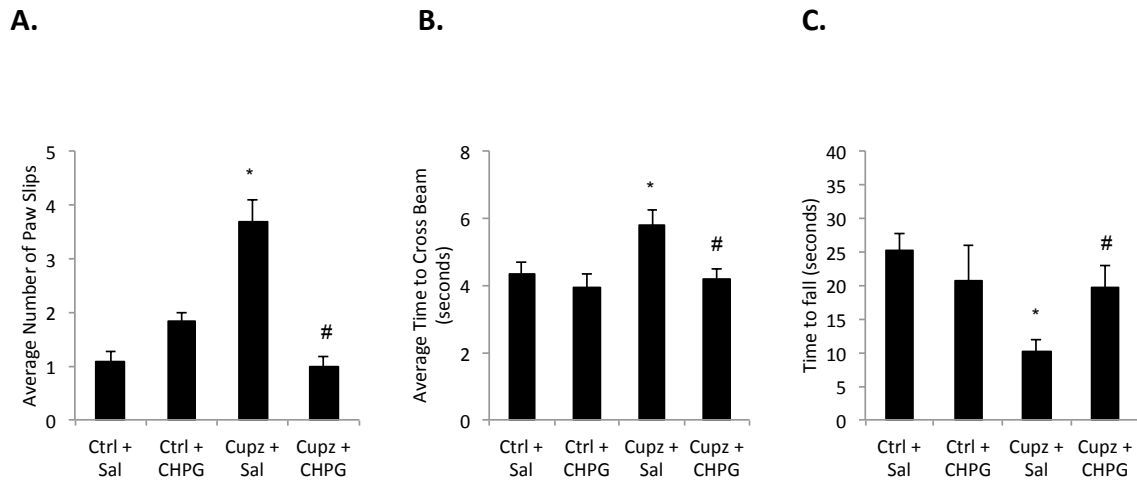
Figure 10. Multiple intraperitoneal injections of CHPG over 2 weeks increases motor coordination and grip strength. Balance beam assay **(A-B)** and wire hang test **(C)** were performed in mice subjected to a 4 week cuprizone lesion and injected ip with 20 mg/kg CHPG or 0.9% saline vehicle every other day for an additional 2 weeks while remaining on cuprizone feed. Analysis was done blinded to treatment groups. *Sig. diff. from saline-injected control-fed mice, *$p<0.05$, #Sig. diff. from saline-injected cuprizone-fed mice, #$p<0.05$, ANOVA. Each experiment consists of one mouse per treatment group and each experiment was repeated twelve times for the balance beam assay and eight times for the wire hang test.
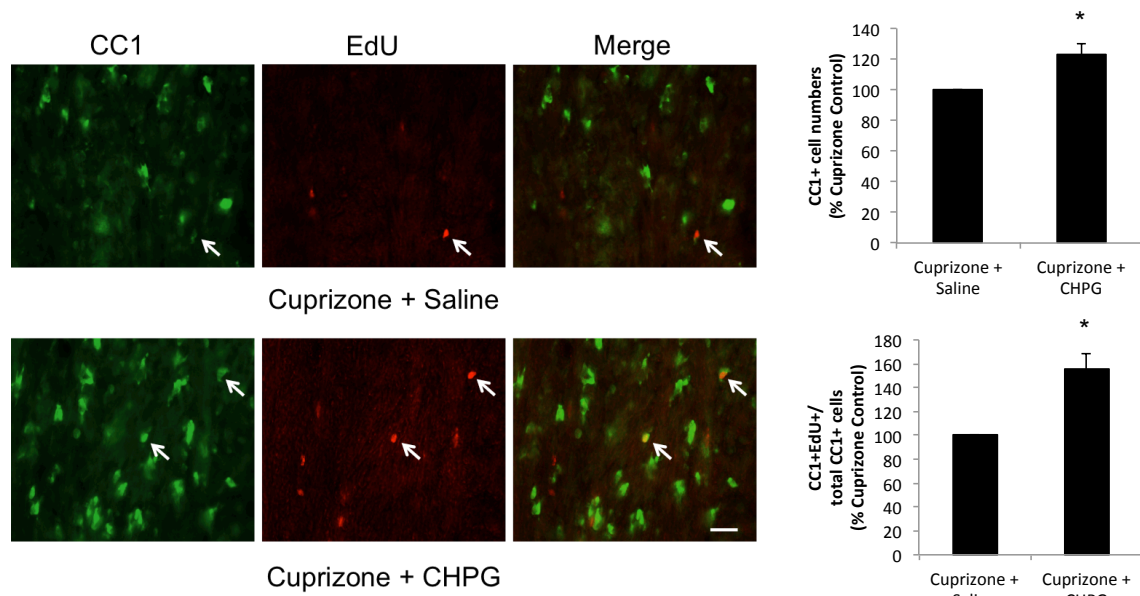
Figure 11. Multiple intraperitoneal injections of CHPG over 2 weeks increases the numbers of CC1+ oligodendrocytes and the percentage of CC1+EdU+ cells. CC1 and EdU staining in the corpus callosum of mice subjected to a 4 week cuprizone lesion and injected ip with 20 mg/kg CHPG or 0.9% saline vehicle every other day for an additional 2 weeks while remaining on cuprizone feed. 20 matched 20 μm sections taken from the corpus callosum were counted per mouse (Experiments performed with Lauren Lercher). Arrows indicate co-localized CC1+EdU+ cells. *Sig. diff. from saline-injected cuprizone-fed mice, *p<0.05, t-test. Each experiment consists of one mouse per treatment group and each experiment was repeated six times. Scale bar, 20 μm.

3.3 Determine the roles of Group I mGluRs on astrocytes, astrocyte-derived BDNF, and oligodendroglial TrkB on CHPG-mediated effects.

3.3.1 CHPG acts in the lesion site and requires mGluR5 to elicit its effects

Since CHPG is a Group I mGluR agonist, its actions may be mediated through mGluR1, mGluR5, or both receptors (Doherty et al., 1997; Kammermeier, 2012). To elucidate the receptor responsible for CHPG-induced elevations in BDNF and myelin proteins, a single stereotaxic injection of the selective mGluR5 antagonist 2-methyl-6-(phenylethynyl)pyridine (MPEP) was injected directly into the lesioned corpus callosum at a concentration of 150 μM one hour before intraperitoneal administration of 40 mg/kg CHPG. This approach has the advantage of not only identifying the receptor mediating the actions of CHPG, but it also determines if the action is at the lesion site. While CHPG again increases BDNF, MBP, and PLP in the absence of MPEP, mice receiving MPEP prior to CHPG fail to elevate these proteins (Fig. 12). This suggests not only that mGluR5 is important for CHPG to elicit its effects, but also that CHPG is acting within the lesion site even after peripheral administration.

3.3.2 CHPG requires mGluR5 on astrocytes to elicit its effects

Previous studies in our lab found that astrocytes were the only cell type in the lesion to contain both mGluR5 and BDNF (Fulmer et al., 2014). To test the possibility that astrocytes are the cell responsible for CHPG's actions, inducible conditional knockout mice were developed that delete mGluR5 from GFAP+ cells upon tamoxifen injection. hGFAP-CreERT2 mice (MMRRC) were crossed with

mGluR5$^{fl/fl}$ mice (a gift from Anis Contractor, Northwestern University, Chicago, Illinois) to generate hGFAP-CreERT2-mGluR5$^{fl/fl}$-eGFP mice. Cre+ mice as well as those lacking cre recombinase as controls (Cre-) were injected with tamoxifen one week before starting cuprizone treatment. Analysis of these mice reveals that tamoxifen treatment does not change numbers of GFAP+ cells but does result in a 51% decrease in numbers of mGluR5+GFAP+ cells (Fig. 13A; experiments performed with Danielle Sainato). Interestingly, these mice also exhibit a 1.6-fold increase in numbers of mGluR1+GFAP+ cells (Fig. 13B; experiments performed with Danielle Sainato and Lauren Lercher), suggesting that mGluR1 may be upregulated when mGluR5 is lost. Following injection of CHPG, only control mice are able to reverse deficits in myelin proteins while this effect is blocked in mice in which astrocytic mGluR5 is deleted (Fig. 13C), suggesting that CHPG requires astrocytic mGluR5 to elicit its effects. Furthermore, this suggests that CHPG does not act through mGluR1 since these receptors are upregulated in the hGFAP-CreERT2-mGluR5$^{fl/fl}$-eGFP mice.

3.3.3 CHPG requires astrocyte-derived BDNF to elicit its effects

To determine if CHPG requires astrocyte-derived BDNF to exert its actions, as was the case with the more general mGluR agonist ACPD, tamoxifen-inducible conditional knockout mice were utilized in which BDNF is deleted from GFAP+ cells upon tamoxifen treatment and compared to controls. These mice were previously reported to exhibit a level of 49% recombination (percentage GFAP cells exhibiting recombination/total GFAP+ cells) (Fulmer et al., 2014). As in previous studies, mice

were injected with 40 mg/kg CHPG intraperitoneally 24 hours and 6 hours prior to dissection and the corpus callosum was examined for changes in myelin proteins. Similar to the results observed when mGluR5 is deleted from astrocytes, mice deficient in astrocytic BDNF are unable to respond to CHPG (Fig. 14). This suggests that not only are astrocytes required to express mGluR5 but that these cells must also contain BDNF for CHPG to elicit its effects.

3.3.4 CHPG requires TrkB on oligodendrocytes to elicit its effects

Previous studies have found that oligodendrocytes in the corpus callosum (VonDran et al., 2011), as well as other brain regions (Du et al., 2003; Du et al., 2006b; VonDran et al., 2010; Xiao et al., 2010), express TrkB receptors and that these receptors can directly mediate effects of BDNF on oligodendrocytes (Wong et al., 2013; Fletcher et al., 2018; Nguyen et al., 2019). Therefore, to determine if CHPG-elicited effects of BDNF are due to direct actions on oligodendrocytes, PLP-CreERT2-TrkB^{fl/fl}-lacZ mice were utilized that exhibit a 69% decrease in TrkB+CC1+ relative to total CC1+ cells upon tamoxifen treatment. As noted in Fig 15, deletion of TrkB from PLP+ cells results in an inability of CHPG to elevate myelin proteins. Interestingly, the increases in BDNF were also blocked, suggesting that oligodendrocytes may also contribute BDNF to CHPG's actions. Future studies will address this intriguing possibility.

