The Role of MAPKs in CNS demyelination

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ABSTRACT OF THE DISSERTATION

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MAPKs are protein kinases that play crucial roles in regulating cell proliferation, survival and differentiation. In the central nervous system (CNS), p38 MAPK and Erk1/2 play an essential role during development of the oligodendrocyte lineage and are necessary for myelination. However, the effects on mature myelin, particularly under pathological conditions remain unclear. In various neurodegenerative diseases, mature myelin deteriorates, leading to myelin loss and neuronal dysfunction. Several growth factors and cytokines are found to be increased in the demyelinated lesions suggesting that the extracellular stimuli may contribute to the myelin pathology and the subsequent changes in oligodendrocyte phenotypes. In this study, we investigated the effects of growth factor signaling on mature oligodendrocytes and the myelin. Specifically, we investigated the role of Erk1/2 and p38 MAPK activation in triggering oligodendrocyte demyelination. Using an *in vitro* model for CNS demyelination, we show that growth factors that are found upregulated in CNS lesions, such as FGF-2 promotes myelin breakdown through activation of the Erk1/2 and p38

MAPK pathways. We also show that ectopic activation of either Erk1/2 or p38 MAPK is sufficient to induce demyelination. Our study also indicates that growth factor or MAPK-induced demyelination was not associated with cell death, indicating the possibility of oligodendrocyte de-differentiation. To further investigate this, in chapter three we characterized the phenotype of mature oligodendrocytes following MAPK activation. Ectopic activation of Erk1/2 in mature oligodendrocytes resulted in the down-regulation of myelin proteins and a drastic change in cell morphology. A similar phenotypic change was also observed upon ectopic activation of p38 MAPK. Interestingly, after inducing the phenotypic changes, Erk1/2 activation but not p38 MAPK was sufficient to direct non-proliferating mature oligodendrocytes to re-enter the cell cycle. This result suggests that mature oligodendrocytes may have the ability to regenerate and remyelinate following the myelin loss. These studies also demonstrate that MAPKs are crucial in regulating this process. In chapter four, we investigated whether Erk1/2 mediates oligodendrocyte demyelination induced under pathological conditions. To this end, we used an *in vitro* model for white matter injury in which diffused axon injury is mimicked by mechanical stretching of the myelinated axons in culture. Our data shows that axonal stretch injury induces oligodendrocyte demyelination independent of axon degeneration and inhibition of Erk1/2 activation has a protective effect on the myelin. Altogether, these results suggest that MAPKs play an essential role in triggering oligodendrocyte demyelination and promoting phenotypic changes that may contribute to the subsequent regenerative process of remyelination.

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3 Significance and Research Aims

Oligodendrocytes are the myelin-forming glial cells of the central nervous system (CNS), and they play a critical role by forming myelin sheaths around axons, providing electrical insulation and enhancing conduction velocity. Myelin is an electrically resistant membrane primarily composed of lipids. It also consists of proteins such as myelin basic protein (MBP), proteolipid protein (PLP), 2',3'-cyclic nucleotide 3′-phosphodiesterase (CNP), myelin-associated glycoprotein (MAG), and myelin-oligodendrocyte glycoprotein (MOG) (Baumann and Pham-Dinh, 2001). Compact myelin sheaths serve as an insulator in the internodal region by preventing leakage of current and by concentrating the voltage-gated sodium channels in the nodes of Ranvier. Conduction velocity is increased as the action potential jumps from node to node, generating rapid saltatory conduction (Arroyo and Scherer, 2000; Buttermore et al., 2013).

Maintenance of a healthy myelin sheath is crucial for neuronal health and proper conduction of action potentials. In the demyelinating disorders of the CNS, myelin sheaths are damaged, leading to the loss of neurological functions. There is increasing evidence that in demyelinating disorders of the CNS, many growth factors and proinflammatory cytokines are upregulated. Although causes for demyelination are not known, signaling from these extracellular stimuli may converge, activating common downstream signaling molecules that can cause demyelination. The goal of this study was to elucidate the mechanisms regulating demyelination of oligodendrocytes. Specifically, I examined the role of mitogen-activated protein kinases (MAPKs) in oligodendrocyte demyelination that are commonly activated by

various growth factors and cytokines. I have investigated these roles through three specific aims as described below.

Specific Aim 1 (Chapter Two): To determine the intracellular pathways mediating oligodendrocyte demyelination.

In this aim, using an *in vitro* myelinated dorsal root ganglion (DRG)-oligodendrocyte precursor cell (OPC) co-culture model, where OPCs differentiate and form myelin on associated axons, I investigated the role of growth factors in triggering demyelination of oligodendrocytes. We found that fibroblast growth factor 2 (FGF-2) causes myelin breakdown in myelinated co-cultures. We further investigated the downstream signaling molecules mediating oligodendrocyte demyelination. Our results indicated that FGF-2 induced demyelination is mediated through Erk1/2 and p38 MAPK pathway. Interestingly, we also found that ectopic activation of Erk1/2 or p38 MAPK in mature oligodendrocytes is sufficient to trigger oligodendrocyte demyelination in DRG-OPC co-cultures.

Specific Aim 2 (Chapter Three): **To determine the fate of oligodendrocytes after demyelination.**

We characterized the phenotype of differentiated mature oligodendrocyte upon activation of Erk1/2 or p38 MAPK. To specifically activate Erk1/2 or p38 MAPK in mature oligodendrocytes, we expressed constitutively active MKK1 and MKK6 (CA-MKK1 or CA-MKK6), upstream activators of Erk1/2 and p38 MAPK, respectively, under the control of doxycycline-inducible promoter. Ectopic activation of Erk1/2 or p38 MAPK in mature oligodendrocytes was sufficient to down regulate myelin

proteins and also induced morphological changes. We also show that activation of Erk1/2, but not p38 MAPK, is sufficient to drive non-proliferative mature oligodendrocytes to re-enter the cell cycle.

Specific Aim 3 (Chapter Four): To determine whether the injury-associated demyelination of oligodendrocytes is mediated by Erk1/2 or p38 MAPK signaling.

In this aim, we investigated the effects of diffuse axon injury (DAI) on myelinated oligodendrocytes, specifically whether stretch-injury induces oligodendrocyte demyelination. We observed that stretch injury to the myelinated axons triggered oligodendrocyte demyelination in the absence of obvious axonal loss. We also addressed whether the injury associated demyelination was mediated through the activation of MAPKs. Our results indicate that Erk1/2 pathway may be involved in the oligodendrocyte demyelination induced by stretch-injury.

4 Chapter One: Introduction

4.1 CNS demyelination and pathology

The myelin sheath on axons is crucial for orchestrating the timely impulses on a target by enhancing conduction velocity. The loss of myelin leads to functional impairment in the nervous system, and may occur as a consequence of injury or disease, where myelin sheaths are either destroyed or damaged, forming demyelinated lesions. The conduction of action potentials is impaired in demyelinated axons due to "short-circuiting" the action current through the denuded axon and prolonging the refractory period (Daroff and Bradley, 2012). These consequences result in the loss of neurological function and manifest as various combinations of physical and cognitive impairments. In many neurological disorders, such as multiple sclerosis (MS), the targets of injury are oligodendrocytes, leading to demyelination. Other major causes of demyelination in the CNS are genetic abnormalities and environmental factors such as trauma, viruses, toxins and inflammatory damage that could subsequently lead to demyelination (Kennedy and Steiner, 1994; Lucchinetti et al., 2000)**.**

Multiple sclerosis (MS) is a severe demyelinating disorder of the CNS where myelin and oligodendrocytes are damaged, causing focal areas of demyelination referred to as "plaques" (Hu and Lucchinetti, 2009). Multiple factors can cause inflammation, which in turn may lead to demyelination. It is a complex disease, as demyelinated lesion patterns vary across patients. Four patterns of MS lesions, categorized as pattern I, II, III and IV, have been identified based on immune cell infiltration and appearance of the lesions. Here it is noteworthy that in lesion patterns I and II, oligodendrocyte cell bodies are well preserved, whereas in pattern III and IV,

loss of oligodendrocytes is observed (Franklin and Ffrench-Constant, 2008; Hu and Lucchinetti, 2009). The reason behind the varied degree of oligodendrocyte loss and heterogeneity among the lesions of MS patients is not clear. However, there is increasing evidence that many cytokines and growth factors are expressed in high levels at the lesions, possibly contributing to the pathology of the disease. For example, cytokines such as interferon-γ (IFN-γ) and interleukin 17 (IL-17), and growth factors such as FGF-2 are found to be upregulated in MS (Lock et al., 2002; Lu et al., 1993; Matusevicius et al., 1999; Sarchielli et al., 2008). The signals from these growth factors and cytokines and their mode of action during the course of the disease have yet to be elucidated.

Demyelination can also be caused by viral or bacterial infections. Progressive multifocal leukoencephalopathy (PML) is an example of a viral mediated demyelinating disorder. PML mostly occurs in people with severe immune deficiency, such as acquired immunodeficiency syndrome (AIDS) patients, and is caused by the JC virus (JCV), which infects oligodendrocytes, leading to cell lysis and myelin destruction (Ferenczy et al., 2012). Studies have discovered that immunodeficiency virus 1 and JCV do interact on a molecular level via the Tat protein, thus explaining the significant increase of PML in patients with acquired immunodeficiency syndrome. Furthermore, the indirect production of cytokines by immunodeficiency virus 1 is responsible for increasing JC virus replication. Cytokines, such as transforming growth factor-beta (TGF-β) via SMAD 3 and 4, were shown to be involved in increasing JC virus replication and thus the development of PML (Atwood et al., 1995; Enam et al., 2004; Stettner et al., 2009). TGF-β and its receptor

were found to be over expressed in astrocytes and oligodendrocytes of the infected brains compared to controls (Enam et al., 2004). These studies indicate that cytokines and their signaling cascades might be involved in causing demyelination and further affecting oligodendrocyte biology.

Physical damage to axons, as seen in traumatic incidences such as motor vehicle accidents and assaults, could be another cause of demyelination. Insulted axons are damaged as a result of force exerted on the white matter of the brain (Adams et al., 1984; Gennarelli, 1993). Traumatic brain injury (TBI) and spinal cord injury can cause to neurological disorders and long term disabilities (Gronwall and Wrightson, 1974; Levin et al., 1990), possibly leading to axon degeneration and subsequent demyelination. Due to the rapid stretching of axons during TBI, axons are injured or damaged throughout the brain, an effect known as diffuse axon injury (DAI) (Adams et al., 1989; Gennarelli, 1993; Grady et al., 1993). DAI is the predominant pathology resulting from TBI (Adams et al., 1989). The effects of DAI on myelin and oligodendrocytes have yet to be investigated.

Many other factors contribute to the demyelination other than autoimmunity, infectious agents and physical insults. Exposure to chemicals like ethidium bromide or lysolecithin can lead to demyelination, either by damaging the myelin sheaths or by causing death of oligodendrocytes (Blakemore, 1982; Blakemore and Franklin, 2008).

4.2 Oligodendrocyte development

4.2.1 Oligodendrocyte origin

During CNS development, oligodendrocytes in the spinal cord and brain arise from multiple sources. The majority of the OPCs in the spinal cord, which eventually differentiate and myelinate, originate from the ventral ventricular zone, and a small portion comes from the dorsal spinal cord. In the forebrain, OPCs originate from different sources in a temporal fashion. The initial source of OPCs is from the medial ganglionic eminence and anterior entopeduncular areas of the ventral forebrain. The second source is the caudal and lateral ganglionic areas, and the last source is the postnatal cortex. OPCs that are derived from multiple sources are functionally redundant as indicated in studies where the distribution of OPCs is unaffected even if one source is destroyed (Kessaris et al., 2006). These different sources of OPCs suggest that cells may compete for the limited available growth factors in the brain (van Heyningen et al., 2001). This is made evident by the fact that very few OPC lines are successful in generating oligodendrocytes, and in fact, the first wave of OPCs is eventually lost and does not contribute to generating oligodendrocytes. Though OPCs arise from multiple sources, in order to reach their final destinations, these OPCs have to migrate long distances in the brain (Bradl and Lassmann, 2010).

4.2.2 OPC migration

During CNS development, OPC migration is essential and regulated by growth factors (PDGF, FGF-2 and HGF), chemotrophic molecules (netrins and semaphorins) and chemokines (CXCL1). Contact mediated migration of OPCs, which includes extracellular matrix proteins, cell surface molecules, and cadherins, have also been shown to regulate OPC migration (Bradl and Lassmann, 2010). Migration of OPCs from the subventricular zone (SVZ) is also observed at demyelinated lesions in

MS and in a rodent model of demyelination induced by lysolecithin (Aguirre and Gallo, 2007; Nait-Oumesmar et al., 2007). After reaching their final destinations, they differentiate into myelinating oligodendrocytes.