Overall, these studies demonstrate that CHPG requires astrocytic mGluR5 and astrocyte-derived BDNF to elevate myelin proteins through oligodendroglial TrkB in a demyelinated lesion in mice.
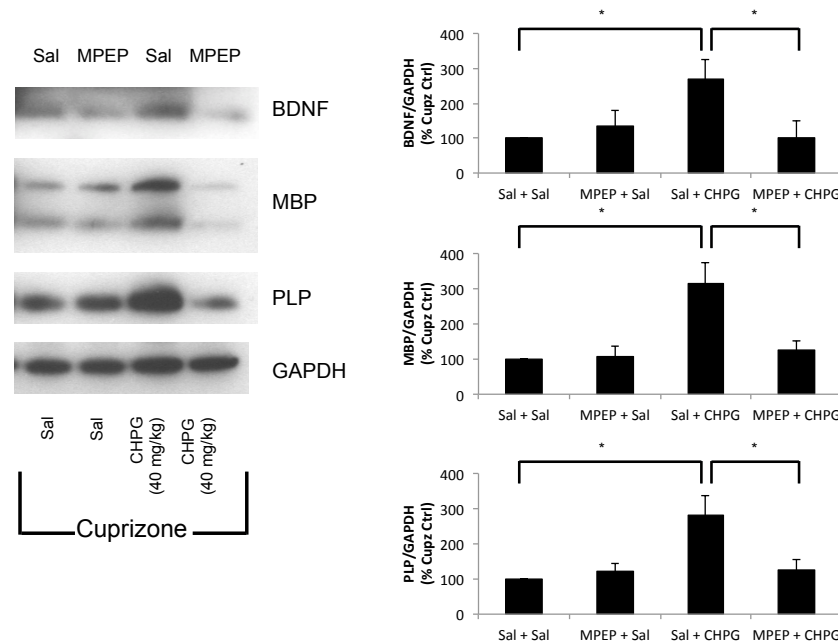
Figure 12. Pharmacological inhibition of mGluR5 blocks the actions of peripherally administered CHPG. Western blots demonstrate BDNF, MBP, and PLP protein levels in the corpus callosum of mice subjected to a 4 week cuprizone lesion and injected ip with 40 mg/kg CHPG or 0.9% saline vehicle 1 hour following stereotaxic injection of the mGluR5 antagonist MPEP (150 uM) or saline. Mice were dissected 24 hours after CHPG injection. Graph represents densitometric analysis of Western blots normalized to GAPDH and presented as percent saline-injected cuprizone-fed mice. *Sig. diff. *p<0.05, ANOVA. Each experiment consists of one mouse per treatment group and each experiment was repeated five times.
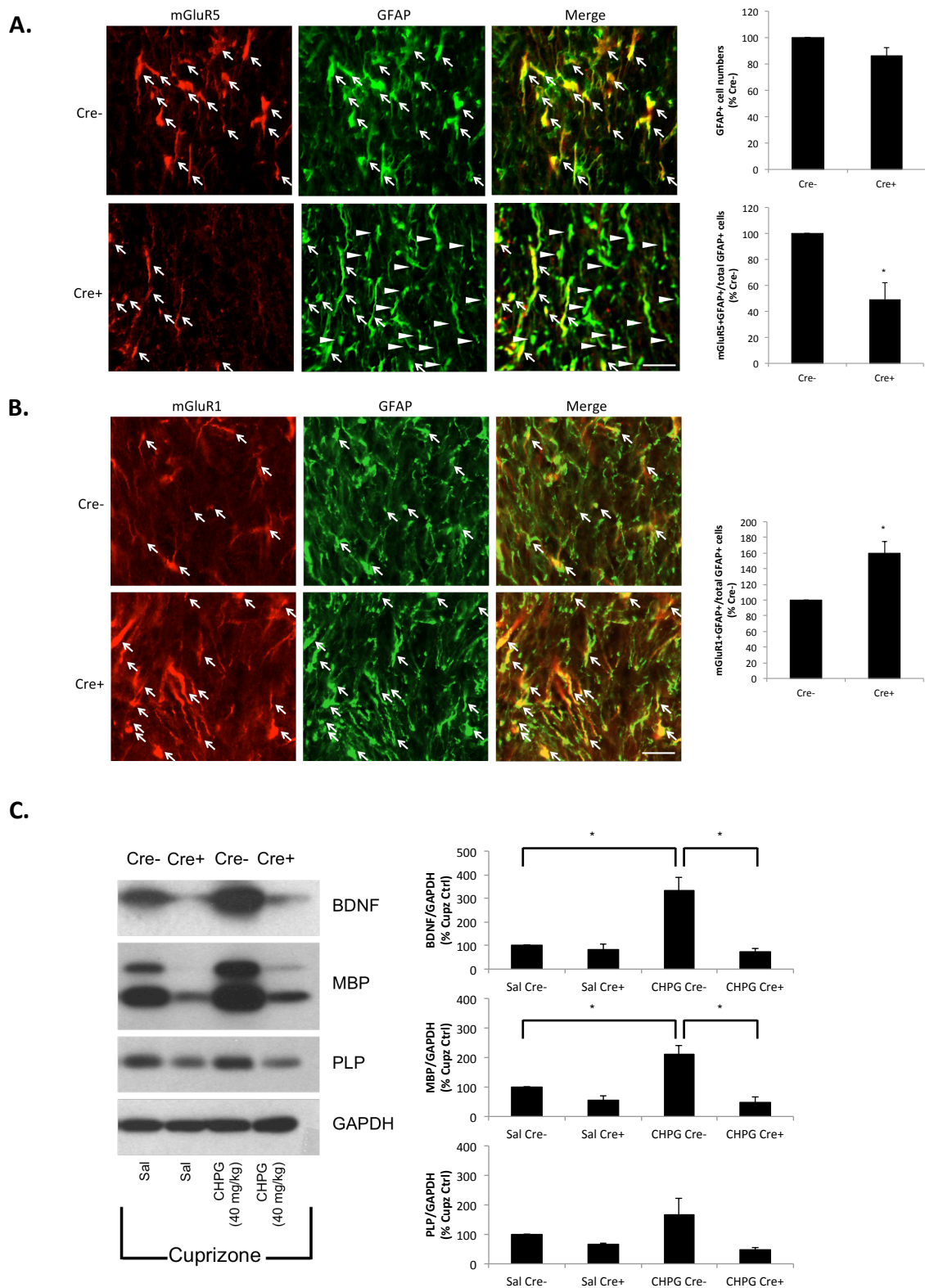
Figure 13. Genetic deletion of mGluR5 from astrocytes blocks the actions of peripherally administered CHPG. mGluR5+GFAP+ **(A)** or mGluR1+GFAP+ **(B)**

staining in the corpus callosum of hGFAP-CreERT2-mGluR5$^{fl/fl}$-eGFP mice (Cre+) or mGluR5$^{fl/fl}$-eGFP mice lacking cre expression (Cre-) subjected to a 4 week cuprizone lesion. 17 matched 14 μm sections taken from the corpus callosum were counted per mouse (mGluR5+GFAP+ experiments performed with Danielle Sainato; mGluR1+GFAP+ experiments performed with Danielle Sainato and Lauren Lercher). Arrows indicate co-localized cells. Arrowheads indicate GFAP+ cells that do not express mGluR5. *Sig. diff. from Cre- cuprizone control, *p<0.05, t-test. Each experiment was repeated three times. Scale bar, 20 μm. **(C)** Western blots demonstrate BDNF, MBP, and PLP protein levels in the corpus callosum of Cre+ or Cre- mice subjected to a 4 week cuprizone lesion and injected ip with 40 mg/kg CHPG or 0.9% saline vehicle. Mice were dissected 24 hours after CHPG injection. Graph represents densitometric analysis of Western blots normalized to GAPDH and presented as percent saline-injected cuprizone-fed mice. *Sig. diff. *p<0.05, ANOVA. Each experiment consists of one mouse per treatment group and each experiment was repeated five times.
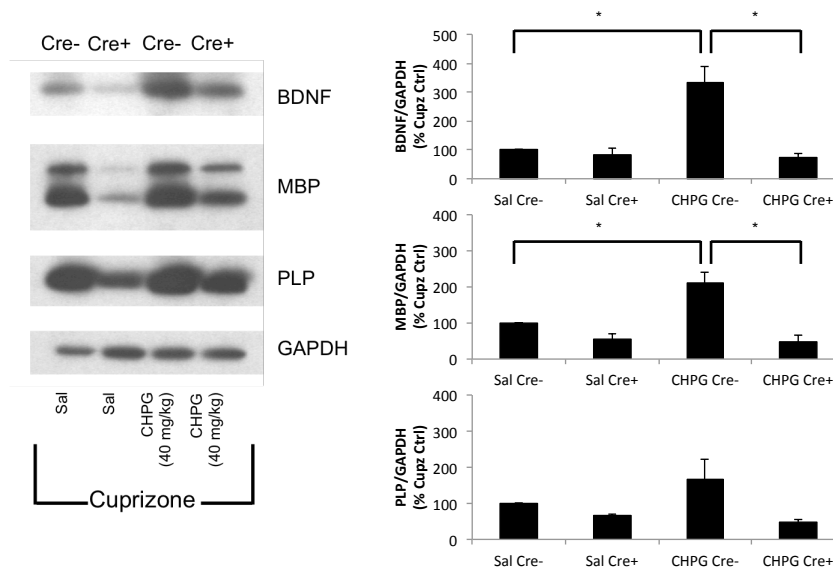
Figure 14. CHPG-elicited increases in BDNF and myelin proteins are blocked when BDNF is deleted from astrocytes. Western blots demonstrate BDNF, MBP, and PLP protein levels in the corpus callosum of hGFAP-CreERT2-BDNF^{fl/fl}-eGFP mice (Cre+) or BDNF^{fl/fl}-eGFP mice lacking cre expression (Cre-) subjected to a 4 week cuprizone lesion and injected ip with 40 mg/kg CHPG or 0.9% saline vehicle 6 and 24 hours prior to dissection. Graph represents densitometric analysis of Western blots normalized to GAPDH and presented as percent saline-injected cuprizone-fed mice. *Sig. diff. *p<0.05, ANOVA. Each experiment consists of one Sal Cre- mouse, one CHPG Cre- mouse, and one CHPG Cre+ mouse and each experiment was repeated four times. Sal Cre+ treatment was repeated two times.
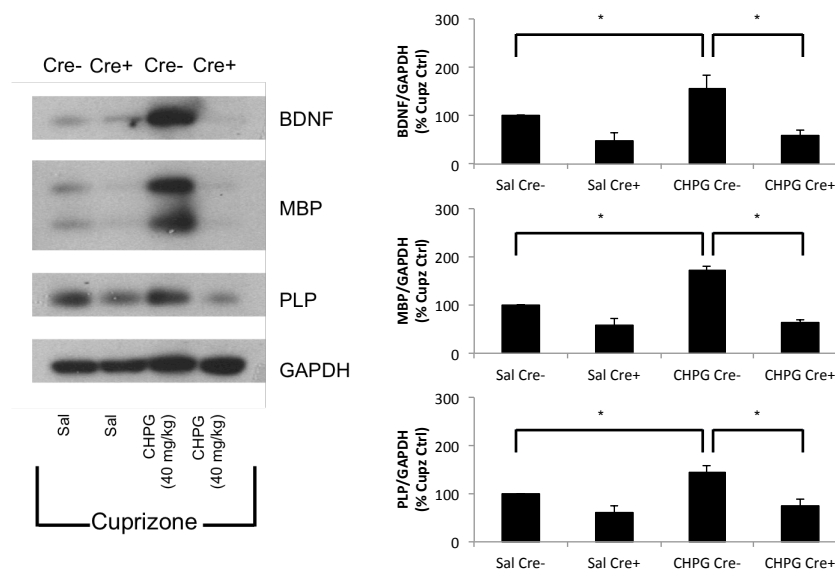
Figure 15. CHPG-elicited increases in BDNF and myelin proteins are blocked when TrkB is deleted from oligodendrocytes. Western blots demonstrate BDNF, MBP, and PLP protein levels in the corpus callosum of PLP-CreERT2-TrkB$^{fl/fl}$-eGFP mice (Cre+) or TrkB$^{fl/fl}$-eGFP mice lacking cre expression (Cre-) subjected to a 4 week cuprizone lesion and injected ip with 40 mg/kg CHPG or 0.9% saline vehicle 6 and 24 hours prior to dissection. Graph represents densitometric analysis of Western blots normalized to GAPDH and presented as percent saline-injected cuprizone-fed mice. *Sig. diff. *p<0.05, ANOVA. Each experiment consists of one mouse per treatment group and each experiment was repeated four times.

Chapter 4: Discussion

In summary, my studies defined the roles of a small molecule mGluR Group I agonist, CHPG, on oligodendrocyte lineage cells following a cuprizone-induced demyelinating lesion in mice. Consistent with preliminary data from our lab, 5 μM CHPG increases myelin proteins when injected directly into the lesioned corpus callosum at 6 hours. This observation was expanded using the clinically more relevant approach of a peripheral injection in which CHPG (20 or 40 mg/kg, ip) increases BDNF, MBP, and PLP at 24 hours. CHPG has no effect on these proteins when administered to control-fed mice, suggesting that CHPG is only effective in the presence of a lesion. Furthermore, these changes at 24 hours occur without changes in numbers of CC1+ mature oligodendrocytes, suggesting that CHPG increases myelin proteins per oligodendrocyte. Total Olig2+ cell numbers remain unchanged following cuprizone or CHPG treatment, suggesting that OPCs increase as CC1+ mature oligodendrocytes decrease.

Western blot analysis reveals that OPC markers PDGFRα and NG2 do indeed increase following cuprizone treatment, which is confirmed with immunofluorescent staining for PDGFRα+ cell numbers. While 20 mg/kg CHPG does not affect these OPC markers at 24 hours, administration of 40 mg/kg CHPG decreases OPC protein levels and cell numbers. These observations were not due to changes in proliferation or death of OPCs, suggesting CHPG may promote the differentiation of these cells. While this was not evident at 24 hours since the numbers of CC1+ mature oligodendrocytes remained unchanged, differentiation is apparent at later time points.

In addition to the effects observed at 24 hours, CHPG-elicited increases in BDNF, MBP, and PLP are maintained up to 3 days following ip injection, but are reduced by 7 days. Moreover, injections of 20 mg/kg CHPG every other day for 2 weeks increases protein levels of BDNF and myelin proteins in cuprizone- but not control-fed mice. This dosing regimen also enhances myelination itself, evident by increased myelin thickness and numbers of myelinated fibers as assessed by transmission electron microscopy. Changes in myelination correspond to improvements in behavior, particularly motor coordination, balance, and grip strength. In contrast to the results observed 24 hours following CHPG injection, 2 weeks of CHPG treatment increases numbers of mature CC1+ oligodendrocytes, which is at least in part due to enhanced differentiation of oligodendrocyte lineage cells. This suggests that at 24 hours, there may be an increase in a middle oligodendrocyte lineage cell marker that was not examined in the current studies.

In elucidating the mechanisms of CHPG-elicited effects, the mGluR5 antagonist MPEP blocked elevations in BDNF and myelin proteins when injected directly into the lesion site prior to ip administration of CHPG. The inducible conditional deletion of mGluR5 specifically from GFAP+ astrocytes also blocks actions of CHPG. Likewise, CHPG requires the actions of astrocyte-derived BDNF and the presence of TrkB on oligodendrocytes to increase myelin proteins as indicated by the inducible conditional deletion of BDNF from GFAP+ cells and of TrkB from PLP+ cells respectively. The data indicate that CHPG binds to mGluR5 on astrocytes, which causes these cells to release BDNF that binds to TrkB on oligodendrocytes to increase myelin proteins. Finally, no pathological findings are noted up to 3 days

following administration of 150 mg/kg CHPG, an important observation for mGluR5 agonists if they were to have future clinical implications.

4.1 Relating the current work to previous studies of our lab

Previous studies of our lab found that activation of Group I/II mGluRs by injecting the agonist ACPD directly into the cuprizone-lesioned corpus callosum elevates levels of astrocyte-derived BDNF, leading to increases in myelin proteins (Fulmer et al., 2014). In the current work, I investigated whether a more specific mGluR agonist, CHPG, which binds only to Group I receptors, can act similarly to ACPD even when injected intraperitoneally. As was the case with ACPD, elevations in BDNF and myelin proteins are at least in part due to astrocyte-derived BDNF. Expanding upon this observation in the current work, however, is that astrocytic mGluR5 and oligodendroglial TrkB likewise mediate these responses. Moreover, multiple intraperitoneal injections of an mGluR agonist enhances myelination itself and reverses behavioral deficits associated with cuprizone treatment. Collectively, these data suggest that astrocytes within a demyelination site may be cells capable of promoting remyelination when properly stimulated or manipulated.

4.2 TrkB deletion blocks CHPG-mediated increases in BDNF

An interesting aspect of this work is that the deletion of TrkB from oligodendrocytes themselves results in a decrease in BDNF. It is important to remember that BDNF is not only synthesized in astrocytes, but is also synthesized and released from oligodendrocytes (Dai et al., 2003; Bagayogo and Dreyfus, 2009).

This raises the possibility that astrocyte-derived BDNF may influence oligodendrocyte-derived BDNF or other aspects of oligodendrocyte function that have not been examined in this study. Similarly, BDNF also exists in microglia (Dougherty et al., 2000; Trang et al., 2009; Trang et al., 2011; Zhang et al., 2014a) and the expression of TrkB receptors on these cells has been identified (Mizoguchi et al., 2009; Spencer-Segal et al., 2011; Zhang et al., 2014a), suggesting that astrocyte-derived BDNF may also impact microglia. The possibility that a positive autocrine feedback loop may exist in oligodendrocytes and microglia is a topic for future investigation.

4.3 The role of the lesion site in promoting or diminishing myelination

Another suggestion of this work is that the state of the lesion environment may determine when CHPG is most effective. Because I show that CHPG acts through mGluR5 on astrocytes, CHPG requires astrocytes to first become reactive before being able to elicit its actions. This is apparent by the fact that CHPG is only effective after a lesion but not in control-fed mice when astrocytes have little to no expression of mGluR5 (Fulmer et al., 2014). Thus, CHPG may not be as effective in early stages of the disease when reactive astrocytes and their mGluR5 expression are not abundant. Moreover, this observation suggests that the same effect of mGluR5 stimulation of astrocytes may be apparent in other neurodegenerative diseases where astrocytes upregulate mGluR5. At that point, the cells may become receptive to mGluR agonists such as CHPG to attenuate disease characteristics.

4.4 Clinical relevance of these studies

The fact that CHPG can be administered through a peripheral injection is another important observation of this work since BDNF itself does not cross the blood-brain-barrier (Poduslo and Curran, 1996). This clinically relevant route of administration therefore allows small molecules such as CHPG to get into the brain and enhance endogenous sources of BDNF. Other studies also have begun to peripherally administer TrkB agonists that cross the blood-brain-barrier (Simmons et al., 2013; Geraghty et al., 2019), though in the cuprizone model specifically, they have only been injected via intracerebroventricular administration thus far (Fletcher et al., 2018; Nguyen et al., 2019). The advantage of targeting astrocytic mGluR5, however, is that this is a disease-specific target not observed under control conditions.

4.5 The role of Group I mGluRs on glia in other models

This work is consistent with culture work that supports the possibility that mGluR5 stimulation elicits actions that may be positive on astrocytes. For example, treatment with the Group I/Group II agonist ACPD leads to the release of neurotransmitters like glutamate (Miller et al., 1995; Nakahara et al., 1997; Pasti et al., 1997; Biber et al., 1999), or neurotrophins like BDNF (Jean et al., 2008). Similarly, studies indicate that activation of Group I mGluRs may also be directly beneficial to other glial cells that are found within the lesion site. For example, the Group I agonist 3,5-dihydroxyphenylglycine (DHPG) reduces oxidative stress and prevents OPC death in culture (Deng et al., 2004; Luyt et al., 2006) and releases

BDNF from cultured oligodendrocytes (Bagayogo and Dreyfus, 2009). Agonists of Group I mGluRs on microglia reverse activation of the microglia (Byrnes et al., 2009b). Moreover, the application of CHPG itself to the BV2 microglial cell line attenuates cell death, oxidative stress, inflammation, and proliferation, yet enhances BDNF expression under toxic conditions (Qiu et al., 2015; Ye et al., 2017; Huang et al., 2018).