4.2.3 OPC proliferation, differentiation and maturation

Several growth factors such as PDGF and FGF-2 regulate OPC proliferation and once they reach an adequate population size they proceed into next stages of development (Fortin et al., 2005; Fruttiger et al., 1999). The transition of OPCs into mature oligodendrocytes is tightly controlled and involves the integration of both extrinsic and intrinsic factors. The extrinsic factors involve signals from growth factors such as PDGF, FGF-2, and insulin-like growth factor 1 (IGF-1), which have all been shown to regulate differentiation (Bansal and Pfeiffer, 1997b; Hsieh et al., 2004; Wolswijk and Noble, 1992). The intrinsic mechanism of OPC differentiation involves OPCs ability to differentiate after a specific number of cell divisions. Though PDGF was shown to prevent OPC differentiation and maturation, after a specific number of cell divisions, OPCs exit the cell cycle and differentiate, even in the presence of PDGF. This is accomplished by accumulating $p57^{kip2}$, which are cyclin-dependent kinase (cdk) inhibitory proteins (CKI) that inhibit cyclinE-cdk2 at the G1-S checkpoint (Dugas et al., 2007). Therefore, these cell cycle inhibitory proteins serve as internal regulators thus regulating the switch from proliferation to differentiation. Another important regulator that promotes differentiation of OPCs is thyroid hormone, which accumulates inhibitory proteins such as $p27^{kip1.}$ Thus, in the presence of thyroid hormone, the differentiation of OPCs is promoted even in the presence of PDGF (Durand and Raff, 2000). Together, extrinsic growth factors and

intrinsic cell cycle inhibitory protein levels work in concert to drive OPC development by regulating the timing of cell cycle exit and the onset of differentiation.

Before interacting with neurons, differentiated oligodendrocytes develop a complex branching network by undergoing morphological changes, including process extension. Differentiated oligodendrocytes with multiple processes are capable of ensheathing multiple axons, and this event is highly coordinated. Ensheathment by oligodendrocytes is temporally regulated and is completed within 12-18 hours. After the ensheathment of the axons, oligodendrocytes myelinate by wrapping their processes multiple times around axons and forming compact myelin (Bradl and Lassmann, 2010). The molecular mechanisms that regulate oligodendrocyte myelination are not well understood. Electrical activity induced by axonal signals, along with growth factors such as NRG1 type III, were shown to regulate the myelination process in the CNS (Brinkmann et al., 2008; Demerens et al., 1996; Kuperman et al., 1964; Stevens et al., 2002).

For the myelin assembly, oligodendrocytes have to synthesize and transport myelin proteins such as MBP and PLP in a short time. Neuronal signals might regulate this complex process of transporting and delivering myelin proteins. For example, in the absence of neurons, PLP is localized to the oligodendrocyte cell bodies and shows little co-localization with MBP (Fitzner et al., 2006). Furthermore, in the absence of neurons, synthesized PLP is internalized by endocytosis (Trajkovic et al., 2006). However, in the presence of neurons, PLP co-localizes with MBP, which

is trafficked to the neuronal membrane. These observations suggest that compact myelin formation is regulated by neuronal signals.

4.2.4 Oligodendrocyte lineage progression

The differentiation of OPCs into mature oligodendrocytes is a multistep process with distinct morphological and antigenic stages of maturation. The developmental stages during the progression of precursor cells into mature oligodendrocytes have been well characterized. Bipolar OPCs first transition into multipolar late progenitors, and then they differentiate into post-mitotic immature oligodendrocytes. Immature oligodendrocytes differentiate into mature oligodendrocytes with complex, well-branched processes (Pfeiffer et al., 1993; Song et al., 2001). OPCs are characterized by the expression of membrane ganglioside A2B5 and PDGFRα; chondroitin sulphate proteoglycan NG2; and transcription factors Olig1/2, Nkx2.2, and Sox10. Differentiated oligodendrocytes express many markers, which include 2, 3'-cyclic nucleotide 3'-phosphodiesterase (CNP), galactosylceramide (GalC), myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), and myelin proteolipid protein (PLP).The stages of oligodendrocyte development during lineage progression can be identified by the expression of the stage-specific antigens. Early progenitors are characterized by the expression of Olig2 and A2B5, and they become late progenitors upon O4 expression. The next stage of maturation is the immature stage, where they initially express O1 (GalC), and HPC7 followed by CNP. Finally, the immature oligodendrocytes transition into mature oligodendrocytes upon expressing MBP, MOG (Emery, 2010a; Emery, 2010b; Miller, 2002; Pfeiffer et al., 1993) (Figure 1).

4.3 Growth factors in oligodendrocyte development

Distinct stages of oligodendrocyte development such as proliferation, migration, differentiation, maturation and myelination of oligodendrocytes are regulated by growth factors that activate one or more pathways. Several growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF-2), insulin-like factor-1 (IGF-1), and glial growth factor (GGF), have been shown to influence oligodendrocyte development.

4.3.1 FGF

FGF-2, a member of the FGF family, elicits diverse responses upon binding to its cognate receptor, including regulating proliferation, differentiation, and process extension during oligodendrocyte development. FGF receptors are expressed by oligodendrocyte lineage cells in a developmentally regulated manner (Bansal et al., 1996; Bansal et al., 2003). OPCs express FGF receptor 3 (FGFR3), with peak levels in the late progenitor stage that gradually decrease thereafter. FGF receptor 2 (FGFR2) levels gradually increase and are found predominantly in differentiated oligodendrocytes. FGF receptor 1 (FGFR1) expression reaches its peak level in terminally differentiated mature oligodendrocytes (Bansal et al., 1996). The developmental regulation of FGF receptor expression provides the molecular basis to elicit multiple responses in developing oligodendrocytes.

Early stages of oligodendrocyte development, such as OPC generation and specification, are influenced by FGF signaling. Studies have shown that OPC specification is mediated through FGF signaling, and inhibiting FGF blocks OPC specification in neural precursor cells (Kessaris et al., 2004; Naruse et al., 2006).

Recent *in vivo* knock out studies found that OPCs were not generated in FGFR1/FGFR2 knockout mice, whereas OPC generation in FGFR3 knock outs was unaffected (Furusho et al., 2011).

FGF-2 acts as a mitogen for OPCs and further promotes proliferation by upregulating PDGF receptor α (PDGFRα), expression in OPCs (Wolswijk and Noble, 1992). But differentiation and maturation of oligodendrocytes is inhibited in the presence of FGF-2 (Bansal and Pfeiffer, 1997b). *In vitro* studies demonstrated that along with FGF-2, FGF-8, and FGF-17 also inhibited differentiation of OPCs via FGFR3 (Fortin et al., 2005). In differentiated oligodendrocytes, process outgrowth was enhanced by FGF-9 (Fortin et al., 2005). *In vivo* studies have demonstrated that, in FGF-2 null mice, an increased number of oligodendrocytes were observed in the adult mice without any significant difference in OPC density, survival, or proliferation, indicating that differentiation of OPCs was promoted in the absence of FGF-2 (Murtie et al., 2005).

Recent *in vivo* studies demonstrated that FGF signaling also affects CNS myelination by regulating the thickness of the myelin sheath (Furusho et al., 2012). In FGFR1/FGFR2 mutant mice, myelin thickness was reduced compared to normal adults, and this affect was associated with reduced Erk1/2 activity in the hypo-myelinated axons (Furusho et al., 2012). The distinct effects of FGF-2 in oligodendrocytes are mediated through several downstream kinases such as Erk1/2, p38 MAPK and p70 S6 kinase (Baron et al., 2000).

Although FGF signaling plays an important role during oligodendrocyte development, it is also implicated in the pathology of demyelinating diseases. Many

studies have reported that FGF-2 has deleterious effects on mature oligodendrocytes. *In vitro* studies have demonstrated that in the presence of FGF-2, mature oligodendrocytes down regulate myelin proteins (Bansal and Pfeiffer, 1997a; Fressinaud et al., 1995). *In vivo* studies have also demonstrated a similar observation, where injection of FGF-2 into the cerebrospinal fluid (CSF) of rat brains caused myelin disruption in the anterior medullary velum (AMV) region (Butt and Dinsdale, 2005; Goddard et al., 2001). Additionally, *in vitro* studies also showed that mature oligodendrocytes lose their membranes upon FGF-2 treatment, and although they can still reenter the cell cycle, they cannot exit (Bansal and Pfeiffer, 1997a; Fressinaud et al., 1995).

4.3.2 PDGF

PDGF is a potent mitogen for OPCs as demonstrated by gain and loss of function studies. *In vivo* overexpression studies demonstrated hyperproliferation of OPCs, which resulted in an excess number of oligodendrocytes. In PDGF-A knockout mice, hypomyelination was observed as a consequence of decreased proliferation of OPCs (Calver et al., 1998; Fruttiger et al., 1999). During development and in the adult CNS, expression of the PDGF receptor, PDGFRα, is restricted to OPCs (Pringle and Richardson, 1993; Pringle et al., 1996). OPC proliferation induced by PDGF is mediated through MAPK, phosphatidylinositol 3-kinase (PI3-K), and phospho-lipase C gamma (PLCγ) pathways (McKinnon et al., 2005). However, the differentiation and maturation of OPCs is inhibited by PDGF in cooperation with FGF-2 (Wolswijk and Noble, 1992).

4.3.3 Neuregulin (NRG)

NRG has been shown to promote oligodendrocyte survival. In the developing optic nerve, oligodendrocyte death was decreased upon delivering NRG, and increased death was observed upon neutralizing endogenous NRG (Fernandez et al., 2000). NRG1 type III was also shown to be involved in regulating the myelination process of oligodendrocytes. NRG1 type III/ErbB signaling plays a crucial role in regulating myelination in the PNS (Maurel and Salzer, 2000; Taveggia et al., 2005). But in the CNS, myelination was unaffected in NRG1 type III knockout mice (Brinkmann et al., 2008). However, hypermyelination was observed in mice overexpressing NRG1 type III (Brinkmann et al., 2008). These studies indicate that myelination is independent of NRG1 signaling in the CNS, but increased levels of NRG1 can promote myelination.

4.3.4 IGF-1

Along with FGF, PDGF and NRG, several other growth factors play crucial roles in oligodendrocyte development. One important positive regulator of OPC proliferation and differentiation is IGF-1. It is reported that IGF-1 synergizes with FGF-2 to promote OPC proliferation, and this effect is mediated through Cyclin D1 activation (Frederick and Wood, 2004; Jiang et al., 2001). FGF-2 through Erk1/2 activity enhances Cyclin D1 expression in OPCs, and IGF-1 promotes its nuclear localization through PI3K pathway (Frederick et al., 2007). Loss of function studies demonstrated that, in OPCs lacking the IGF-1 receptor (IGF1R), the overall numbers of OPCs and mature oligodendrocytes were decreased (Zeger et al., 2007). However, this study did not distinguish between whether mature oligodendrocytes were reduced as a result of decreased OPCs or impaired differentiation. This result is clarified by an

over expression study that found that OPC differentiation is promoted upon over expressing IGF-1 under a metallothionein 1 promoter (Mathews et al., 1988; Ye et al., 1995).

4.4 Molecular regulators of oligodendrocyte development

MAPKs belong to the serine/threonine kinase family and respond to many growth factor signals. Upon growth factor stimulation, MAPK signaling comprises a three tier signaling cascade. First, MAPK kinase kinase (MKKK) phosphorylates downstream MAPK kinase (MKKs/MEKs), and then activated MKKs phosphorylate the MAPKs. Activated MAPKs then phosphorylate a wide range of substrates and promote various biological effects in a tissue specific manner. The MAPK family includes p38 MAPK, Erk1/2 and JNK, which are activated in response to stress or growth factors (Roskoski, 2012).

4.4.1 Erk1/2 functions in oligodendrocyte development

Erk1 and Erk2 are serine/threonine kinases that are activated in response to the signal relayed from either receptor tyrosine kinases or G-protein-coupled receptors. The signaling cascade includes Ras-Raf-MEK-Erk. Both MEK1 and MEK2 are known to activate Erk1/2 at Tyr204/187 and then Thr202/185. Activated Erk1/2 further phosphorylates a wide range of downstream substrates, such as transcriptional factors and regulatory molecules. Depending on the cellular context, Erk1/2 is known to play a role in a wide range of cellular processes, including proliferation, migration, differentiation, cell survival (Roskoski, 2012).

In oligodendrocytes, Erk1/2 mediates various growth factor effects and regulates diverse biological functions during different stages of lineage progression. For example, Erk1/2 is required in mediating PDGF- and FGF-induced proliferation of OPCs. *In vitro* studies showed that inhibition of Erk1/2 is sufficient to inhibit the proliferation of OPCs induced by PDGF- and FGF (Baron et al., 2000). *In vivo* overexpression and loss of function studies also reported Erk1/2 role in OPC proliferation. In transgenic mice expressing constitutively-active MKK1, an upstream activator of Erk1/2, a transient increase of OPC number was observed. Additionally, deleting Erk1/2 in Olig1-positive OPCs lead to reduced proliferation. Therefore, these studies demonstrate that Erk1/2 is required for OPC proliferation (Ishii et al., 2013).

Although several growth factors are implicated in activating the Ras/MEK/Erk1/2 pathway, FGF and BDNF have been specifically identified to affect myelination via Erk1/2 signaling. Reduced myelin sheath thickness was observed in mice lacking FGFR1/2 receptors, and similar effect was also observed upon deleting Erk1/2 from oligodendrocytes (Furusho et al., 2012; Ishii et al., 2012). Disrupted FGF signaling by deleting FGFR1/2 receptors in oligodendrocytes is also associated with decreased Erk1/2 activation. The correlation between arrested myelin growth in both FGFR1/2-/- and Erk1/2-/- mice, along with decreased Erk1/2 activity in FGFR1/2-/- mice, suggests that FGF-mediated myelin growth occurs through Erk1/2 (Furusho et al., 2012; Ishii et al., 2012). BDNF was also shown to induce myelin protein expression and enhance myelination in oligodendrocyte-neuron co-cultures mediated through Erk1/2 signaling (Xiao et al., 2012). Erk1/2 is also reported to have an effect on oligodendrocyte process extension, which can be inhibited as a consequence of

Erk1/2 inactivation (Furusho et al., 2012; Stariha et al., 1997; Younes-Rapozo et al., 2009).