4.6 Importance to MS

Interestingly, expression of glial mGluR5 may be a disease-specific target with importance to human tissue. Studies have shown that mGluRs are upregulated primarily on astrocytes and microglia in human MS lesions compared to control tissue (Geurts et al., 2003; Geurts et al., 2005; Newcombe et al., 2008). In the case of astrocytes, upregulated mGluR2/3 as well as 5 is reported on virtually all reactive astrocytes within lesion sites, while mGluR1, 4 and 8 are associated only with a subpopulation of those cells (Geurts et al., 2003; Geurts et al., 2005; Newcombe et al., 2008). Microglia also express mGluR1, 2/3 and 8 within active MS lesions (Geurts et al., 2003; Geurts et al., 2005; Newcombe et al., 2008) and cells morphologically resembling young oligodendrocytes have been reported to exhibit mGluR2/3 (Newcombe et al., 2008). Unlike astrocytes, however, microglia and young oligodendrocytes do not upregulate mGluR5 in human samples, suggesting that astrocytic mGluR5 is particularly relevant to human demyelinating lesions. Therefore, astrocytic mGluR5 may be a novel therapeutic target for treating demyelinating diseases and other neurodegenerative diseases known to upregulate

astrocytic mGluR5 by taking advantage of the disease environment to promote regeneration and repair.

Chapter 5: Future Directions

With the ultimate goal of finding more effective treatments for demyelinating diseases in humans through the use of remyelinating agents, future studies are needed to further define effects of Group I mGluR agonists like CHPG for clinical relevance and therapeutic potential. This includes the potential influence of BDNF derived from oligodendrocyte lineage cells and microglia, further defining effects of CHPG on OPCs, determining the optimum time to administer CHPG, examining possible gliotransmitters and growth factors other than BDNF that may be affected by CHPG, exploring the effects of CHPG and related compounds in other models of demyelination, and assessing the pharmacokinetic properties of these molecules. Possible experiments to begin to address these questions are described below.

5.1 Effects of BDNF derived from oligodendrocyte lineage cells and microglia

My studies suggest that astrocytic mGluR5, astrocyte-derived BDNF, and TrkB on PLP+ cells are required for CHPG to increase myelin proteins (Fig. 14 and Fig. 15). Unexpectedly, however, was that the deletion of TrkB on PLP+ cells also prevented CHPG-induced elevations in BDNF protein levels since CHPG would still be able to bind to mGluR5 on astrocytes and release BDNF from these cells. These data suggest that oligodendrocyte lineage cells or microglia may also contribute BDNF to CHPG's actions. As noted above, both of these cell populations contain BDNF and express TrkB receptors. Therefore, these cells have the potential to respond to astrocyte-derived BDNF to release their own sources of BDNF that can then act in an autocrine manner. Although BDNF expression has been identified in

oligodendrocyte lineage cells in culture (Dai et al., 2003; Bagayogo and Dreyfus, 2009), *in vivo* during development in the basal forebrain, corpus callosum, and frontal cortex (Dai et al., 2003), as well as *in vivo* in the spinal cord of adult animals (Dougherty et al., 2000), less is known about its expression on these cells within a cuprizone-induced lesion. Therefore, initial studies will need to examine if BDNF is in fact expressed in oligodendrocyte lineage cells within the cuprizone lesion. If BDNF is found in these cells, the potential role of oligodendrocyte lineage cell-derived BDNF could be assessed following CHPG treatment. To determine the possibility that oligodendrocyte-derived BDNF can contribute to remyelination, BDNF$^{fl/fl}$ mice could be crossed with PLP-CreERT2 or NG2-CreERT2 mice to generate inducible conditional knockout mice that delete BDNF from PLP+ or NG2+ cells respectively. Although the deletion of BDNF from astrocytes completely blocks the effects of CHPG (Fig. 14), astrocyte-derived BDNF may influence oligodendrocyte lineage cells directly to release their own sources of BDNF. If this is the case, administration of CHPG to BDNF-deficient oligodendrocyte lineage cells may still have a partial increase in myelin proteins due to the BDNF released from astrocytes, but would not be as profound as the increase observed in wild-type mice. These differences can then be compared to determine the relative contribution of BDNF from oligodendrocyte lineage cells. Alternatively, oligodendrocyte lineage cells could release an intermediate factor that directly affects microglia.

Microglial BDNF has been identified in culture and in the *in vivo* spinal cord (Dougherty et al., 2000; Trang et al., 2009; Trang et al., 2011; Zhang et al., 2014a), yet studies are lacking regarding its expression in the adult lesioned brain.

Therefore, initial studies should examine the expression of BDNF in these cells in cuprizone-fed mice. If microglial BDNF is identified within a cuprizone lesion, BDNF$^{fl/fl}$ mice could be crossed with Cx3cr1-CreERT2 mice to generate inducible conditional knockout mice that delete BDNF from Cx3cr1+ cells. While Cx3cr1 is expressed on T-cells, dendritic cells, and monocytes in the periphery, this receptor is exclusively expressed on microglia within the CNS (Ludwig and Mentlein, 2008), making Cx3cr1-CreERT2-BDNF$^{fl/fl}$ mice advantageous in studying the role of microglial BDNF in the cuprizone model. Similarly to the proposed experiments above, if astrocyte-derived BDNF influences microglia to release their own sources of BDNF, mice deficient in microglial BDNF may still have a partial increase in myelin proteins following CHPG treatment due to astrocyte-derived BDNF. Differences in the magnitude of myelin protein increases could again be compared to wild-type mice to determine the relative contribution of microglial BDNF to CHPG's effects.

5.2 Role of CHPG on OPCs

Astrocyte-derived BDNF is at least in part required for CHPG to increase BDNF and myelin proteins (Fig. 14). To determine if it is also responsible for the CHPG-induced decreases in OPC markers, these OPC proteins could be analyzed in the same hGFAP-CreERT2-BDNF$^{fl/fl}$ mice that were used to determine BDNF and myelin protein levels after CHPG administration. To begin to address this question, preliminary studies analyzed protein levels of PDGFRα and NG2 in these hGFAP-CreERT2-BDNF$^{fl/fl}$ mice. When BDNF is deleted from astrocytes, CHPG-induced

decreases in PDGFRα and NG2 are blocked (Fig. 16), suggesting that astrocyte-derived BDNF may promote the differentiation of OPCs. These studies will need to be repeated to determine statistical significance.

It is possible that oligodendrocyte lineage cells may be directly stimulated by CHPG as mGluR5 has been identified on OPCs and young oligodendrocytes (Luyt et al., 2003; Deng et al., 2004; Luyt et al., 2004; Luyt et al., 2006; Bagayogo and Dreyfus, 2009; Jantzie et al., 2010). Its expression on these cells within a demyelinating lesion, however, is not known. Therefore, these cells must first be co-stained with mGluR5 to determine if these cells have the ability to respond to CHPG within a cuprizone lesion. Preliminary data suggests that SOX10+ cells express mGluR5 within a cuprizone-induced lesion (Fig. 17; experiments performed with Danielle Sainato). Since SOX10 labels all oligodendrocyte lineage cells, additional experiments may wish to determine the stage-specific markers of these cells. For example, if mGluR5 is confirmed to co-localize with NG2+ cells, NG2-CreERT2-mGluR5$^{fl/fl}$ mice can be utilized to determine if CHPG mediates any effects through OPC-derived mGluR5. Previous studies found that the Group I mGluR agonist, DHPG, can stimulate cultured oligodendrocyte lineage cells to release BDNF (Bagayogo and Dreyfus, 2009). Therefore, changes in protein levels of BDNF should be assessed in mice that are deficient in oligodendroglial mGluR5. If differences in BDNF levels are observed, potential changes in myelin proteins should be assessed afterwards. Alternatively, future studies may wish to delete both mGluR5 and BDNF from oligodendrocyte lineage cells to determine the overall roles of these cells in CHPG-mediated effects.

5.3 Identify the optimum time to administer CHPG

Following 4 weeks of cuprizone treatment, CHPG works through astrocytic mGluR5, astrocyte-derived BDNF, and oligodendroglial TrkB to increase myelin proteins at 24 hours. What is not known, however, is the time point in which CHPG is most effective. The disease environment of a cuprizone-induced lesion is dynamic and complex with several glial populations undergoing changes, both in cell number and in the expression of various proteins. For example, mature oligodendrocytes decrease in number whereas OPCs, astrocytes, and microglia increase in the lesion site (Matsushima & Morell, 2001). With respect to astrocytes, expression of both BDNF and mGluR5 increases after cuprizone treatment (Fulmer et al., 2014; An et al., 2019). Therefore, a time course of administering CHPG following 1, 2, 3, 4, 5, or 6 weeks of cuprizone would aid in addressing the optimum time when CHPG is most effective. Since CHPG requires astrocytic mGluR5 and oligodendroglial TrkB to increase myelin proteins, both of these receptors should be highly expressed at the time of CHPG administration. Initial studies would begin with a time course that shows changes in the expression of these receptors using Western blot analysis. If differences are noted in mGluR5 and TrkB expression, subsequent studies would identify co-labeled mGluR5+GFAP+ or TrkB+CC1+ cells by immunofluorescent staining to determine which cells express these receptors and at what time points. A time course using 6 weeks of cuprizone feed followed by 1, 2, 3, or 4 weeks of control feed as a recovery period may be of interest to assess if CHPG enhances remyelination after a demyelinating insult. An optimal time point for CHPG

administration during the recovery phase would then be able to be determined as well.

Due to the ongoing cellular changes within a cuprizone-induced lesion, it is not so straightforward to determine if CHPG works through the same mechanism of astrocytic mGluR5, astrocyte-derived BDNF, and oligodendroglial TrkB when continuously administered over time. Initial studies could examine this possibility by injecting CHPG every other day for 2 weeks in the three conditional knockout mouse models used in the 24 hour studies and measuring changes in BDNF and myelin proteins. If increases in myelin proteins are blocked in the knockout models, changes on myelination itself could also be evaluated using transmission electron microscopy. One study injecting a BDNF mimetic into cuprizone-fed mice found that the percentage of myelinated axons was still increased when TrkB was deleted from CNPase+ cells although increases in myelin thickness were blocked, suggesting that that TrkB on cells other than oligodendrocyte lineage cells may exist for BDNF-elicited effects (Fletcher et al., 2018).

5.4 Potential of astrocytic mGluR5 to influence gliotransmitters and growth factors other than BDNF

In addition to BDNF, astrocytes express several other gliotransmitters and growth factors that promote myelination (Eroglu, 2009; Moore et al., 2011; Zamanian et al., 2012; Barnett and Linington, 2013; An et al., 2019). Furthermore, astrocytes can release some of these factors following mGluR stimulation. As noted above, the Group I/Group II agonist ACPD leads to the release of glutamate (Miller et

al., 1995; Nakahara et al., 1997; Pasti et al., 1997; Biber et al., 1999) as well as BDNF (Jean et al., 2008; Fulmer et al., 2014). While specific stimulation of Group II mGluRs can also enhance the release of other factors like GDNF (Battaglia et al., 2015) and TGF-β (Bruno et al., 1998; Caraci et al., 2011), it is not known if these other factors can be influenced by stimulation of Group I mGluRs.

Cultured astrocytes can be treated with CHPG before being lysed and used for Western blot analysis to determine if GDNF and TGF-β are upregulated following CHPG treatment. Additionally, the culture medium from these cells could be analyzed for these factors to determine if they are actually being released from the astrocytes. If so, their effects on the demyelination and remyelination processes must also be evaluated. In this case, inducible conditional knockout mice can again be utilized to selectively delete GDNF or TGF-β from astrocytes. Following CHPG injection, myelin protein levels would be assessed. If GDNF or TGF-β does have an effect on myelin proteins in the cuprizone model, then Cre+ mice would be expected to show a decrease in MBP and PLP. Alternatively, if they do not have an effect on myelin proteins, MBP and PLP levels in Cre+ mice should be similar to those of Cre-mice.

Alternatively, it is possible that other astrocyte-derived factors may be impacted by CHPG. A future direction of this work would be to identify these factors using a transcriptomic approach. Activated astrocytes express a host of molecules that are known to positively affect regeneration (Eroglu, 2009; Moore et al., 2011; Zamanian et al., 2012; Barnett and Linington, 2013; An et al., 2019). For these studies, mice with GFP-associated astrocytes would be subjected to the cuprizone

lesion for 4 weeks and then treated with CHPG or saline. After 24 hours the astrocytes would be sorted and assessed for activated genes. The upregulation of these genes would have to be confirmed using RT-PCR. These future studies could more extensively evaluate roles of astrocyte-derived molecules in affecting myelination and may identify additional therapeutic targets.

5.5 Effects in other demyelinating models and neurodegenerative diseases

In addition to the cuprizone model, effects of CHPG in other demyelinating models, such as EAE and lysolecithin, are not known. Evaluating actions of CHPG in other demyelinating models is important to determine that CHPG effects are not specific to only the cuprizone model and to determine if other mechanisms of CHPG may be involved that are absent in the cuprizone model. For example, the EAE model has an immune component and primarily affects the spinal cord. Therefore, mechanisms in the spinal cord may be different from those that occur in the brain. The immune system may also play a role in CHPG's effects in the EAE model that are not evident following cuprizone treatment. Lysolecithin, on the other hand, induces a focal demyelinating lesion at the site of injection. While this lesion is more specific to the area of injection compared to treatment with cuprizone, in which several areas besides the corpus callosum have now been identified as being affected (Groebe et al., 2009; Skripuletz et al., 2010; Goldberg et al., 2015), one advantage of using lysolecithin is that it can be injected into the brain or spinal cord. This allows for the comparison of CHPG-elicited mechanisms in multiple CNS regions using the same model of demyelination.

Other neurodegenerative diseases in which oligodendrocytes and myelin also play a role in their disease progression can also be examined following CHPG treatment. This is also important in determining CHPG is only effective in one specific disease or if its application can be expanded to other neurodegenerative diseases and disorders that may have a common underlying pathophysiology. Finally, both male and female mice should be evaluated for effects of CHPG in these models to determine any potential sex-specific endpoints or mechanisms.

5.6 Pharmacokinetic properties

To the best of my knowledge, my work is the first that administers CHPG through an intraperitoneal injection. Therefore, pharmacokinetic properties such as its half-life, distribution throughout the body, and clearance and excretion from the body are not currently known. Furthermore, future studies may wish to administer CHPG orally to determine additional properties such as its bioavailability, potential to be metabolized, and how and where it gets absorbed. To begin to address these questions, CHPG can be administered to mice both orally and intravenously at the same dose, with blood samples collected over time. LC-MS-MS can be used to determine plasma concentrations of CHPG at each time point. This data plotted as plasma concentration versus time will allow several parameters to be identified, including the maximal plasma concentration (C_{max}) and the time at which this occurs (T_{max}), the area under the curve (AUC), and half-life ($T_{1/2}$). The absolute bioavailability (F) can then be determined using the AUC of orally administered CHPG divided by the AUC of intravenously administered CHPG. If bioavailability of

CHPG is low, this could suggest poor absorption or poor first-pass metabolism, or both. Therefore, another set of mice could be co-administered an inhibitor of drug metabolizing enzymes, such as 1-aminobenzotriazole (ABT), a pan-CYP inhibitor, with CHPG, both given orally. If the bioavailability of CHPG were still low in combination with ABT, this would suggest poor absorption. Alternatively, a high bioavailability in combination with ABT would suggest that CHPG is highly absorbed but undergoes extensive metabolism. If ABT also inhibits the clearance of intravenously administered CHPG, this would suggest that metabolism of CHPG mostly occurs in the liver rather than the gastrointestinal tract. These are important characteristics to consider before determining the potential of any new therapeutic to be effective in humans.
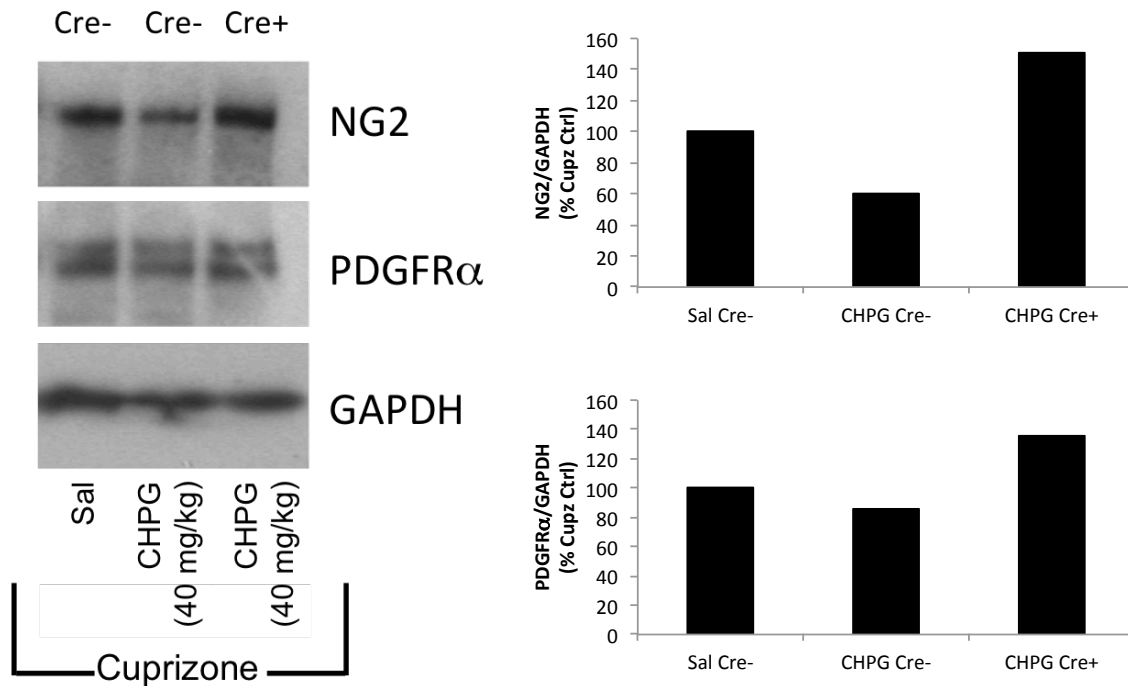
Figure 16. Preliminary data suggests CHPG-elicited decreases in OPCs are blocked when BDNF is deleted from astrocytes. Western blots demonstrate NG2 and PDGFRα protein levels in the corpus callosum of mice subjected to a 4 week cuprizone lesion and injected ip with 40 mg/kg CHPG or 0.9% saline vehicle 6 and 24 hours prior to dissection. Graph represents densitometric analysis of Western blots normalized to GAPDH and presented as percent saline-injected cuprizone-fed mice. N = 1.
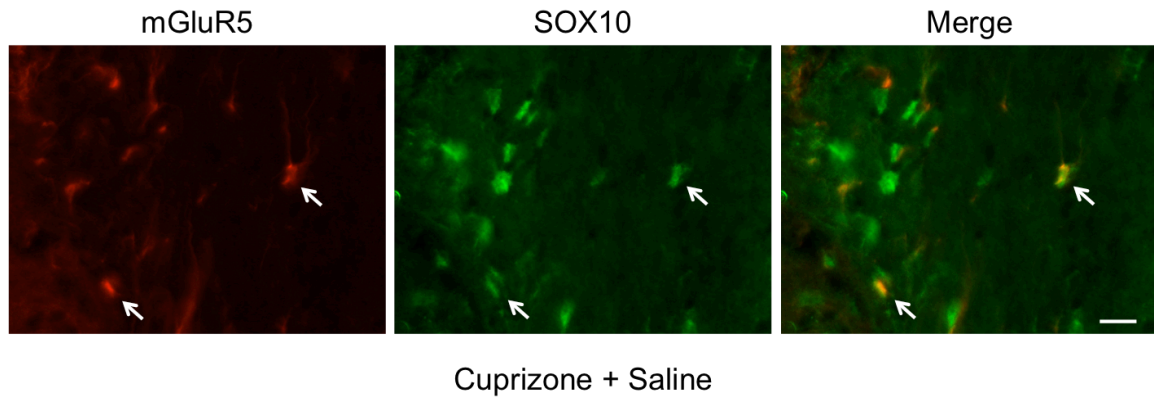
Cuprizone + Saline

Figure 17. Preliminary data suggests SOX10+ cells express mGluR5 in the lesioned corpus callosum. mGluR5 and SOX10 staining in the corpus callosum of mice subjected to a 4 week cuprizone lesion and injected ip with 0.9% saline vehicle 6 and 24 hours prior to dissection (Experiments performed with Danielle Sainato). Arrows indicate co-localized mGluR5+SOX10+ cells. Scale bar, 20 μm. N = 1.