Erk1/2 is also involved in regulating oligodendrocyte lineage progression. During oligodendrocyte lineage progression, a role for Erk1/2 was found in the transition of early progenitor to late progenitor stage. *In vitro* studies using pharmacological inhibitors demonstrated that inhibiting Erk1/2 in early progenitors blocked progression into late progenitors. However, Erk1/2 inhibition during the immature state did not affect progression into mature oligodendrocytes (Guardiola-Diaz et al., 2012). Thus, these studies suggest that Erk1/2 serves as a crucial integrator of multiple growth factor signals, with diversified roles throughout oligodendrocyte development.

Although many studies have shown Erk1/2 is a positive regulator during oligodendrocyte development, a negative role for Erk1/2 has also been reported. Along with growth factors, Erk1/2 is also activated by various cytokines such as interferon-γ. Studies have shown that interferon-γ-induced Erk1/2 affects OPC survival. In this study, the death induced by interferon-γ was prevented upon inhibiting Erk1/2 activity, suggesting that Erk1/2 mediates interferon-γ induced death in OPCs (Horiuchi et al., 2006). In the brains of experimental autoimmune encephalomyelitis (EAE) mice, an animal model for MS, enhanced Erk1/2 activation was observed. However, its implications in disease pathology are not well understood (Shin et al., 2003).

In the PNS, studies have also shown Erk1/2 to be a crucial regulator in driving Schwann cell demyelination and de-differentiation. *In vitro* studies demonstrated that

in cyclic adenosine monophosphate (cAMP) differentiated Schwann cells, Erk1/2 activation induced Schwann cell de-differentiation and inhibited expression of myelin genes. Similarly, ectopic activation of Erk1/2 induced Schwann cell demyelination and de-differentiation in myelinated Schwann cell-DRG neuron co-cultures. Inhibiting Erk1/2 also blocked neuregulin-induced Schwann cell demyelination. Additionally, during peripheral nerve injury, sustained activation of Erk1/2 was observed in the associated Schwann cells. These studies suggest that the diversified biological effects mediated through Erk1/2 are either induced by growth factors or cytokines, and Erk1/2 plays a crucial role in integrating multiple signals.

4.4.2 p38 MAPK functions in oligodendrocyte development

p38 MAPK is often referred to as stress-activated kinase, as it is activated in response to various stress stimuli such as UV radiation and inflammatory cytokines (Ono and Han, 2000; Zarubin and Han, 2005). Various growth factors are also capable of activating p38 MAPK. p38 MAPK exists as four isoforms: p38α, p38β, p38γ, and p38δ. The predominant isoform detected in the nuclei and cytoplasmic processes of oligodendrocytes is p38α (Haines et al., 2008). p38 MAPK is activated by the specific upstream kinases MKK6 and MKK3 by phosphorylating threonine and tyrosine residues. The conformational change induced in the activation loop upon phosphorylation enables the binding of downstream substrates (Ashwell, 2006; Cuadrado and Nebreda, 2010). p38α and p38γ, and p38δ are activated by MKK3, whereas MKK6 is capable of activating all four isoforms. MKK3 and MKK6 are in turn activated by further upstream kinases MKKK (TAK1, Ask1, MKKK4 and MLK3) in response to external stimuli (Ono and Han, 2000; Zarubin and Han, 2005).

Upon activation, p38 MAPK regulates diverse cellular processes such as proliferation, migration, differentiation, apoptosis and inflammation in various cell types (Nebreda and Porras, 2000).

In the CNS, p38 MAPK mediates the effects of growth factors, thereby regulating the cellular processes that are essential for proper oligodendrocyte development. For example, PDGF and FGF dependent progenitor proliferation is mediated through p38 MAPK, and inhibiting p38 MAPK activity in OPCs resulted in decreased proliferation (Baron et al., 2000). Further, p38 MAPK also regulates the timing of cell cycle exit by accumulating the cell cycle inhibitor, $p27^{Kip1}$ in progenitor cells, thus promoting differentiation. Studies have shown that p38 MAPK inhibition resulted in decreased levels of $p27^{Kip1}$ in OPCs, thus preventing the initiation of differentiation (Casaccia-Bonnefil et al., 1997).

p38 MAPK also promotes oligodendrocyte differentiation and myelination by modulating several transcriptional factors such as cyclic-AMP response element-binding protein (CREB) and Sry-related HMG-BOX gene 10 (SOX10) (Bhat et al., 2007; Chew et al., 2010). Studies have demonstrated that cAMP-dependent oligodendrocyte differentiation was mediated through p38 MAPK regulating PKA (protein kinase A) - CREB activity (Bhat et al., 2007). Furthermore, p38 MAPK promotes expression of myelin genes by regulating the activity of the transcription factor, Sox10. Sox 10 binding to the MBP promoter is attenuated upon p38 MAPK inhibition, whereas its binding was promoted upon over expressing MKK6, an upstream activator of p38 MAPK (Chew et al., 2010). In the same study, cross talk between the MAPKs were demonstrated in which p38 MAPK was shown to suppress

Erk1/2 and c-jun, a negative regulator of myelination (Chew et al., 2010). Thus, p38 MAPK promotes oligodendrocyte differentiation and myelination by regulating both positive and negative effectors of myelination.

Studies have implicated p38 activity to be involved in numerous inflammatory diseases such as rheumatoid arthritis and inflammatory bowel syndrome. Various inflammatory cytokines mediate their effects through the activation of p38 MAPK. Production of many pro-inflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor-α (TNF-α) are also regulated by the activation of p38 MAPK (Foster et al., 2000; Hollenbach et al., 2005; Lopez-Santalla et al., 2011; Schieven, 2005; Zarubin and Han, 2005), suggesting p38 MAPK plays a role in various inflammatory diseases.

p38 MAPK is also implicated in inducing inflammation and demyelination in the CNS. In EAE, an animal model of CNS demyelination, inhibition of p38 MAPK directly suppressed the symptoms of EAE compared to the control (Namiki et al., 2012). Additionally, blocking apoptosis signal-regulating kinase 1 (ASK1), an upstream kinase that activates p38 MAPK, resulted in suppressing EAE-associated inflammation (Guo et al., 2010). p38 MAPK has also been found to be activated in the brains of EAE rats (Shin et al., 2003), suggesting that p38 MAPK is an important regulator in driving CNS disease pathology. However, the mode of action of p38 MAPK during EAE is not clear. Although p38 MAPK mediates diverse effects of inflammatory cytokines during the disease progression, the specific effects on oligodendrocytes are not well understood.

Several studies have shown p38 MAPK function in inducing cellular de-differentiation in various cell types such as chondrocytes, Muller glia and Schwann cells. p38 MAPK promotes de-differentiation in chondrocytes by inhibiting the differentiating signal PKC (Kim et al., 2002). In Muller glia, FGF-2-induced p38 MAPK was shown to drive de-differentiation and proliferation by activating notch signaling (Ghai et al., 2010). Recently, *in vitro* and *in vivo* studies in the PNS have shown that p38 MAPK is an important regulator in driving Schwann cell demyelination and de-differentiation (Yang et al., 2012). These studies suggest that p38 MAPK is a crucial regulator for oligodendrocyte development and is also involved in the pathology of demyelinating diseases. In chapter two, we focused on characterizing the role of p38MAPK in oligodendrocyte demyelination.

4.4.3 c-jun N-terminal kinase (JNK) in oligodendrocyte development

JNK is activated in response to stress, cytokines and growth factors. Specifically, JNK is activated by MKK4 and MKK7 upstream kinases (Roskoski, 2012). JNK activates downstream transcriptional factors, such as c-jun, by phosphorylating the N-terminus. c-jun is shown to be a negative regulator of myelination in both the CNS and PNS. In OPCs, inhibition of myelin genes was observed in the presence of high phosphorylated c-jun (Chew et al., 2010; Jessen et al., 2008; Mirsky et al., 2008).

4.4.4 Other signaling pathways regulating oligodendrocyte development

Besides MAPKs, many other signaling pathways have been shown to regulate various aspects of oligodendrocyte development. For example, growth factor (PDGF/ IGF-1) dependent oligodendrocyte cell survival and proliferation is mediated through

PI-3-K/Akt pathway (Cui and Almazan, 2007; Ebner et al., 2000; Ness and Wood, 2002; Zaka et al., 2005). *In vivo* gain- and loss of function studies have demonstrated IGF-1 as an important regulator in CNS myelination (Beck et al., 1995; Carson et al., 1993; Ye et al., 1995; Ye et al., 2002). More importantly, these effects of IGF-1 are thought to be mediated through PI-3-K/Akt signaling via mammalian target of rapamysin (mTOR) (Ness et al., 2002; Ness and Wood, 2002; Wood et al., 2013). Further*, in vivo* studies in the CNS observed increased myelination in the transgenic mice overexpressing Akt in oligodendrocytes, which also showed increased expression of mTOR. Inhibiting mTOR with rapamycin prevented the hypermyelinating phenotype in this transgenic mouse (Flores et al., 2008). Similar results affecting differentiation and myelination were also observed by inhibiting mTOR with rapamycin (Tyler et al., 2009). Recent *in vitro* studies also identified a role for mTOR in regulating oligodendrocyte lineage progression, demonstrating a specific function in regulating the transition of immature oligodendrocytes to mature oligodendrocytes (Guardiola-Diaz et al., 2012). These studies suggest that growth factor signals such as IGF-1 are mediated through PI-3-K/Akt via the mTOR pathway during oligodendrocyte development.

4.5 Remyelination in the CNS

In demyelinating diseases, efficient remyelination is essential to regain functional recovery after the insult. After the demyelination, remyelination is a natural regenerative process in the nervous system, where new myelin sheaths are synthesized by the oligodendrocytes to wrap the exposed axons. In experimental models of

demyelination, induced by toxins such as cuprizone or by injecting lysolecithin, spontaneous remyelination happens after the insult, and a complete recovery is observed. But in diseases such as MS, appearance of shadow plaques during early stages indicates that remyelination can spontaneously occur, but it is often incomplete and aberrant, with thinner and shorter myelin segments, and eventually fails as the disease progresses into the latter stages. Impaired remyelination after the demyelinating insult is due to the involvement of many factors that might act synergistically. For example, the lesion environment might not be conducive to remyelination, with inhibitory effects on OPC recruitment that may eventually deplete the OPCs. Other factors that prevent remyelination include a lack of signals in the lesion environment to promote oligodendrocyte progression, transient demyelinating episodes after remyelination, axonal degeneration and the inability of axons to be remyelinated. However, in some MS patients, complete remyelination is evident in most of the lesions, although the reason for this discrepancy is not clear.

Formation of new myelin sheaths requires the availability of functional mature oligodendrocytes, which can come from two potential sources. First, OPCs can be recruited to the lesion site to eventually generate new mature oligodendrocytes. Second, intact oligodendrocytes that survived the demyelinating insult may also contribute to remyelination. A role for OPCs in remyelination is supported by observations that greater numbers of oligodendrocytes were observed at the site of remyelination, and remyelination occurs even in the areas where oligodendrocytes are experimentally depleted (Prayoonwiwat and Rodriguez, 1993; Sim et al., 2002). These studies indicate that resident OPCs proliferate, then migrate towards the lesion

area where they differentiate, mature and remyelinate the denuded axons. Neural stem cells (NSCs) from the SVZ may also contribute to myelinating oligodendrocytes. After the demyelinating injury, NSCs migrate to the injury site and develop into myelinating oligodendrocytes (Aguirre et al., 2007; Nait-Oumesmar et al., 1999; Picard-Riera et al., 2002).

Intact oligodendrocytes may also contribute to remyelination. In patterns I and II lesions of MS, a higher proportion of oligodendrocyte cell bodies are preserved, and these lesions also exhibit enhanced remyelination compared to patterns III and IV. The correlation between increased remyelination and preserved oligodendrocytes implicate that they might have a role in remyelination. However, two separate experiments show that surviving oligodendrocytes might not contribute to remyelination. One study showed that transplanting mature oligodendrocytes in the experimental models did not overcome the remyelination failure (Targett et al., 1996). A second study tested the ability of intact oligodendrocytes to myelinate using an injury model that induced demyelination with galacterocerebroside antibodies, which preserves most of the cell bodies after the insult. They then used X-irradiation to remove the OPCs that might contribute to remyelination. In both experiments, remyelination by resident oligodendrocytes was not observed (Keirstead and Blakemore, 1997). However, mature oligodendrocytes in culture or upon exposure to X-irradiation might not behave the same as oligodendrocytes that survive after demyelination. To enhance the remyelinating potential of oligodendrocytes, it is important to understand the molecular mechanisms regulating the plasticity of

oligodendrocytes, thus providing insight towards the development of new therapeutic strategies to enhance the remyelination capability of native oligodendrocytes.

4.6 Dedifferentiation ability of oligodendrocytes

Many cell types, such as Schwann cells, are capable of maintaining their plasticity even after their maturation. After injury, or upon treatment with high doses of neuregulin, Schwann cells have the ability to dedifferentiate into immature Schwann cells and re-enter the cell cycle (Zanazzi et al., 2001). Activation of the Ras/raf/Erk pathway triggers de-differentiation and demyelination in Schwann cells. *In vitro* studies showed that Erk1/2 activation induces dedifferentiation of Schwann cells and blocks myelin gene expression (Harrisingh et al., 2004; Ogata et al., 2004). Over-expression of Erk1/2 in Schwann cells caused dedifferentiation and demyelination in myelinated Schwann cell-DRG co-cultures. Inhibition of Erk1/2 blocked neuregulin-induced demyelination (Harrisingh et al., 2004). Besides Erk1/2, p38 MAPK has also been shown to be involved in regulating Schwann cell de-differentiation (Yang et al., 2012).