Conclusion

My studies expand upon previous work from our lab that indicate mGluR agonists can be effective in reversing characteristics of a cuprizone-induced demyelinating lesion in mice through the actions of astrocyte-derived BDNF. An intraperitoneal injection of the Group I mGluR agonist, CHPG, effectively reverses characteristics associated with a cuprizone-induced lesion, including changes in myelination and behavior. Furthermore, I show that CHPG's effects are dependent on astrocytic mGluR5, astrocyte-derived BDNF, and oligodendroglial TrkB (Fig. 18). Future studies will build upon this work in determining the roles of BDNF and mGluR5 on other cell types, as well as other astrocyte-derived gliotransmitters and growth factors, in response to CHPG. Overall, astrocytic mGluR5 may be a novel therapeutic target for treating demyelinating diseases and other neurodegenerative diseases known to upregulate astrocytic mGluR5 by taking advantage of the disease environment to promote regeneration and repair. It is my hope that this research can contribute towards finding better therapeutic treatments for demyelinating diseases such as MS that currently have no cure.
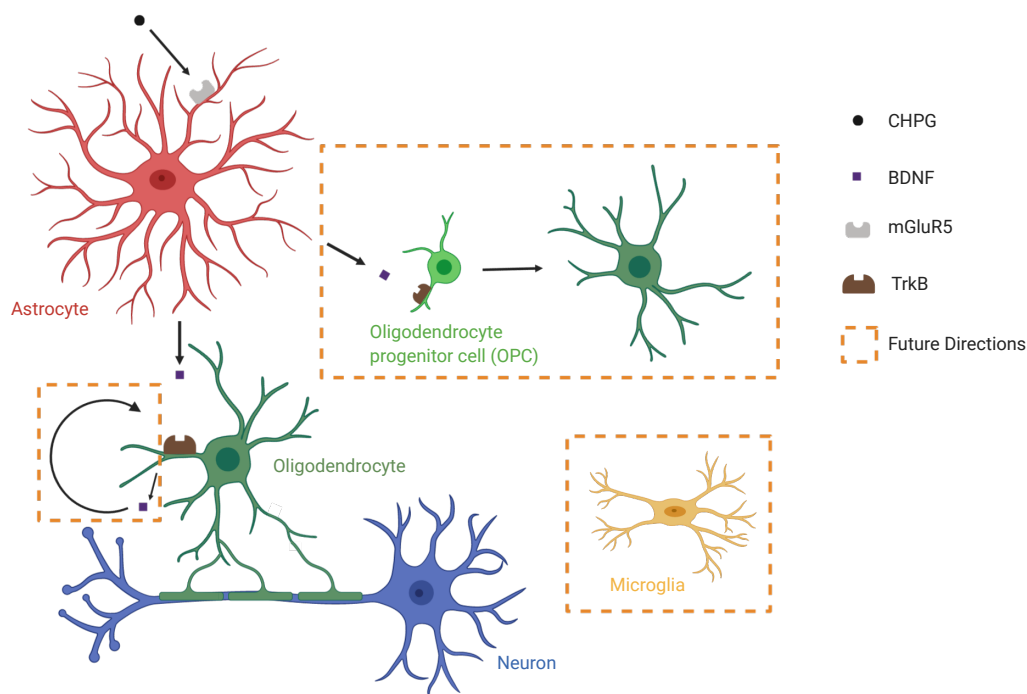
Figure 18. Proposed mechanisms of CHPG-elicited effects. CHPG stimulates mGluR5 on astrocytes, causing these cells to release BDNF. BDNF then binds to TrkB receptors on oligodendrocytes to increase myelin. Future directions will explore the potential role of oligodendrocyte-derived BDNF to act in an autocrine manner, possible direct effects on OPCs, and contributions of microglia. Created with BioRender.

Appendix: Reactive astrocytes as therapeutic targets for brain degenerative diseases: Roles played by metabotropic glutamate receptors

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Abstract

Astrocytes are well known to play critical roles in the development and maintenance of the central nervous system (CNS). Moreover, recent reports indicate that these cells are heterogeneous with respect to the molecules they express and the functions they exhibit in the quiescent or activated state. Because astrocytes also contribute to pathology, promising new results raise the possibility of manipulating specific astroglial populations for therapeutic roles. In this mini-review, we highlight the function of metabotropic glutamate receptors (mGluRs), in particular mGluR3 and mGluR5, in reactive astrocytes and relate these to three degenerative CNS diseases: multiple sclerosis, Alzheimer's disease and Amyotrophic Lateral Sclerosis. Previous studies demonstrate that effects of these receptors may be beneficial, but this varies depending on the subtype of receptor, the state of the astrocytes, and the specific disease to which they are exposed. Elucidating the role of mGluRs on astrocytes at specific times during development and disease will provide novel insights in understanding how to best use these to serve as therapeutic targets.

Key words: astrocyte heterogeneity, Group I/II metabotropic glutamate receptors, multiple sclerosis, Alzheimer's disease, Amyotrophic Lateral Sclerosis

Introduction

It is well recognized that astrocytes play a number of critical roles that support the developing and mature brain. In response to injury, however, astrocytes exhibit profound changes in these roles that can result in both negative and positive influences on surrounding cells. We suggest that these roles can be harnessed to aid in the recovery from injury. In particular, we focus this mini-review on roles played by metabotropic glutamate receptors (mGluRs) that are expressed on astrocytes during disease. Recent studies suggest that stimulation of these receptors in some cases may elicit protective effects on neighboring cells and may represent a new therapeutic approach to brain dysfunction. In other cases, however, antagonism may be preferable. Therefore, caution is warranted when evaluating effectiveness of mGluR stimulation (see Fig. 1). As indicated in this mini-review, astrocytes whether quiescent or reactive are highly heterogeneous populations with respect to their response to the local central nervous system (CNS) region in which they reside, and the specific diseases or injuries to which they are exposed. Therefore, the utility of application of specific agonists or antagonists may vary depending on the specific astrocytic populations under investigation and how they are impacted by their environment.

Before beginning it should be noted that we limit this review to astrocytes, their response to injury and effects of mGluR agonists. We recognize that other glial cells react to mGluR stimulation as well as other activating influences. For a more

comprehensive analysis of responses of these cells we refer the reader to excellent additional reviews of this subject (D'Antoni et al., 2008; Byrnes et al., 2009a; Bradley and Challiss, 2012; Loane et al., 2012; Spampinato et al., 2018).

Astrocytic function in the unlesioned brain

Astrocytes are the most abundant glial cell in the CNS and are specialized to perform many functions to support neuronal activity in the developing and adult nervous system. These include ion homeostasis, uptake of neurotransmitters, release of growth factors, participation in synaptic transmission, regulation of the blood-brain barrier and contribution to the CNS immune system (Sofroniew and Vinters, 2010). Astrocytes also present a dynamic environment for axon guidance during development by providing appropriate cell surface receptors and adherent molecules (Powell et al., 1997).

Interestingly, many of the functions of astrocytes are regulated by neuron-to-astrocyte crosstalk. Astrocytes are able to respond to several neurotransmitters, including glutamate (Perea and Araque, 2005; Di Castro et al., 2011), adenine triphosphate (ATP) (Bowser and Khakh, 2004; Perea and Araque, 2007), gamma-aminobutyric acid (GABA) (Perea et al., 2016; Mariotti et al., 2018), acetylcholine (Chen et al., 2012) and endocannabinoids (Navarrete and Araque, 2008). In response to these transmitters, astrocytes elevate intracellular calcium levels, release a number of gliotransmitters as well as a host of growth factors that impact

neuronal function (Martin, 1992; Nedergaard et al., 2003). As a result of such signaling, astrocytes then modulate synaptic function, maintenance, pruning and remodeling and express ion channels and neurotransmitter receptors and transporters (Kang et al., 1998; Guthrie et al., 1999; Navarrete and Araque, 2008). These physiological roles, manipulated by gliotransmitters and growth factors in normal astrocytes, are also observed during pathophysiological states of the nervous system, as discussed below.

Astrocytes are heterogeneous cells that not only differ in morphology and expression of intermediate filament levels, but also in the roles they play (Wilkin et al., 1990). For example, morphological differences are reported when a subtype of astrocytes from the human cortex and hippocampus are compared. High levels of glutamine synthetase (GS) and excitatory amino-acid transporters -1 and -2 (EAAT1, EAAT2) are observed in the hippocampus with long-process astrocytes, while cortical astrocytes are more heterogeneous with cells that are protoplasmic but exhibit reduced numbers of small processes and a low expression of GS, EAAT1, and EAAT2 (Sosunov et al., 2014). As suggested here, the heterogeneity in morphology may extend to heterogeneity in function. This is indicated for example in the observation that astrocytes of different regions release different substances that may influence neighboring neurons distinctly. For example, cultured astrocytes of the substantia nigra are better at supporting dopamine neuron survival than are astrocytes of the hippocampus (O'Malley et al., 1992). Such differences are also noted when substantia nigra astrocytes are compared to those of the ventral

tegmental area. In this case, recent studies of effects of astrocytes on local dopaminergic neurons suggest that growth and differentiation factor 15 (GDF15), a member of the transforming growth factor beta (TGF-β) superfamily, may be responsible for differences in survival and protection when dopaminergic neurons from the two brain regions are compared (Kostuk et al., 2019).

Heterogeneity also is observed in the markers of astrocyte function within brain regions. For example, astrocytic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, glutamate transporter 1, and potassium channel Kir4.1 expression are differentially expressed within regions as well as between specific regions in the brain (Poopalasundaram et al., 2000; Emsley and Macklis, 2006; Regan et al., 2007; Hoft et al., 2014). With respect to expression of mGluR5, examined in this mini-review, heterogeneity is also evident. Thus, few astrocytes of the spinal cord exhibit this receptor (Silva et al., 1999), while the majority of cortical astrocytes do (Biber et al., 1999). It is important to consider what roles these differences may play when thinking of how regional astrocytes may differ in response to mGluR agonists in distinct disease states.

Astrocytic response to injury

In the case of brain injury or disease, astrocytes become reactive. In this process, many of the actions of quiescent cells become enhanced or reduced to influence proximate cells. Traditionally, it was thought that these changes are

negative. For example, astrocytes can form a physical barrier to axon growth and produce a variety of molecules that serve as an impediment to nerve cell survival (Ohtake and Li, 2015; Adams and Gallo, 2018). Moreover, reactive astrocytes express a wide variety of inflammation-associated molecules and are capable of antigen presentation. These changes have profound pro-inflammatory effects that present an inhibitory environment for glial differentiation and endogenous remyelination (Hammond et al., 2014; Hammond et al., 2015). In a more specific example, studies by the Gallo laboratory demonstrated that when effects of endothelin-1, a secreted intercellular signaling molecule were characterized after focal demyelination of the corpus callosum, it acted as a negative regulator of NG2 glial differentiation and functional remyelination (Hammond et al., 2014). Moreover, ablation or inhibition of endothelin receptor-B accelerates oligodendrocyte progenitor differentiation and remyelination (Hammond et al., 2015). Similarly, other proteins, such as bone morphogenetic proteins, have negative effects on oligodendrocytes following spinal cord injury (SCI) (Wang et al., 2011) and in the case of the mouse model of ALS, mutated astrocytes can release toxic factors that kill up to 90% of co-cultured motor neurons (Diaz-Amarilla et al., 2011; Trias et al., 2013). This has relevance to the response to disease states.

On the other hand, recently, it has become more widely recognized that astrocytes can also have neuroprotective effects and enhance axonal and neuronal regeneration (Faulkner et al., 2004; Anderson et al., 2016). Reactive astrocytes in some cases suppress immune responses following CNS injury, maintain

extracellular homeostasis and produce growth factors (Belanger and Magistretti, 2009). Thus, newly proliferated astrocytes may interact and organize into scars that surround and isolate tissue lesions and protect or enhance regeneration. For example, after SCI (Wanner et al., 2013), signal transducer and activator of transcription 3 (STAT3), expressed by reactive astrocytes, has a key role in regeneration that includes control of inflammation (Zhong et al., 1994; Hong and Song, 2014). Selective deletion of the Stat3 driver after a wound leads to a significant increase of immune cell infiltration and neurodegeneration (Bush et al., 1999; Anderson et al., 2016). These observations suggest that astrocytes may be critical for the recovery of function and survival after injury.

To evaluate the effects of injury on astrocyte function and their production of specific molecules, gene transcriptome approaches have been used to characterize subtypes of astroglial cells in response to brain damage. GeneChip analysis of reactive astrocytic populations was evaluated in two brain injury mice models: neuroinflammation induced by a single intraperitoneal injection of lipopolysaccharide and focal ischemic stroke produced by transient middle cerebral artery occlusion (Zamanian et al., 2012). In both models, glial fibrillary acidic protein immunoreactivity is observed after 1 day and persists at least 1 week in combination with increased activated microglia. A core set of genes is upregulated in reactive astrocytes of both injury models, however, at least 50% of the altered gene expression is specific to a given injury type. These data suggest that there are distinct subtypes of reactive astrocytes, reminiscent of distinct types of quiescent

astrocytes. In the case of reactive astrocytes, these have been termed as A1 and A2 based on their detrimental or beneficial effects, respectively, during injury and repair (Zamanian et al., 2012; Liddelow and Barres, 2017; Liddelow et al., 2017).

A1 reactive astrocytes may have negative effects on surrounding cells in response to inflammation. For example, they may secrete molecules that are inhibitory to neurite outgrowth. In addition, swelling of these astrocytes after injury may result in the release of excessive amounts of glutamate. Liddelow et al (Liddelow et al., 2017) suggests that this A1 activation may be induced by activated microglia through the secretion of cytokines. After induction, A1 astrocytes secrete a neurotoxin of uncertain identity that induces rapid death of neurons and oligodendrocytes. A2 astrocytes, in contrast, are commonly induced by ischemia and their responses to the ischemia are beneficial. This population is geared toward restoring trophic support and synapse repair and to promote the survival and growth of neurons (Hayakawa et al., 2014). A2 astrocytes express high levels of neurotrophic factors and cytokines, including brain-derived neurotrophic factor (BDNF), cardiotrophin-like cytokine factor 1 (CLCF1), interlukin-6 (IL-6), and GDF15, as well as thrombospondins that promote synapse repair (Eroglu, 2009; Zamanian et al., 2012). Determining the cellular and molecular basis underlying A2 induction remains an issue to address and is important with respect to degenerative disease.

Use of mGluRs to regulate astrocytes after injury

A number of studies have focused on astrocytic mGluRs as targets that can be manipulated to enhance repair after injury (Fulmer et al., 2014; Liddelow and Barres, 2017; Adams and Gallo, 2018; Miller, 2018; Spampinato et al., 2018). mGluRs, particularly mGluR3 and mGluR5, are the two most abundant mGluRs found on astrocytes (Biber et al., 1999; Sun et al., 2013). In response to injury, these receptors are upregulated at the lesion site, suggesting that astrocyte function can be influenced in the specific location where effects may be important. However, as noted previously, discretion is merited with respect to the function of these receptors. While some studies have described positive effects of astrocytic mGluR activation after injury through the actions of neurotrophins and growth factors (Bruno et al., 1998; Caraci et al., 2011; Fulmer et al., 2014; Battaglia et al., 2015; Durand et al., 2017), others have reported that they may elicit harmful effects through the production of cytokines and inflammatory mediators (Sofroniew, 2014). These differences in effect may be due to the state of activation of astrocytes, the region being assessed and the type of lesion being examined (Biber et al., 1999; Sofroniew, 2005; Anderson et al., 2014; Liddelow and Barres, 2017; Liddelow et al., 2017). Consequently, environmental distinctions must be taken into consideration when assessing astrocytic mGluRs as potential pharmacological targets and included in determining whether stimulation of these receptors should be enhanced or inhibited.

Signaling by and regional expression of astrocytic mGluR3 and mGluR5

mGluRs are G protein-coupled receptors consisting of seven transmembrane domains that are subdivided into Group I, II, and III based on their signaling transduction pathways, amino acid sequence homology, and selectivity of agonists and antagonists (Pin and Duvoisin, 1995; Dingledine et al., 1999; D'Antoni et al., 2008; Byrnes et al., 2009a; Spampinato et al., 2018). mGluR5, in addition to mGluR1, is classified as a Group I mGluR, while mGluR3 is part of the Group II mGluRs along with mGluR2. Group I mGluRs function through G_q-proteins, resulting in activation of phospholipase C (PLC), hydrolysis of phosphoinositides, release of calcium, and activation of protein kinase C (PKC). Further downstream signaling pathways include casein kinase 1, cyclin-dependent protein kinase 5, Jun kinase, mitogen-activated protein kinase/extracellular receptor kinase, and mammalian target of rapamycin/p70 S6 kinase (Karim et al., 2001; Hou and Klann, 2004; Warwick et al., 2005; Page et al., 2006; Li et al., 2007; Saugstad and Ingram, 2008). On the other hand, Group II mGluRs are associated with G_i- and G_0-proteins, and are negatively coupled to adenylate cyclase. Activation of Group II mGluRs inhibits voltage-gated calcium entry into the cell. In addition, these receptors can activate MAPK and phosphatidyl inositol 3-kinase pathways (Pin and Duvoisin, 1995; Iacovelli et al., 2002; D'Antoni et al., 2008; Byrnes et al., 2009a; Niswender and Conn, 2010; Spampinato et al., 2018).

Gene expression analysis has been done to study the presence of mGluR3 and mGluR5 specifically on astrocytes isolated from mouse hippocampus or cortex.

These studies reveal that while all mGluRs are found at least at low levels in adult tissue in mice, the most abundant receptor is mGluR3 followed by mGluR5 (Sun et al., 2013). These two mGluRs are also present in humans under normal conditions, while others are undetectable (Geurts et al., 2003; Geurts et al., 2005; Newcombe et al., 2008; Sun et al., 2013). It is of interest that in some cases expression of the receptors changes with development, but not in others, suggesting that the role of specific receptors may be altered as the brain matures. Astrocytic mGluR3 expression remains relatively stable at 1-, 2-, 3-, and 12-weeks of age (Sun et al., 2013), while mGluR5 expression is highest at postnatal day 7 (Cai et al., 2000; Sun et al., 2013) before rapidly declining through adulthood (Cai et al., 2000; Sun et al., 2013).

To examine the presence of these receptors in astrocytes *in vitro*, cells are removed from developing animals and grown in culture. This approach has the advantage of evaluating the isolated cells, examining their receptors and defining their function. In general, as was the case *in vivo*, mGluR3 and mGluR5 show strong expression when compared to all the other mGluRs (Biber et al., 1999). Interestingly, culture studies also reveal that regional differences are apparent. While mGluR3 and mGluR5 are found in astrocytes isolated from thalamus, tegmentum, cortex, hippocampus, and striatum (Biber et al., 1999), there are almost undetectable levels of these receptors within the cerebellum (Biber et al., 1999) and spinal cord (Silva et al., 1999). It is interesting to consider what these regional differences may signify. Transcriptome analysis has indicated that cultured

astrocytes exhibit a phenotype akin to A2 reactive astrocytes of the ischemic brain (Zamanian et al., 2012). These studies suggest that regional differences in astrocyte expression of mGluRs in culture may foretell regional differences that while not evident *in vivo*, will be evident after specific injuries.

Rodent brain slices have been studied to bridge the gap between *in vitro* and *in vivo* studies. In particular, specific agonists of Group I and/or Group II mGluRs induce transient increases in intracellular calcium levels within astrocytes of hippocampal slices as they do *in vivo* (Pasti et al., 1997; Shelton and McCarthy, 1999; Nett et al., 2002; Zur Nieden and Deitmer, 2006; Copeland et al., 2017). In concordance with *in vivo* studies also is the fact that astrocytic mGluR5 is developmentally regulated in slices with the highest expression occurring in slices isolated from P1-10 rodents before declining into adulthood (Cai and Kimelberg, 1997; Kimelberg et al., 2000).