The plasticity of oligodendrocytes to de-differentiate and remyelinate is not well understood, nor are the factors regulating the capability of oligodendrocytes to dedifferentiate. Previous studies suggested that oligodendrocytes might retain their plasticity to de-differentiate by demonstrating their ability to reenter the cell cycle. Studies *in vitro* have shown that, upon treating mature oligodendrocytes with FGF-2, myelin protein expression is downregulated, and oligodendrocytes change their morphology and re-enter the cell cycle, but they do not complete mitosis (Bansal and

Pfeiffer, 1997a; Fortin et al., 2005). Treatment with glial growth factor (GGF) was also reported to induce loss of MBP expression and trigger phenotypic reversion in differentiated oligodendrocytes (Canoll et al., 1999). Further understanding the factors that regulate oligodendrocyte plasticity might be important to enhance the remyelinating potential of oligodendrocytes in demyelinating diseases. In chapter three, I investigated whether mature oligodendrocytes retain the plasticity to re-enter the cell cycle.

5 Materials and Methods

Antibodies and growth factors.

For immunofluorescence analysis, monoclonal antibody (SMI94) to myelin basic protein (Covance) and FLAG antibody to DYKDDDDK-tag (Genscript) were used at 1:300 and 1:500, respectively. Polyclonal antibodies to phospho-p38 MAPK (Cell Signaling Technology) and neurofilament NF-M (Covance) were used at 1:200 and 1:1000, respectively. PDGFR-α (Cell Signaling Technology), and phospho-Erk1/2 (Promega) were used at 1:100. Ki-67 (Neo markers) was used at 1:500 dilution. Dylight 488, 594 and 649 fluorophore conjugated secondary antibodies were all obtained from Jackson Immuno Research Laboratories and used at 1:400 dilutions. For Western blot analysis, monoclonal antibodies to phospho-Akt (Cell Signaling Technology) and polyclonal antibody to phospho-Erk1/2 (Promega) were used at 1:1000 and 1:5000, respectively. Polyclonal antibodies to Akt (Cell Signaling Technology) and Erk1/2 (Promega) were used at 1:1000 and 1:5000, respectively. Polyclonal antibodies to phospho-p38 (Cell Signaling Technology), p38 (Cell Signaling Technology), phospho-JNK (Cell Signaling), JNK (Cell Signaling) were all used at 1:1000. Monoclonal antibody to α-actin (Sigma-Aldrich) was used at 1:5000 dilutions. HRP conjugated secondary antibody and ECL substrate were purchased from PIERCE.

The growth factors used in the demyelination studies: Fibroblast growth factor 2 (FGF2), and platelet-derived growth factor (PDGF) were purchased from R&D Systems. Recombinant human glial growth factor-II (rhGGF-II, type II Nrg1) was from Acorda. For inhibition experiments, pharmacological inhibitors SB203580 (p38

MAPK inhibitor) (LC labs), U1026 (Erk1/2inhibitor), LY29402 (PI3-K inhibitor), and SP600125 (JNK inhibitor) were prepared in DMSO and used at 2.5 μM, 5μM and 10 μM concentrations.

Culture media.

Culture media were as follows: NB media, neurobasal medium with B-27 supplement, 0.08% glucose, 1% glutamine, and 0.1mg/ml penicillin/streptomycin, and 50ng/ml NGF. C media, minimal essential medium (Invitrogen) supplemented with 10% FBS, 1% Glutamine, 0.4% glucose, 0.1mg/ml penicillin/streptomycin and 50 ng/ml NGF. MEM-C consisted of minimal essential medium (MEM) supplemented with 10% FBS, L-glutamine, and 1% pen-step. N2B2 media, DMEM/F-12 supplemented with 0.6mg/ml BSA, 10ng/ml d-biotin, 20nM progesterone, 100nM Putrescine, 5ng/ml selenium, 50μg/ml apotransferrin, and 0.1mg/ml penicillin/streptomycin. N2S media was composed of 66% N2B2 media, 34% B104 conditioned media, 5ng/ml FGF-2 and 0.5% fetal bovine serum. Differentiation media was composed of neurobasal medium with B-27 supplement, 10ng/ml d-biotin, 30ng/ml Triiodothyronine (T3) and 0.1mg/ml penicillin/streptomycin.

Purified dorsal root ganglion neuron cultures

Dorsal root ganglion (DRG) neurons were dissected from E14.5- E16.5 rat embryos and were dissociated in 0.25 % trypsin (Gibco) for 30 min at 37° C. The dissociated DRGs were plated onto 12-mm glass coverslips coated with growth factor reduced matrigel (150-200ng/ml) (Invitrogen) (1 DRG/ coverslip) in NB media in a

140 µl droplet. After 6-8 hours after plating, the cultures were flooded in NB media containing 15 µM fluorodeoxyuridine (FUDR) (Sigma-Aldrich) and 5 µM uridine (Sigma-Aldrich). To remove non neuronal proliferating cells, cultures were maintained in FUDRr and uridine mixture for 3-4 days and then switched to fresh NB media. Cultures were then maintained in NB media until the DRG axons reached the periphery of the coverslips.

Primary cortical oligodendrocyte progenitor cultures

OPCs were purified from cortical mixed glial cultures by established methods (McCarthy and de Vellis, 1980). Cortices were dissected from the brains of postnatal day 1-2 rat pups. Cortical pieces were then briefly digested in trypsin 0.25%, DNase1 and mechanically dissociated. Then cells were plated onto T75 flasks in MEM-C. Mixed glial cultures were maintained for 10 days. Purified OPC cultures were prepared by differential shaking (McCarthy and de Vellis, 1980). Purified OPCs were plated onto poly-D-lysine coated T75 flasks at a density of 2 X 10^4 cells/cm^2 in N2S medium. Purified OPCs were passaged once using papain and plated onto DRGs for other experiments.

DRG-OPC co-cultures

Oligodendrocyte progenitor cells (OPC) were purified from mixed glial cortical cultures as described above. The OPCs were plated onto DRG neurons at a density of 100,000-200,000 cells/coverslip in C media. The DRG-OPC co-cultures were maintained for 18-21 days, with fresh medium supplemented every two days.

The myelinated co-cultures were then either treated with growth factors or for inhibition studies using the pharmacological inhibitors.

Immunocytochemistry

After treatment, coverslips were fixed in 4% PFA for 20 minutes washed twice in PBS, permeabilized in ice-cold methanol for 20 minutes, and then the coverslips were rehydrated in PBS. Samples were then blocked in 5% normal goat serum (NGS) + 0.3% triton X-100 in PBS for 1 hour at room temperature. Cover slips were incubated at 4º C overnight with primary antibodies prepared in blocking solution. After PBS washes, coverslips were incubated with secondary antibodies for 1 hour. Cell nuclei were stained with 4', 6'-diamidino-2-phenylindole dihydrochloride (DAPI) at 1:1000. Coverslips were then washed 3 times in PBS and mounted. To assess apoptosis, after immunostaining with MBP, the coverslips were fixed again with 4% PFA and TUNEL (Promega) assay was performed. For staining filamentous actin, Alexa Fluor 488® conjugated phalloidin (Invitrogen) was used at 1:100. The cultures were fixed with 4% PFA, and were not permeabilized with methanol. A similar procedure for primary and secondary antibody incubation was performed. Cultures were then incubated with phalloidin stain for 20 minutes, rinsed with PBS and coverslipped.

Quantification of demyelination in DRG-OPC co-cultures

The degree of demyelination was quantified using two different methods: by counting the MBP-positive demyelinated clusters with a 40x objective and by counting the

MBP-positive demyelinated segments with a 100X objective. In both methods, cell counts were performed on75 independent fields (25 fields/coverslip/treatment). In the first method using a 40x objective, within each field, MBP-positive demyelinated clusters and the total number of MBP-positive clusters were counted. The level of demyelination was determined as the total number of MBP-positive demyelinated clusters as a percentage of the total number of MBP-positive myelinated clusters. In the second method using a 100x objective, within each field, MBP-positive demyelinated segments and the total number of MBP-positive segments were counted. The level of demyelination was determined as the total number of MBP-positive demyelinated segments as a percentage of the total number of MBP-positive myelinated segments. One way ANOVA followed by Turkey's post hoc analysis was performed using SigmaStat 3.5 software.

Quantification and statistical analysis

Images of oligodendrocytes were taken on a Nikon E800 microscope. To quantify MBP-positive cells, TUNEL-positive cells, and Ki-67-positive cells in oligodendrocytes, 15 randomly selected fields were counted per coverslip, with 3 coverslips per experiment, for a total of 3 experiments. To assess the branching morphology of oligodendrocytes, images were taken using a 60X objective lens, and the branching complexity was quantified using Sholl analysis (Image J software). 20 fields were counted per coverslip, with 3 coverslips per experiment for a total of 3 experiments. One way ANOVA followed by Turkey's post hoc analysis was performed using SigmaStat 3.5 software.

Western blotting

After treatments, total cell lysates from DRG-OPC cocultures were harvested in lysis buffer containing 20mM Tris pH7.4, 1%NP-40, 10% glycerol, 2.5 mM EGTA, 2.3mM EDTA, 1mM sodium orthovanadate, 1mM PMSF, 10μg/ml aprotinin and 20μM leupeptin. The lysates were centrifuged at 12,000g at 4ºC. BCA protein assay (Pierce) was performed to determine protein concentration. 25 μg of total protein per sample was aliquoted, boiled for 5 minutes and separated by SDS-PAGE on 10% polyacrylamide gels. Separated proteins were transferred onto PVDF membrane and blocked in 5% non-fat milk in TBS for 1 hour. Membranes were then incubated in primary antibodies diluted in 5% BSA and 0.1% Tween-20 in TBS overnight at 4ºC. The following day, membranes were washed 3 times in TBS-0.1% Tween for 5 minutes. Then the membranes were incubated in goat anti-rabbit or goat anti-mouse HRP conjugated secondary antibodies diluted in 5% non-fat milk, 0.1% Tween 20 in TBS at a dilution of 1:5000. After 1 hour incubation at room temperature, the membranes were washed, and secondary antibody signals were detected using enhanced chemiluminescence western blotting substrate and developed on X-ray films.

Generation of lentivirus and infecting OPCs

The following procedure was used to generate the lentivirus encoding either constitutively activate-MKK1 (CA-MKK1) or constitutively activate-MKK6 (CA-MKK6). Constitutively activated MKK6 expression plasmid pcDNA3-Flag MKK6,

and constitutively activated MKK1 expression plasmid pcDNA- MKK1 were obtained from Addgene, cloned into pEN_TTmcs (Addgene) and inserted into a modified pSLIK lentiviral vector via LR clonase II (Invitrogen) to generate pSLIK-CA-MKK6 and pSLIK-CA-MKK1. The lentiviral vector was transfected into 293FT cells together with psPAX2 (packaging vector) and pMD2.G (envelop plasmid) using a CaPO4 transfection kit (Invitrogen). Briefly, 293 FT cells (Invitrogen) were grown to 80% confluency in 100mm plates. 293FT Cells were then transfected with a total of 60-75μg of DNA using CaPO4 (Invitrogen) in advanced media [Advance DMEM (Invitrogen), 10% Fetal Bovine Serum (Atlas), 5% CD lipid concentrate (Invitrogen), cholesterol)]. 5-6 hours post transfection, media was changed to fresh advanced media for viral collection. Cells were incubated in 7.5% CO2 at 37 C° for 48-60 hrs. The viral-containing advanced medium was collected into 15 ml conical tubes and centrifuged at 5000g for 5 minutes at room temperature to allow for packing of cellular debris. 6.5ml of the supernatant media was collected and centrifuged at 3500RPM for 5 minutes. 6ml of the 293FT conditioned media was collected and used for infecting OPCs within 24 hours. Viral media supplemented with 5ng/ml PDGF and 5ng/ml FGF-2 was added onto purified OPCs. 8-12 hours later, the media containing the virus was switched to fresh N2S media and cells were grown to confluency, then plated onto either DRGs or on poly-D-lysine/laminin coated coverslips for further experiments.

Preparation and assembly of the injury wells

PEEK rings were washed thoroughly using a laboratory detergent and sonicated. Assembly of injury wells consisted of a large and small PEEK ring. Silicone membrane (0.005" Gloss/Gloss silicone sheeting Specialty Manufacturing, Saginaw, MI) was cut into 1.5 x 1.5cm squares, washed with dH_2O and dried. The square pieces of silicone membrane were assembled to make a tissue culture well using the two PEEK rings and an O-ring to create a tight fit between the two PEEK rings, preventing leakage of the media. Assembled wells are thoroughly washed and autoclaved for further use in tissue culture.

DRG neuron-oligodendrocyte co-cultures for stretch injury

Assembled wells with silicone membrane were coated with growth factor reduced matrigel (150-200ng/ml) in sterile dH_20 and allowed to dry overnight. Isolated DRG neurons from E14.5- E16.5 rat embryos were dissociated in 0.25 % trypsin (Gibco) for 30 min at 37° C. The dissociated DRGs were plated on one side of the injury well (6-7DRGs/ well) by using a plating insert to isolate the cell bodies and create a cell free zone. Plating inserts were molded out of polydimethylsiloxane elastomer (PDMS NuSil Technology LLC, Carpinteria,CA). The plating insert prevents the neuron cell bodies to adhere to silicone membrane in the cell free zone. After 6-8 hrs of plating, NB media containing 15 μM fluorodeoxyuridine (FUDR) and 5 μM uridine was added in the cell free zone. The plating insert was removed 24 hours after plating to allow maximal attachment of the DRG neurons. Two days later, the FUDR and uridine mixture was switched to fresh NB media. Cultures were maintained in NB media for 3 weeks to allow the axons to transverse the cell free

zone. After 3 weeks, isolated OPCs were plated at a density of 250,000-300,000cells/well in C-media. Cultures will be maintained by supplementing C-media every other day for another 2 weeks allowing the OPC to differentiate into oligodendrocytes and form myelin segments.

In vitro DAI (stretch-injury) model

The *in vitro* DAI model comprised a pressure chamber attached to an air pulse generating system. The cultures on the silicone membrane were rapidly deformed by applying a pressure pulse into the chamber, thus deforming the membrane. The stretching of the silicone membrane deformed the attached myelinated axons. A rigid mask with a 2mm gap at the center was placed underneath the silicone membrane, allowing the stretch to be restricted to the specified gap area. The parameter that induces stretch to cause deformation of the membrane is reported in terms of strain, which is defined as the ratio of total deformation to its original form. For example, a 60% strain means that membrane has been deformed 60% relative to its original confirmation. The rate of injury is measured in terms of strain rate, which is defined as the rate at which a particular strain is used to injure the cultures. For example, strain rate of $30s^{-1}$ means 60% strain is delivered over 30 seconds of time.

6. Chapter Two: To determine the intracellular pathways mediating oligodendrocyte demyelination

6.1 Introduction

MS is a severe demyelinating disorder of the CNS, where myelin sheath-forming oligodendrocytes are damaged, creating demyelinated lesions. The heterogeneity of these lesions suggests that multiple causes may trigger oligodendrocyte demyelination. In demyelinating disorders of the CNS, many proinflammatory cytokines and growth factors are upregulated. For example, fibroblast growth factor-2 (FGF-2) has been found to be increased in the cerebrospinal fluid (CSF) of MS patients (Sarchielli et al., 2008). In chronic active and inactive plaques of MS patients, an increase in FGF-2 expression is observed in microglia and macrophages (Clemente et al., 2011). Studies have also shown that expression of FGF-2, FGFR1 and FGFR3 are increased near and within lesions in experimental demyelination models (Liu et al., 1998; Messersmith et al., 2000). These findings suggest that cytokines and growth factors may play a role in the pathology of demyelinating diseases.

It is well established that growth factors such as FGF-2 play a role during oligodendrocyte development. FGF-2 function is implicated in diverse cellular processes of oligodendrocytes including the proliferation, differentiation, and myelination. FGF-2 acts as a mitogen for OPCs and regulates migration (Simpson and Armstrong, 1999). In the presence of FGF-2, OPC differentiation is inhibited (Bansal and Pfeiffer, 1997b; Zhou et al., 2006). Recently, FGF-2 was also shown to play a role in regulating the thickness of the myelin sheath (Furusho et al., 2012). These studies suggest that oligodendrocytes elicit multiple responses to FGF-2 depending on their stages of development. It is unknown whether FGF-2 plays a role in initiating

demyelination of oligodendrocytes. The possibility for FGF-2 to induce demyelination is suggested by *in vitro* studies, in which FGF-2 was shown to down regulate expression of myelin proteins such as proteolipid protein (PLP), myelin basic protein (MBP), and myelin-associated glycoprotein (MAG), as well as FGFR-2 itself (Bansal and Pfeiffer, 1997a; Butt and Dinsdale, 2005; Fressinaud et al., 1995). The study also showed that following down-regulation of the myelin proteins, oligodendrocytes alter their morphology and re-enter the cell cycle, but fail to complete mitosis (Bansal and Pfeiffer, 1997a).

In the peripheral nervous system (PNS), the role of growth factors in inducing demyelination has been well established. For example, Schwann cell mitogens such as neuregulin (NRG) and FGF-2, when added to myelinated cultures, trigger myelin break down (Yang et al., 2012; Zanazzi et al., 2001). *In vivo* activation of ErbB2 receptor, a receptor for NRG has been linked to injury-induced demyelination (Guertin et al., 2005). Furthermore, the demyelinating effects of these growth factors have been shown to be mediated by activation of the Erk1/2 and p38 MAPK pathways in Schwann cells (Guertin et al., 2005; Harrisingh et al., 2004; Yang et al., 2012).

FGF-2, PDGF, and NRG are known mitogens for OPCs. In this aim, we investigated whether these growth factors could trigger oligodendrocyte demyelination. Our studies used DRG-OPC co-cultures, in which OPCs differentiate and form mature myelin segments. We also used various pharmacological inhibitors to identify signaling pathways that mediate the growth factor effects on myelin.

Here, we show that FGF-2 triggers myelin breakdown in myelinated DRG-OPC co-cultures. Results from this study also demonstrate that the FGF-2-induced

oligodendrocyte demyelination is mediated through activation of the Ras/Raf/Erk1/2 or p38 MAPK pathways. Furthermore, ectopic activation of either Erk1/2 or p38 MAPK in oligodendrocytes is sufficient to induce demyelination.

6.2 Results

FGF-2 induces demyelination in DRG-OPC co-cultures

Studies in the PNS have shown that demyelination can be induced by Schwann cell mitogens such as NRG and FGF-2 (Yang et al., 2012; Zanazzi et al., 2001). But in the CNS, the effects of OPC mitogens on oligodendrocyte demyelination are not well understood. In this study, we addressed the effect of growth factors on oligodendrocyte demyelination, and further we investigated the mechanisms underlying the process. FGF-2, PDGF and NRG are known mitogens for OPCs making them ideal candidates for the study. To determine their effects on myelinated oligodendrocytes, I employed dorsal root ganglion (DRG) neuron-oligodendrocyte precursor cell (OPC) co-cultures, in which OPCs differentiate in the culture and form myelin on neighboring DRG neurons.

OPCs purified from neonatal rat cortex were plated onto DRG neurons and maintained until the oligodendrocytes myelinated the axons (18-21 days). The cultures were then treated with PDGF (3.3nM), NRG (3.3nM), and FGF-2 (3.3nM), and forty-two hours later, the cultures were fixed and immunostained for MBP. A single oligodendrocyte has the capacity to form multiple myelin segments, and the myelin segments formed by the oligodendrocyte are normally grouped together in what is referred to as a myelin cluster. Figure 2A shows an image of a myelin cluster with smooth intact myelin segments in control conditions. FGF-2 treatment induced extensive breakdown of myelin as shown in Figure 2A. Quantification of the results showed a fourfold increase in demyelination after FGF-2 treatment (Figure 2B). Compared to the control, demyelination was also observed in cultures treated with

NRG, however to a lesser extent compared to FGF-2. The FGF-2 effect on demyelination was also studied with increasing doses of the growth factor (0.3nM, 1nM, 3nM, 5nM). As shown in Figure 3B, FGF-2 induced demyelination in a dose-dependent manner: a significant increase in demyelination was observed in cultures treated with 3nM and 5nM FGF-2 (Figure 3).

In vivo studies have reported oligodendrocyte death at demyelinated lesions, suggesting that the death of oligodendrocytes may be a cause of demyelination (Lucchinetti et al., 2000). *In vitro* studies also reported that differentiated oligodendrocytes undergo apoptosis in the presence of FGF-2 (Muir and Compston, 1996). To determine whether FGF-2 induced demyelination is associated with oligodendrocyte death, a TUNEL assay was performed on co-cultures 42 hours after FGF-2 treatment. In the absence of FGF-2, 6% of the cells were positive for TUNEL (Figure 4). There was no significant increase in cell death following FGF-2 treatment, indicating FGF-2 induced demyelination was not associated with cell death.

We also examined the effect of FGF-2 on myelination of oligodendrocytes. Studies have shown that FGF-2 inhibits differentiation of OPCs into mature oligodendrocytes (Zhou et al., 2006). However it is unknown whether FGF-2 would block myelination independent of its effect on oligodendrocyte differentiation, OPCs were co-cultured with DRG neurons in the absence of FGF-2 for 7-8 days to allow the OPCs to differentiate normally into mature oligodendrocytes. The co-cultures were then treated with increasing doses (0.3nM, 1nM, 3nM, 5nM) of FGF-2 for 7 days. After myelination was completed, the co-cultures were fixed and immunostained for MBP. First, we analyzed the effect of FGF-2 on differentiation of oligodendrocytes in

the co-cultures by determining the percentage of MBP-positive oligodendrocytes, whether myelinating or non-myelinating. Then, we analyzed the effects on myelination among the differentiated oligodendrocytes by the percentage of myelin clusters formed among the MBP-positive oligodendrocytes. At low doses, FGF-2 had no effect on oligodendrocyte differentiation, but attenuated myelination significantly. At higher doses, the overall number of MBP-positive oligodendrocytes decreased in FGF-2 co-cultures and consequently, myelination was also blocked. These results indicated that low doses of FGF-2 (0.3nM and 1nM) inhibited myelination independent of oligodendrocyte differentiation (Figure 5). Altogether, these studies suggest that FGF-2 induces demyelination in myelinated co-cultures and also have an inhibitory role during myelination. Based on these results, I further investigated the mechanisms by which FGF-2 initiates oligodendrocyte demyelination *in vitro.*

Activation of Erk1/2 and p38 MAPK mediates FGF-2 induced oligodendrocyte demyelination

When bound to FGF-2, FGF receptor complex activates various downstream signaling pathways (Baron et al., 2000; Fortin et al., 2005). Among these, we examined the activation kinetics of Erk1/2, JNK1/2, Akt, and p38 MAPK in myelinated DRG-OPC co-cultures. Twenty-one-day-old myelinated DRG-OPC co-cultures were treated with 3.3nM FGF-2, a dose sufficient to induce demyelination. Lysates were collected at various time points (15', 30', 1hr, 2hr, 4hr, and 6hr), and the activation states of Erk1/2, JNK1/2, Akt, and p38 MAPK were determined by Western blot analysis. Activation of Erk1/2 and JNK1/2 was observed at 15 minutes after FGF-2 treatment and persisted up to 6 hours (Figure 6). In contrast, FGF-2

induced Akt activation was transient, showing maximal activation at 1hr and decreases afterwards. Activation of p38 MAPK was slow and occurred gradually, with significant activity observed at 4 hr following FGF-2 treatment. These results suggest that all four major signaling pathways are activated by FGF-2 in DRG-OPC co-cultures, however, with different kinetics.

To investigate whether these pathways play a role in mediating demyelination induced by FGF-2, pharmacological inhibitors were used to block the activity of the signaling molecules. The inhibitors used in this study are listed in Figure 7B. Myelinated DRG-OPC co-cultures pretreated with the inhibitors then stimulated with FGF-2 in the continuous presence of the inhibitors. Thirty-six to forty-two hours later, the cultures were fixed and immunostained for MBP. Treatment with U1026 and SB203580, which inhibit Erk1/2 and p38 MAPK, respectively, significantly decreased the levels of demyelination induced by FGF-2 (Figure 7). In contrast, inhibition of PI-3kinase or JNK1/2 pathways had no effect on FGF-2 induced demyelination (Figure 7).

To further confirm the roles of Erk1/2 and p38 MAPK in mediating FGF-2 induced demyelination, we investigated whether U1026 and SB203580 elicited inhibitory effects in a dose-dependent manner. Myelinated co-cultures were treated with increasing doses (2.5μM, 5μM and 10μM) of the inhibitors. The inhibitory effect on demyelination was mostly observed at 5μM and 10μM for both U1026 and SB203580 (Figures 8 and 9). Interestingly, in control cultures, which normally exhibit 20-25% basal level demyelination in the absence of FGF-2, the inhibitor treatment did not have a protection effect on the myelin, suggesting the specific functions of the

MAPK pathways in mediating FGF-2 induced demyelination. In a parallel experiment, the efficiency of U1026 on inhibiting FGF-2-induced Erk1/2 was tested by Western blot analysis. In Figure 8C, 10μM U1026 completely inhibited Erk1/2 activation in FGF-2 treated co-cultures. Altogether, these results suggest that the Ras/Raf/Erk1/2 and p38 MAPK pathways play a role in mediating FGF-2 induced demyelination. In the following section, we investigated the roles of Erk1/2 and p38 MAPK in inducing oligodendrocyte demyelination.

Ectopic activation of MEK1 initiates oligodendrocyte demyelination

The DRG-OPC co-cultures used in this study were maintained in the presence of serum, under which the OPCs differentiate either into oligodendrocytes or astrocytes. Therefore, by the time the myelin is formed, co-cultures comprise a mix of myelinating oligodendrocytes and astrocytes in combination with DRG neurons. Hence, it is not clear whether functions of Erk1/2 or p38 MAPK were localized to the myelinating oligodendrocytes or in other cell types that elicited an indirect effect that triggered oligodendrocyte demyelination. To address this, we induced Erk1/2 or p38 MAPK activation specifically in myelinated oligodendrocytes in co-cultures and determined the effects on myelin. We generated and infected OPCs with lentiviruses encoding constitutively-active MKK1 (CA-MKK1), an upstream activator of Erk1/2, under the control of a doxycycline-inducible promoter. First, the doxycycline-inducible system was verified in the infected OPCs by immunostaining for p-Erk1/2. Compared to the CA-MKK1 cultures in absence of doxycycline, p-Erk1/2 immunostaining was observed in the infected OPCs treated with doxycycline, suggesting Erk1/2 was activated in a doxycycline-dependent manner (Figure 10).