In models of disease and in human disease tissue, levels of astrocytic mGluRs are upregulated in or near lesions. Therefore, we propose that the roles of these receptors may be most apparent during development, become downregulated during adulthood, but emerge to play critical roles during CNS disease. The models in which mGluR5 is elevated include multiple sclerosis (MS) (Fulmer et al., 2014), Alzheimer's disease (AD) (Shrivastava et al., 2013), amyotrophic lateral sclerosis (ALS) (Vermeiren et al., 2006), epilepsy (Aronica et al., 2000; Ulas et al., 2000; Ferraguti et al., 2001; Umpierre et al., 2016) and SCI (Nicholson et al., 2012; Kim et

al., 2016; Michot et al., 2017). Similarly, astrocytic mGluR5 is upregulated in human tissue from patients with MS (Geurts et al., 2003; Newcombe et al., 2008), AD (Casley et al., 2009; Lim et al., 2013), ALS (Aronica et al., 2001a; Anneser et al., 2004) and epilepsy (Aronica et al., 2001b; Tang et al., 2001; Aoki et al., 2019). In regards to mGluR3, its expression is enhanced on astrocytes in animal models of epilepsy (Aronica et al., 2000; Ferraguti et al., 2001) and in human tissue taken from patients with MS (Geurts et al., 2003; Newcombe et al., 2008), ALS (Aronica et al., 2001a; Anneser et al., 2004) and epilepsy (Aronica et al., 2001b).

Roles of mGluRs on reactive astrocytes

In general, stimulation of Group I and/or Group II mGluRs on reactive astrocytes leads to the release of neurotransmitters, including glutamate (Miller et al., 1995; Nakahara et al., 1997; Pasti et al., 1997; Biber et al., 1999), as well as other factors such as BDNF (Jean et al., 2008; Durand et al., 2017), glial-derived neurotrophic factor (GDNF) (Battaglia et al., 2015), and TGF-β (Bruno et al., 1998; Caraci et al., 2011). Astrocytic mGluR activation can also lead to enhanced glutamate uptake through Group I or II receptors (Yao et al., 2005; Umpierre et al., 2019). These data suggest that astrocytic mGluRs have the potential to play positive roles in the diseased brain. Nevertheless, these effects may vary based on the different environments of the different diseases. For example, as will be discussed in the next section, mGluR stimulation may elicit positive astrocytic effects in diseases such as MS and AD, while eliciting mixed effects in other diseases like ALS.

Roles of mGluR3 and mGluR5 in response to disease

Multiple sclerosis: mGluR5 is increased in reactive astrocytes specifically within the lesion sites of the cuprizone model of MS (Fulmer et al., 2014). This increase is not found on microglia or CC1+ mature oligodendrocytes. In the experimental autoimmune encephalomyelitis (EAE) model of MS, studies of tissue samples taken from EAE rodents indicate an increase in mGluR5 in the whole brain and forebrain. However, the cells expressing these receptors were not identified (Sulkowski et al., 2009; Sulkowski et al., 2013). In an attempt to determine roles of mGluR agonists and antagonists in rodent models of MS, these drugs have been injected either locally within the lesion site or systemically. The Group I/Group II mGluR agonist *trans*-(1S,3R)-1-amino-1,3-cyclopentanedicarboxylic acid (ACPD) injected directly into the cuprizone-induced lesion increases synthesis and release of BDNF, an effect that is blocked when BDNF was selectively deleted from astrocytes, suggesting that the mGluRs mediate the increase in this trophic factor in astrocytes (Fulmer et al., 2014).

In the case of the EAE models, effects of the mGluR agonists and antagonists were injected into the whole animal making the relative contribution of these receptors on astrocytes compared to other cell types unknown. In this model, mGluR5 antagonists have no effect on motor function (Fazio et al., 2008), nor do they affect myelin ultrastructure compared to EAE animals receiving vehicle

(Sulkowski et al., 2013; Sulkowski et al., 2014; Dabrowska-Bouta et al., 2015), suggesting that actions of mGluRs in EAE may be different from those in the cuprizone model. It should be noted however, that application of the agonists to the EAE CNS as a whole may miss a subtle difference elicited through the actions of astrocytes that can be enhanced. Clearly, additional studies are needed to identify which cells express mGluRs. This makes it possible to elucidate roles of agonists and determine their potential to signal though mGluRs on astrocytes or other cell types.

Alzheimer's disease: *In vivo* studies of mGluRs and astrocytes in AD are quite limited, however it is interesting to note that amyloid-beta (Aβ) increases expression of mGluR5 in an AD transgenic model (Shrivastava et al., 2013). This effect also occurs when Aβ is added to astrocytes in culture (Casley et al., 2009; Grolla et al., 2013; Lim et al., 2013; Shrivastava et al., 2013), indicating that when this agent is elevated, roles of astrocytic mGluR5 may be enhanced. However, information is lacking as to what the consequence is of this upregulation.

Culture models of AD are most informative in defining effects of mGluRs on astrocytes. These indicate that mGluRs are present on astrocytes in these models. In general, stimulation of the receptors has had beneficial results. This is most well known with respect to Group II receptors. For example, astrocytic Group II activation reduces Aβ production (Durand et al., 2014), and increases Aβ uptake in astrocytes, as well as releases BDNF from these cells (Durand et al., 2017). BDNF in this study enhances neuron survival when neurons are challenged by treatment

with Aβ. In complementary work, stimulation with ACPD that stimulates both Group I and Group II receptors also increases BDNF synthesis and release (Jean et al., 2008). Other studies indicate that stimulation of mGluR3 reduces Aβ-induced neurodegeneration in mixed neuronal-glia cultures (Caraci et al., 2011). This effect is blocked when the receptor function is inhibited or when astrocytes are derived from mGluR3 deleted mice. In this case, mGluR3 rescues the neurons from Aβ-elicited death through the action of astrocyte-derived TGF-β. Overall, these studies in culture models of AD indicate that both astrocyte-derived BDNF and TGF-β may play positive protective roles in this disease and that this may be regulated by mGluRs.

Amyotrophic Lateral Sclerosis: In ALS, studies of the role of astrocytic mGluR5 have focused on astrocytes cultured from animal models of the disease, particularly the hSOD1^{G93A} mouse or rat models. These studies find that mGluR5 is expressed at three-fold greater levels in hSOD1^{G93A} astrocytes than in wild-type cells (Vermeiren et al., 2006). In this disease however, upregulation of astrocytic mGluR5 appears to have negative consequences. Stimulation of Group I mGluRs on hSOD1^{G93A} astrocytes results in the death of these cells and this effect is blocked with an mGluR5 antagonist (Rossi et al., 2008). Moreover, while wild-type astrocytes treated with a Group I agonist enhances aspartate uptake, astrocytes derived from hSOD1^{G93A} rats fail to increase aspartate uptake, indicating that the mutant gene blocks protective roles of the Group I agonist (Vermeiren et al., 2006; Vergouts et al., 2018). Proper removal of excitatory transmitters such as aspartate and glutamate

can be important in preventing excitotoxicity in diseases such as ALS, where increased glutamate levels and reduced glutamate transporter expression is evident in tissue from ALS patients (Perry et al., 1990; Rothstein et al., 1995). Inhibiting mGluR5 activity on hSOD1^{G93A} astrocytes is then a strategy that may be pursued to enhance protective astrocytic functions.

In contrast to activation of Group I receptors, potential actions of Group II mGluRs on hSOD1^{G93A} astrocytes have not yet been studied in culture. However, effects of a Group II agonist injected subcutaneously have been studied in hSOD1^{G93A} mice (Battaglia et al., 2015). Injection results in reduced neuronal death and elevated GDNF levels in the spinal cord with corresponding improvements in motor performance and neurologic signs. The same study shows that the Group II agonist enhances GDNF release from cultured wild-type astrocytes through mGluR3. It is not yet known if astrocytes are responsible for the effects observed in hSOD1^{G93A} mice.

Conclusions

This mini-review has documented a number of studies that suggest the possible importance of astrocytes as therapeutic targets in treatment of CNS disease. In particular, we summarize roles of quiescent astrocytes and how they alter their functions in response to injury. In discussing these events it becomes obvious that astrocytes are not simple homogeneous populations. Their critical

impact on the maintenance of CNS function has been increasingly recognized. However, what is still generally unappreciated is their heterogeneity in function and in response to disease. We focus this review on the roles played by mGluR3 and mGluR5, recognizing that these receptors are only representative of multiple receptors that influence function. What has been obvious, however, is that these receptors are upregulated on astrocytes at or near lesion sites, putting them in optimal position to have important influences under these conditions. Moreover, manipulation of signaling through these receptors is beginning to emerge as a strategy worth pursuing in at least some disease conditions.

One note about the studies that have been discussed: Although descriptive work assesses how astrocytes respond to injury and where mGluRs are expressed on astrocytes, it has been difficult to attribute the results of manipulation of these cells and these receptors to function *in vivo*. Critical work is clearly necessary to extend studies of function by using new animal models where astrocytes specifically can be manipulated by the deletion of a particular protein at distinct time points as is now being done in a number of studies (Fulmer et al., 2014; Umpierre et al., 2019).
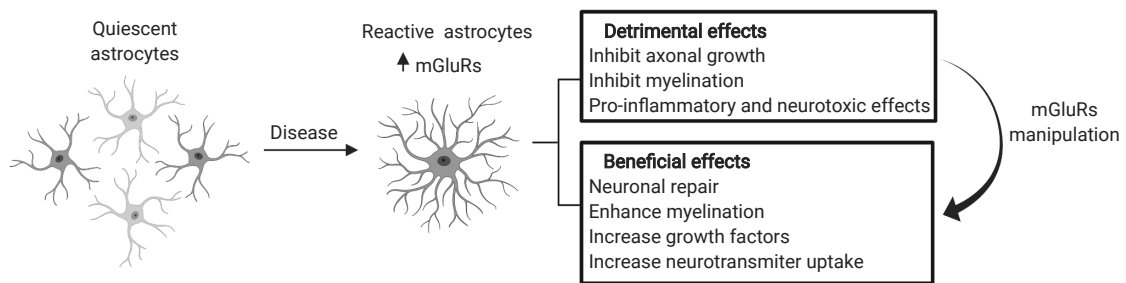
Acknowledgements

Fig. 1 Response of astrocytes to disease can be manipulated by mGluRs in a positive or negative direction depending on the state of astrocyte activation, the local astrocyte environment and the disease that is responsible for reactivity. Created with BioRender.

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