CA-MKK1 OPCs were then co-cultured with DRG neurons for 15-18 days in the absence of doxycycline. Once the OPCs differentiated and formed myelin, doxycycline was added to induce the transgene activation. Control cultures were established using oligodendrocytes that were either non-infected or infected with lentiviruses carrying the vector alone (pSLIK). The doxycycline-dependent activation of Erk1/2 in co-cultures was verified by Western blot analysis. Figure 11A shows that the addition of doxycycline was sufficient to activate Erk1/2 in co-cultures. Forty-two hours after doxycycline treatment, extensive breakdown of myelin clusters were observed in the CA-MKK1 infected co-cultures, but not in control cultures (Figure 11B). Quantification of the results showed a four-fold increase in demyelination in doxycycline-treated CA-MKK1 cultures compared to the controls (Figure 11C). Figure 10D shows a reduction in the levels of MBP and MOG in doxycycline-treated CA-MKK1 co-cultures. These results suggest that ectopic activation of Erk1/2 in myelinating oligodendrocytes is sufficient to cause demyelination and down regulate myelin proteins.

Ectopic activation of MKK6 initiates oligodendrocyte demyelination

To investigate whether p38 MAPK activation in oligodendrocytes is sufficient to induce demyelination in co-cultures, we infected OPCs with lentiviruses encoding constitutively-active MKK6 (CA-MKK6-FLAG), an upstream activator of p38 MAPK under the control of a doxycycline inducible promoter. Initially, we verified the doxycycline-dependent activation of p38 MAPK in infected OPCs by immunostaining for FLAG and p-p38 MAPK. The addition of doxycycline induced CA-MKK6 (FLAG-positive) expression and was sufficient to activate p38 MAPK in

infected OPCs, but not in CA-MKK6 OPCs in doxycycline free media (Figure 12). Infected OPCs were co-cultured with DRGs in the absence of doxycycline to complete the myelination. Doxycycline was then added to the myelinated co-cultures to express CA-MKK6. Co-cultures with non-infected oligodendrocytes or oligodendrocytes infected with lentiviral vector alone (pSLIK) or infected with CA-MKK6 in the presence or absence of doxycycline were used as controls. Figure 13A shows that the addition of doxycycline induced CA-MKK6 (FLAG+) expression and was sufficient to activate p38 MAPK in infected co-cultures. Upon doxycycline treatment, myelin clusters with signs of demyelination were evident in CA-MKK6 co-cultures, whereas myelin clusters were unaffected in control cultures (Figure 13B). Quantification of the results show a significant increase in the percentage of demyelinated clusters in doxycycline-treated CA-MKK6 infected co-cultures compared to the controls (Figure 13C). These data suggested that ectopic activation of p38 MAPK in oligodendrocytes is also sufficient to cause demyelination in myelinated co-cultures.

6.3 Discussion

In this chapter, we investigated whether growth factors could initiate demyelination of oligodendrocytes. Among the growth factors tested, FGF-2 triggered extensive demyelination in DRG-OPC co-cultures. We also showed that MAPKs play a role in mediating the effect of FGF-2 on demyelination. Finally, we demonstrated that ectopic activation of Erk1/2 or p38 MAPK was sufficient to induce oligodendrocyte demyelination in co-cultures. Our findings on FGF-2 are in line with a previous report, which showed that injecting FGF-2 into the cerebrospinal fluid (CSF) induced myelin break down in the caudal anterior medullary velum (AMV) of rat pups aged P6 and P9 (Goddard et al., 2001). On the contrary, injecting PDGF-AA, another mitogen for OPCs, had no effect on the myelin, an effect that is likely due to the fact that PDGFR-α expression is down regulated in mature oligodendrocytes (Butt et al., 1997; Goddard et al., 2001).

PDGF and FGF-2 were shown to inhibit the differentiation of OPCs into mature oligodendrocytes (Bansal and Pfeiffer, 1997b; Wolswijk and Noble, 1992; Zhou et al., 2006). To address the effects of growth factors on mature myelin, 18-21 day old co-cultures were stimulated with growth factors after the formation of myelin segments so as not to interfere with the process of oligodendrocyte differentiation; hence we conclude that the effects of FGF-2 were on mature myelin in the co-cultures. *In vitro* monoculture studies also indicated that FGF-2 disrupts myelin in mature oligodendrocytes, where the myelin proteins such as proteolipid protein (PLP), myelin basic protein (MBP), and myelin-associated glycoprotein (MAG) were downregulated by FGF-2 (Bansal and Pfeiffer, 1997a). *In vivo* studies by Goddard et

al. reported that cellular localizations of MBP mRNA and PLP protein were disrupted upon injecting FGF-2 into the CSF (Goddard et al., 2001). Therefore, both down regulation of myelin proteins and disrupted localization of MBP mRNA and PLP protein in previous studies could be possible explanations for the FGF-2 induced myelin break down observed in our *in vitro* system.

Mature oligodendrocytes undergo cell death eventually as a consequence of aberrant reentry into the cell cycle induced by FGF-2 (Muir and Compston, 1996). Analyses of MS lesions reported oligodendrocyte loss in actively demyelinating sites (Lucchinetti et al., 1999; Lucchinetti et al., 2000), suggesting that oligodendrocyte death might be a cause of demyelination. We investigated the possibility of cell death in FGF-2 treated co-cultures. There was no significant increase in apoptosis following FGF-2 treatment suggesting that the demyelination observed in FGF-2 treated cultures was not associated with cell death (Figure 4). This is in agreement with the *in vivo* findings, in which no cell death was observed after myelin disruption induced by FGF-2 injection (Goddard et al., 2001). These findings suggest that demyelination induced by FGF-2 is a primary effect and not as a consequence of oligodendrocyte death. FGF-2 was found to be upregulated in the CSF and also in demyelinated lesions of MS patients (Clemente et al., 2011; Sarchielli et al., 2008). Increased expression of FGF-2, FGFR1 and FGFR3 has been observed near and within lesions in experimental demyelination models (Liu et al., 1998; Messersmith et al., 2000). Enhanced FGF-2 receptor signaling components during these diseases is generally interpreted in relevance to remyelination; however, these studies raise the possibility

that growth factors such as FGF-2 might be directly involved in causing myelin loss and also impair remyelination.

Therefore, we further investigated FGF-2 effect on myelination of oligodendrocytes. FGF-2 is shown to inhibit differentiation of OPCs into mature oligodendrocytes (Bansal and Pfeiffer, 1997b; Zhou et al., 2006), however the direct effect of FGF-2 on myelination of differentiated oligodendrocytes is unknown. In our study, we allowed OPCs to differentiate fully in co-cultures then stimulated with FGF-2 to investigate the effect of FGF-2 on the myelination process. Our data show that FGF-2 does elicit an inhibitory effect on myelination by the differentiated oligodendrocytes. This is in contrast to the finding from a recent study, which has demonstrated that myelin sheaths in the CNS of FGF-receptor (FGFR1/FGFR2) knockout mice are thinner than their normal counterpart (Furusho et al., 2012). This discrepancy may be attributed to the differences in the *in vitro* and *in vivo* systems. Alternatively, it is possible that the concentration of FGF-2 used in our studies may not correspond to the physiological levels *in vivo* or the FGF-2 effects on myelin depending on the doses and the stages of oligodendrocyte development may vary.

In our study, another growth factor that was capable of inducing oligodendrocyte demyelination was NRG, albeit to a lesser extent than FGF-2. High doses of soluble NRG were also shown to affect oligodendrocytes by causing phenotypic reversion and down regulation of myelin genes (Canoll et al., 1999). These studies suggest that the effects of FGF-2 and NRG may be mediated through common downstream signaling pathways. A similar example has been demonstrated in the PNS where both FGF-2 and NRG block Schwann cell myelination and trigger

demyelination. The demyelinating effect of these growth factors has been shown to be mediated through activation of the downstream Erk1/2 and the p38 MAPK pathways (Yang et al., 2012; Zanazzi et al., 2001).

The diverse functions of MAPKs have been extensively studied implicating their crucial roles during oligodendrocyte development. During development, Erk1/2 plays a role in mediating PDGF induced proliferation of OPCs and is also involved in the transition from early progenitor to late progenitor during the lineage progression (Baron et al., 2000; Guardiola-Diaz et al., 2012). As mentioned above recently, Erk1/2 role in regulating myelin sheath thickness has been demonstrated (Ishii et al., 2012). Various studies have also demonstrated p38 MAPK function in mediating differentiation and myelination of oligodendrocytes (Bhat et al., 2007; Chew et al., 2010; Fragoso et al., 2007; Haines et al., 2008). Although it is clear that MAPKs play essential roles during development and myelination of the oligodendrocyte lineage, their roles during demyelination is unknown. We further investigated role of MAPKs in mediating the demyelination of oligodendrocytes. We show both Erk1/2 and p38 MAPK pathways mediate FGF-2 induced demyelination, whereas JNK1/2 and PI-3 kinase pathways inhibition had no effect (Figures 7, 8 and 9).

The direct roles of the Erk1/2 and p38 MAPK pathways were further investigated in the context of demyelination. Activating Erk1/2 or p38 MAPK ectopically in myelinated oligodendrocytes was sufficient to initiate myelin break down in co-cultures (Figures 11 and 13). These results raise the possibility that MAPKs may be involved in mediating myelin breakdown in demyelinating disorders of CNS. Following mechanical insults to the CNS or in demyelinating diseases, there

is an increase in the expression of various inflammatory cytokines and growth factors that could contribute to the MAPK activation to trigger demyelination. It would be interesting to test whether inhibiting the MAPK pathways could protect myelin sheaths in the animal models where demyelination is induced experimentally.

Our data suggest that oligodendrocytes after demyelination remain viable. It is unknown whether these cells re-enter the cell cycle or able to de-differentiate into myelin forming oligodendrocytes. *In vitro* studies reported that oligodendrocyte monocultures treated with FGF-2 show aberrant entry into the cell cycle after the down regulation of myelin proteins (Bansal and Pfeiffer, 1997a; Fressinaud et al., 1993; Fressinaud et al., 1995), suggesting that demyelinated oligodendrocytes may potentially de-differentiate into competent, myelin forming oligodendrocytes. In the next chapter, we investigated the possibility of the demyelinated oligodendrocytes reentering the cell cycle and dedifferentiating into precursor cells.

7. Chapter three: To determine the fate of oligodendrocytes after demyelination

7.1 Introduction

In demyelinating disorders of the CNS, loss of myelin sheaths leads to impaired nerve conduction and subsequent degeneration of axons. Therefore, efficient remyelination is essential for functional recovery after insult. Remyelination occurs as a default process after myelin loss in both experimentally-induced demyelination models and in diseases such as MS. In experimental demyelination models, where demyelination is induced by either cuprizone or lysoslecithin, remyelination proceeds after the myelin break down. During early stages of MS, remyelination occurs spontaneously and is evident by the appearance of thinly myelinated axons (shadow plaques). In experimental autoimmune encephalomyelitis (EAE), an animal model of MS, as well as in MS patients, remyelination is often incomplete and aberrant, and eventually fails as the disease progresses. Failure to properly rebuild myelin may be attributed to multiple factors that make the microenvironment non-conducive to promote remyelination (Franklin and Ffrench-Constant, 2008; Franklin and Kotter, 2008).

The source of the oligodendrocytes that contribute to remyelination is not clear. Two potential sources are OPCs in the adult brain and intact mature oligodendrocytes that remain viable after myelin loss. Active demyelination and early remyelination occur simultaneously within some MS lesions, where newly formed myelin sheaths and OPCs appear and repopulate, suggesting that the OPCs eventually differentiate into mature oligodendrocytes and form new myelin sheaths (Franklin and Ffrench-Constant, 2008). During the onset of remyelination, the cells express various markers, indicating the transition of OPCs into mature oligodendrocytes (Fancy et al.,

2004). Furthermore, transplanting OPCs to demyelinated lesions can result in remyelination (Groves et al., 1993; Zhang et al., 1999). These findings suggest that OPCs are the main source of remyelination.

It is possible that the oligodendrocytes that remain viable after demyelination contribute to remyelination. The presence of oligodendrocytes with lost myelin is evident in some MS lesions and in experimental models. MS lesions categorized as patterns I and II show preserved oligodendrocyte cell bodies after myelin loss. These lesions also exhibit higher remyelination compared to patterns III and IV (Franklin and Ffrench-Constant, 2008; Hu and Lucchinetti, 2009). This correlation suggests that preservation of oligodendrocytes cell bodies after loss of myelin sheaths during MS pathology may be a key for efficient remyelination.

In contrast, there is also evidence that resident mature oligodendrocytes cannot contribute to remyelination. In an experimental model of demyelination, when mature oligodendrocytes are transplanted in the lesion, they fail to remyelinate the axons (Targett et al., 1996). However, mature oligodendrocytes differentiated in culture may not be representative of the surviving oligodendrocytes at lesion sites. Further understanding the biology of surviving oligodendrocytes *in vivo* could contribute to developing therapeutic strategies to enhance their remyelination potential.

Earlier studies have indicated that mature oligodendrocytes retain the ability to re-enter the cell cycle, suggesting a regenerative plasticity after the myelin loss. *In vitro* studies using OPC monocultures have demonstrated that mature oligodendrocytes are capable of re-entering the cell cycle following FGF-2 treatment but do not complete mitosis and fail to de-differentiate into OPCs (Bansal and

Pfeiffer, 1997a; Fressinaud et al., 1993). It is possible that FGF-2 signaling alone may not be sufficient to drive the mature oligodendrocytes to divide and regain the ability to de-differentiate.

To elucidate the mechanisms underlying the regenerative ability of mature oligodendrocytes, we investigated oligodendrocyte fate after the myelin loss. In the studies described in chapter 2, the data show that activation of Erk1/2 or p38 MAPK is sufficient to trigger demyelination and down regulate myelin proteins. In this aim, we characterized the phenotype of differentiated oligodendrocyte following ectopic activation of Erk1/2 or p38 MAPK.

We demonstrate that Erk1/2 or p38 MAPK activation in mature oligodendrocytes leads to down regulation of myelin proteins and changes in the morphology of the mature oligodendrocytes. We also show that following activation of Erk1/2, but not p38 MAPK, oligodendrocytes regain the capability to re-enter the cell cycle and complete cytokinesis. However, these oligodendrocytes fail to express markers specific to OPC stage.

7.2 Results

In the previous chapter, we demonstrated that activation of Erk1/2 or p38 MAPK was sufficient to trigger demyelination in co-cultures. But the fate of the oligodendrocyte after myelin loss is unknown. To investigate this, we used differentiated oligodendrocyte monocultures to facilitate clearer observations of oligodendrocyte phenotype changes, which would be difficult to interpret in the dense, heterogeneous cellular environment of a co-culture system. The strategy was to differentiate CA-MKK1 or CA-MKK6 infected OPCs in the absence of doxycycline and once fully differentiated we induce doxycycline-dependent activation of Erk1/2 or p38 MAPK, respectively. The phenotype of CA-MKK1 or CA-MKK6 oligodendrocytes was characterized 48-72 hours after doxycycline treatment. Using this inducible system, we investigated whether MAPK activation allows the mature oligodendrocytes to de-differentiate into the earlier progenitor cell stage, or promotes reentry into the cell cycle. De-differentiation was assessed by the loss of myelin protein expression, changes in cell morphology, and expression of OPC markers. Cell cycle entry was determined by the expression of Ki-67 and mitotic index.

Ectopic activation of Erk1/2 or p38 MAPK down regulates MBP expression in mature oligodendrocytes

Doxycycline was added to the differentiated mature oligodendrocytes to induce CA-MKK1 or CA-MKK6 expression, which in turn activated downstream Erk1/2 or p38 MAPK, respectively. The immunoblot in Figure 14A and Figure 15A shows Erk1/2 and p38 MAPK activation, respectively, in a doxycycline-dependent manner. Forty-eight hours later, the cultures were fixed and immunostained for MBP.

Non-infected oligodendrocytes treated with doxycycline, vector alone (pSLIK) infected oligodendrocytes with doxycycline, and CA-MKK1 or CA-MKK6 infected oligodendrocytes without doxycycline were established as controls.

Prior to doxycycline treatment, 75-85 percent of the CA-MKK1 (0 hr) infected oligodendrocytes were positive for MBP, indicating the level of MBP-positive mature oligodendrocytes before doxycycline treatment. The MBP-positive cells were drastically decreased by 48 hours following doxycycline treatment in CA-MKK1 (48hr) cultures versus CA-MKK1 (0 hr) infected oligodendrocytes prior to doxycycline treatment, suggesting that mature differentiated oligodendrocytes down regulated MBP upon activation of Erk1/2 (Figure 14). The differentiated CA-MKK1 mature oligodendrocytes without doxycycline, as well as other established controls showed no loss of MBP expression whereas MBP expression was lost in doxycycline-treated CA-MKK1 cultures (Figure 14). Figure 14B shows that mature oligodendrocytes expressing active Erk1/2 are MBP-negative. Quantification of MBP-positive cells indicated that doxycycline treated CA-MKK1 infected oligodendrocytes showed a threefold decrease in MBP immunoreactivity compared to controls (Figure 14C). Additionally, the MBP expression in doxycycline-treated, differentiated CA-MKK1 mature oligodendrocytes was verified by Western blot analysis. Figure 14D shows reduced MBP in doxycycline-treated CA-MKK1 mature oligodendrocytes compared to controls. These findings suggest that ectopic activation of Erk1/2 is sufficient to down regulate MBP expression in mature oligodendrocytes. A similar MBP loss was also observed in CA-MKK6 (FLAG-positive) expressing mature oligodendrocytes compared to controls (Figures 15B, and C), suggesting that

p38 MAPK is also sufficient to down regulate MBP expression in mature oligodendrocytes.

Ectopic activation of Erk1/2 or p38 MAPK induces morphological changes in mature oligodendrocytes

One of the hallmarks of differentiated mature oligodendrocyte is a large, membranous network with complex branching. In comparison, OPCs are bi-polar with a simple morphology. We investigated the effects of MAPK activation on oligodendrocyte morphology. CA-MKK1 and CA-MKK6 infected mature oligodendrocyte cultures were established as mentioned above. Forty-eight hours after doxycycline treatment, the cultures were fixed and immunostained with phalloidin to visualize filamentous actin. Upon doxycycline treatment, there was a drastic reduction in the levels of branching in both CA-MKK1 and CA-MKK6 (FLAG-positive) cultures compared to all the controls (Figures 16B and 17B). Morphological changes in CA-MKK1 or CA-MKK6 infected oligodendrocytes were represented quantitatively by plotting the average number of intersections for each Sholl ring (concentric circles with increasing radii are generated form the center of the cell body towards the periphery) against its radius measurement (Sholl profile). A higher number of intersections, or crossings, with the Sholl ring indicate a greater degree of complexity. Sholl profiles were generated using Image J software analysis.

Prior to the doxycycline treatment, CA-MKK1or CA-MKK6 cells averaged 40-45 crossings, similar control cells, indicating an identical morphology with mature oligodendrocytes. Treatment with doxycycline resulted in a drastic reduction of branching in CA-MKK1 cultures compared to the controls, suggesting that the

complex membranous network was lost upon ectopic activation of Erk1/2 in mature oligodendrocytes (Figure 16). A similarly reduced Sholl profile was also observed upon ectopic activation of p38 MAPK in mature oligodendrocytes (Figure 17), however, the morphology attained upon Erk1/2 activation and p38 MAPK activation was different. Erk1/2 activation resulted in a dramatic network simplification, retaining only 2-3 branches, whereas FLAG-positive cells demonstrated a more mild reduction (Figures 16 and 17). This observation indicates that the Erk1/2 and p38 MAPK pathways may act differently to induce morphological changes in mature oligodendrocytes.

Ectopic activation of Erk1/2 or p38 MAPK does not induce cell death in mature oligodendrocytes

Previous studies have reported conflicting results regarding whether FGF-2 may cause oligodendrocyte cell death (Bansal and Pfeiffer, 1997a; Muir and Compston, 1996). Additionally, these opposing findings do not address whether Erk1/2 or p38 MAPK, which are commonly activated by FGF-2, could induce death in mature oligodendrocytes. In order to investigate whether ectopic activation of Erk1/2 or p38 MAPK induces oligodendrocyte death, a TUNEL assay was performed in CA-MKK1 or CA-MKK6 expressing mature oligodendrocytes 48 hours following doxycycline treatment. Following transgene activation in both CA-MKK1 and CA-MKK6 cultures, no significant difference in cell death was observed compared to control, non-infected cultures (Figures 18 and 19). These results suggest that Erk1/2 or p38 MAPK activation did not cause significant cell death in mature oligodendrocytes.

p38 MAPK is activated upon MKK1 activation

So far, our data show that activation of Erk1/2 and p38 MAPK elicit similar effects on oligodendrocytes. They both induced MBP down regulation and changes in oligodendrocyte morphology, suggesting that Erk1/2 and p38 MAPK elicit similar effects, either in parallel or perhaps through the same pathway. In chapter two (Figure 7), we showed that when Erk1/2 or p38 MAPK are inhibited, there is a complete rescue of FGF-2 induced demyelination, suggesting that both work through the same pathway. To ascertain this idea, we tested whether activation of MKK1, an upstream activator of Erk1/2 is sufficient for activation of p38 MAPK. The effect of MKK6 activation, an upstream activator of p38 MAPK, on Erk1/2 activation was also investigated. In mature oligodendrocytes, activation of MKK1 resulted in activation of p38 MAPK along with Erk1/2 (Figure 20A, 14A). Activation of MKK6, however, did not activate Erk1/2 (Figure 20B), indicating that both Erk1/2 and p38 MAPK function within the same pathway, and that Erk1/2 activation occurs upstream of p38 MAPK.

Mature oligodendrocytes reenter the cell cycle upon ectopic activation of Erk1/2

The loss of complex morphology and down regulation of myelin proteins in the absence of cell death indicate that MAPK activation may be driving mature oligodendrocytes to de-differentiate into proliferating OPCs. CA-MKK1 or CA-MKK6 OPCs were allowed to differentiate into mature oligodendrocytes in serum-free media containing T3 in the absence of doxycycline for 7 days. To enrich cultures with differentiated oligodendrocytes prior to activating the MAPKs, any remaining OPCs were killed by treating the cultures with cytosine arabinoside (Ara C) during

the last 48 hours of the differentiation process. To determine the basal level of proliferation in the cultures, we established CA-MKK1 or CA-MKK6 (0 hr) infected oligodendrocytes as a control prior to doxycycline treatment. Prior to doxycycline treatment about 2% of the cells, were Ki-67-positive indicating that most of the cells were in the G0 phase of the cell cycle. The remaining conditions were treated with doxycycline for seventy-two hours, and were fixed and immunostained for Ki-67. Following the MAPK activation (+doxycycline) the number of Ki-67-positive cells increased by 15-18% in MKK1 cultures. Control cultures did not show an increase in Ki67-positive cells (Figure 21). Interestingly, activation of MKK6 (p38 MAPK activation) was not sufficient to promote cell cycle entry (Figure 23). Accordingly, inhibiting p38 MAPK (using SB203580) in MKK1 cultures with doxycycline treatment did not block the entry into cell cycle (Figure 21B). In order to investigate whether these Ki-67-positive cells were able to complete cell division, we determined the mitotic index of these cells. In doxycycline-treated CA-MKK1 cultures, 8% of the Ki-67-positive cells showed signs of mitosis (Figure 22). Additionally, we visualized the cytokinesis of Ki-67-positive cells in doxycycline-treated CA-MKK1 cultures by immunostaining for α-tubulin (Figure 22C). Altogether, this results show that Erk1/2 activation, but not p38 MAPK activation, was sufficient to drive mature oligodendrocyte into a proliferating phenotype.

To determine whether these cells had de-differentiated into OPCs, the cultures were immunostained for PDGFR-α, a marker for progenitors. No significant increase in PDGFR-α-positive cells was observed following Erk1/2 activation (Figure 24). Activation of Erk1/2 did not induce the re-expression of PDGFR-α, suggesting that

Erk1/2 activation is not sufficient to drive the de-differentiation of mature oligodendrocytes into the progenitor stage.

7.3 Discussion

MAPKs are crucial regulators of oligodendrocyte development. Specifically, Erk1/2 and p38 MAPK directly regulate oligodendrocyte proliferation, differentiation and myelination. Erk1/2 has been shown to be important during the lineage progression from early progenitor to late progenitor as well as in regulating myelin sheath thickness in the CNS (Guardiola-Diaz et al., 2012; Ishii et al., 2012). p38 MAPK promotes oligodendrocyte differentiation and myelination, by accumulating p27 kip1, and enhancing the activity of promyelinating transcriptional factors such as SOX10 (Casaccia-Bonnefil et al., 1997; Chew et al., 2010). However, their effect on mature oligodendrocytes is unknown. Several studies have implicated the MAPKs role in demyelinating diseases, where increased activation of Erk1/2 and p38 MAPK was observed in the spinal cords and the brains of EAE-induced mice compared to controls (Shin et al., 2003). In another study, the symptoms of EAE were reduced in p38 MAPK +/- mice compared to control (Namiki et al., 2012). These observations suggest that MAPKs might be involved in myelin pathology by affecting oligodendrocyte biology.

Erk1/2 and p38 MAPK signaling has been reported to cause phenotypic changes in other cell types. For example, in the PNS, both Erk1/2 and p38 MAPK were shown to induce de-differentiation of Schwann cells and revert them into an immature phenotype (Harrisingh et al., 2004; Yang et al., 2012).

To study oligodendrocyte fate upon Erk1/2 or p38 MAPK activation, it is important to induce Erk1/2 or p38 MAPK activation specifically in mature oligodendrocytes, as activation in earlier stages might disrupt oligodendrocyte

development. Therefore, we activated Erk1/2 or p38 MAPK in mature oligodendrocytes by using a doxycycline-inducible promoter system (Figures 14A and 15A). This system enabled us to study the effect of Erk1/2 or p38 MAPK on mature oligodendrocytes without interfering with development.

During OPC differentiation, the cells were allowed to differentiate for 6-7 days before transgene activation. However, it is important to note that oligodendrocyte populations might not be synchronized, and thus undifferentiated OPCs might remain in the cultures, possibly skewing results. Therefore, the CA-MKK1or CA-MKK6 infected mature oligodendrocytes (0hr) served as a control to determine the level of differentiation and also to determine the basal level of preexisting OPCs in the cultures. Hence, we claim that the results shown here reflect the effects on mature oligodendrocytes and are not due to the preexisting OPCs.

Studies by Bansal et al. demonstrated that differentiated oligodendrocytes elicited various responses such as down regulation of myelin genes, loss of membranous sheets and extension of long processes upon FGF-2 treatment. Further, they also reported that mature oligodendrocytes re-enter the cell cycle but do not complete mitosis, and instead are converted into a novel phenotype without dedifferentiating into progenitor stage (Bansal and Pfeiffer, 1997a). In contrast to these findings, Grinspan et al. showed that FGF-2 induced de-differentiation of oligodendrocytes into progenitors. They observed an increase in A2B5 progenitor population when FGF-2 was added to immunopanned O1-positive oligodendrocytes (Grinspan et al., 1996). The discrepancies in these findings may be due to differences in the source and stage of the oligodendrocytes. In the study performed by Grinspan et

al. the oligodendrocytes were less mature, whereas in the Bansal et al. study, the oligodendrocytes may have belonged to a more mature lineage. Studies from Fressinaud et al. also demonstrated 5-bromo-2'-deoxyuridine (BrdU) incorporation by MBP-positive oligodendrocytes upon FGF-2 treatment (Fressinaud et al., 1993). These studies suggest that growth factor signals regulate the plasticity of mature oligodendrocytes. But the mechanisms driving the mature oligodendrocytes to regain the ability to proliferate and de-differentiate are not well defined.

To understand the underlying mechanisms, we investigated additional downstream effectors that might be activated by growth factors such as FGF-2, or by cytokines that are upregulated during demyelinating diseases. We found that ectopic activation of Erk1/2 or p38 MAPK in mature oligodendrocytes down regulated MBP expression, and these effects were accompanied by a loss of membranous networks (Figures 14, 15, 16 and 17). The reported effects of FGF-2, such as myelin gene down regulation and reduction in membranous networks (Bansal and Pfeiffer, 1997a; Fressinaud et al., 1995), are likely mediated through the downstream effectors Erk1/2 or p38 MAPK. The altered morphology of mature oligodendrocyte might be due to cytoskeletal rearrangements induced by Erk1/2 or p38 MAPK. It is possible that the morphology of mature oligodendrocytes following p38 MAPK activation might be similar to the novel oligodendrocyte phenotype observed upon FGF-2 treatment, though these results have yet to be confirmed by staining for O4 and O1. Here, it is noteworthy that the morphological changes induced by Erk1/2 or p38 activation were not exactly similar. We noticed that Erk1/2 activation, but not p38 activation, in

mature oligodendrocytes resulted in a drastic reduction of membrane processes (Figures 16 and 17), indicating differences in their biological roles.

Studies have reported that mature oligodendrocytes undergo cell death upon exposure to growth factors such as FGF-2 (Muir and Compston, 1996). In our study, we did not observe any significant cell death upon Erk1/2 or p38 MAPK activation by 48 hours (Figures 18 and 19). However, our results do not rule out the possibility that cell death might occur upon prolonged Erk1/2 or p38 MAPK activation.

Mature oligodendrocytes are terminally differentiated cells and are not capable of proliferation. However, upon Erk1/2 activation we observed that 15-18% of cells were Ki-67 –positive, indicating that mature oligodendrocytes regained the ability to re-enter cell cycle. In the CA-MKK1 (0 hr) control condition that was fixed at the time of doxycycline treatment, about 2% of cells stained positive for Ki-67, indicating that 98% of the cells were differentiated (Figure 21). This result suggests that Ki-67 expression was induced in mature oligodendrocytes by Erk1/2 activation and was not simply present in preexisting OPCs. Although we observed MBP down regulation and altered morphology upon p38 activation, we did not find any Ki-67-positive cells upon p38 activation (Figure 23). These results indicate that Erk1/2, but not p38 MAPK, is essential to induce oligodendrocyte cell cycle entry.

In our studies, we showed that p38 MAPK is activated downstream of Erk1/2 or MKK1, but Erk1/2 is not activated upon p38 MAPK activation (Figure 20). This finding explains the similar changes observed in mature oligodendrocytes upon Erk1/2 or p38 MAPK activation, suggesting that these molecules act within the same pathway to down regulate MBP expression and alter cell morphology. However,

mature oligodendrocytes did not re-enter the cell cycle upon p38 MAPK activation, whereas Erk1/2 activation did induce reentry into the cell cycle (Figures 21 and 23). Furthermore, inhibition of p38 MAPK activity in the CA-MKK1 cultures did not block mature oligodendrocytes from entering the cell cycle, indicating that Erk1/2-induced cell cycle reentry was not mediated through p38 MAPK (Figure 21B). These results also suggest that Erk1/2 might act through another pathway to induce the proliferative phenotype in mature oligodendrocytes. Taken together, this data suggests that Erk1/2 and p38 MAPK down regulate MBP and reduce branching complexity in mature oligodendrocytes by acting within the same pathway, however, Erk1/2 may induce the proliferative phenotype in mature oligodendrocytes through other pathways.

Bansal et al. observed BrdU incorporation in oligodendrocytes following FGF-2 treatment for 5 days, suggesting entry into S-phase, but the cells remain stalled, unable to enter the M phase of the cell cycle. Contrary to these findings, we observed about 8 percent increase in mitotic indices upon Erk1/2 activation for 72 hours and also noticed cells showing the signs of cytokinesis in these cultures (Figure 22), indicating that these cells not only re-enter the cell but also complete the cell division. A greater number of cells might enter into mitosis upon prolonged activation of Erk1/2.

Although oligodendrocytes re-entered the cell cycle upon Erk1/2 activation, they did not de-differentiate into PDGFR-α expressing OPCs (Figure 24). However, it is possible that they reverted into an immature phenotype capable of entering the cell cycle. In accordance with our findings, studies conducted by Butt and Dinsdale. found

that, upon injecting FGF-2 into the CSF, myelin was disrupted and increased numbers of immature oligodendrocytes and OPCs were observed, indicating that these immature oligodendrocytes might be accumulated as a result of the phenotypic reversion caused by FGF-2 treatment (Butt and Dinsdale, 2005). Our studies along with these observations imply that extracellular signals that activate Erk1/2 might revert mature oligodendrocytes into an immature phenotype. These studies suggest that mature oligodendrocytes have certain degree of plasticity to revert into an immature phenotype and possibly regain the ability of regeneration.

8. Chapter Four: To determine whether the injury-associated demyelination of oligodendrocytes is mediated by Erk1/2 or p38 MAPK signaling

8.1 Introduction

Traumatic brain injury (TBI) is one of the leading causes of death in the United States. Patients who survive may suffer from long-term physical and cognitive impairments caused by TBI-induced neurodegeneration (Gronwall and Wrightson, 1974; Levin et al., 1990). Furthermore, TBI patients are at higher risk of developing Alzheimer's disease (Rasmusson et al., 1995; Roberts et al., 1991). Traumatic incidences, such as motor vehicle accidents, falls and assaults are the leading causes of TBI. The rapid head movements involved in TBI create shearing forces that stretch the white matter axons of the brain, resulting in diffuse axon injury (DAI) (Adams et al., 1989; Adams et al., 1984; Gennarelli, 1993; Grady et al., 1993). Although most axons are intact and viable following DAI, functional abnormalities and degeneration will manifest over time, likely resulting from secondary cellular responses that were triggered by the initial injury.

Previous studies using animal models have reported progressive white matter atrophy associated with TBI. In the cortex, the white matter atrophy can proceed for up to 1 year after the TBI (Bramlett and Dietrich, 2002; Smith et al., 1997). This degeneration of myelinated axons may be due to secondary axonal pathology or occur as a consequence of myelin degeneration. Since, myelin is important for neuronal health, injury-induced myelin damage could result in axonal loss. To understand the pathology of white matter damage, it is important to address how myelin is affected after the injury.

In vitro DAI models enable us to study the effects of DAI on myelin and oligodendrocytes. Various TBI researchers have developed their own stretch injury

device, using mechanical force to induce DAI on cultured cells. Our strategy utilizes an *in vitro* DAI model established by Dr. Bryan J. Pfister at the New Jersey Institute of Technology (NJIT) similar to the one developed in Dr. Douglas Smith's laboratory (University of Pennsylvania) with some modifications (Pfister et al., 2003; Smith et al., 1999). The model uses a pressure-pulse to stretch-injure myelinated axons on a deformable substrate. The system consists of a pressure chamber attached to an air pulse generating system. The cultures are grown on a silicone membrane that is attached to an injury well made from two PEEK rings (Figure 27A). The injury well is placed in the pressure chamber, and the pressure from the system deforms the substrate, subsequently stretch-injuring the associated axons. A mask with a 2mm gap in the center is used underneath the injury well to restrict the stretch-injury to a specified area on the silicone membrane (Figure 27B). After establishing the injury parameters on DRG neurons, we examined the effects of DAI on the myelinated axons. We also determined whether Erk1/2 plays a role in triggering myelin damage associated with DAI.

Our data demonstrate that mechanical stretch-induced injury triggers oligodendrocyte demyelination without obvious axonal loss or degeneration. Furthermore, our results show that the injury-induced oligodendrocyte demyelination is mediated through activation of the Ras/Raf/Erk1/2 pathway.

8.2 Results

Studies have reported degeneration of myelinated axons in humans and animal models following TBI (Bramlett and Dietrich, 2002; Smith et al., 1997). Axonal pathology and degeneration are well-characterized in these studies. However, the specific effects of DAI on myelin are not well understood. In this aim, we investigated the effects of stretch-injury on myelinated oligodendrocytes and identified the mechanisms underlying the process.

In order to localize stretch-injury to the myelinated axons without affecting the cell bodies, we induced uni-directional growth of the DRG axons on matrigel-coated silicone membrane. Dissociated DRG neurons were plated on one side of the plating insert as shown in Figure 27C. Twenty-four hours after the plating, when the neurons have attached to the substrate, the insert was removed to allow the axons to extend and grow towards the well edge. This strategy encouraged the axons to extend towards the opposite side of the neuronal cell bodies, generating a semi-directional axonal growth. Once the axons reached the edges of the well, OPCs were added to the cultures, where they differentiated and eventually myelinated the axons. An image of the myelinated axons cultured in the DAI device is shown in Figure 27D.

After myelination was completed, each culture was inserted into a pressure chamber to induce stretch-injury. In our previous study, we had optimized the injury parameters for DRG axons. This was important since most of the previous studies using the DAI device were performed on cortical neurons, therefore the strain rate (pressure-pulse intensity) that would cause the stretch-injury response on DRG axons was unknown. We had identified 60% strain as the optimal intensity to induce axon

undulation without causing axon degeneration a characteristic stretch-injury response which was accompanied by a rapid influx of calcium into the axon. Axons injured with 90% strain degenerated by 48 hours. Therefore, in the following studies we utilized 60% and 90% strain to determine the effects of stretch-injury on myelinated axons.

Twenty-four hours following stretch-injury, cultures were fixed and immunostained for MBP and neurofilament. At the injury site, myelin clusters with signs of demyelination were observed in both 60% and 90% strain injury conditions. However, cultures injured with 60% strain induced 75% oligodendrocyte demyelination, whereas cultures injured at 90% strain caused 100% demyelination, indicating that stretch-injuring axons triggered oligodendrocyte demyelination (Figure 28). The axons after the stretch remained intact and there was no obvious disconnection. However, in cultures injured with 90% strain, the axons appeared to be more sparsely distributed compared to the non-injured conditions, indicating that axon degeneration might have occurred. Axons that were injured with 60% strain conditions appeared very similar to non-injured conditions (Figure 28).

In specific aim 1, we showed that FGF-2-induced oligodendrocyte demyelination was mediated by Erk1/2 pathway (Figures 7 and 8). Therefore, we next investigated whether the Erk1/2 pathway plays a role in stretch-injury-induced demyelination. Myelinated cultures were pretreated and maintained with the Erk1/2 inhibitor U1026 (10μM), then subjected to 90% strain stretch-injury. Twenty-four hours later, the cultures were fixed and immunostained for MBP and neurofilament. In the absence of U1026, 100% of the myelinated clusters showed signs of

demyelination. In U1026 treated cultures, only 60-70% of the clusters underwent demyelination (Figure 29), indicating that inhibition of Erk1/2 had a protective effect on myelin. In uninjured cultures approximately 30% of clusters showed signs of demyelination, U1026 treatment did not have an effect (Figure 29). There was no obvious disconnection of the axons after the stretch injury, however, axons appeared sparse in 90% strain conditions compared to U1026 treated or non-injured conditions (Figure 29). These results suggest that injury caused by mechanical stretch induces oligodendrocyte demyelination, and Erk1/2 is involved in mediating this process.

8.3 Discussion

In this chapter, we investigated whether stretch-injury to axons could trigger oligodendrocyte demyelination. We successfully established a novel *in vitro* model for white matter injury by combining the mechanical axonal stretch devise with the DRG-OPC myelinated co-cultures (Figure 27). Here, we showed that axonal injury caused by mechanical stretching (using 60% and 90% strains) induced oligodendrocyte demyelination (Figure 28). Furthermore, we demonstrated that Erk1/2 plays a role in mediating oligodendrocyte demyelination following axonal stretch (Figure 29).

The rapid movement of brain during TBI is thought to injure the axons resulting in DAI (Adams et al., 1989; Adams et al., 1984; Gennarelli, 1993; Grady et al., 1993). Although severe brain injury might cause axon disconnection and neuronal death, recent evidence suggests that, in most cases, axons remain connected and are viable after injury. Similarly, in our experiments, stretching the myelinated axons using a pressure pulse did not disconnect the axons, but did lead to the myelin loss. Previous studies have shown that stretch-injured axons degenerate overtime (Bramlett and Dietrich, 2002; Smith et al., 1997). Although axonal pathology might cause degeneration, myelin loss after the injury could be another potential cause. Since myelin is important for neuronal health and provides trophic support to the neurons, the loss of myelin may lead to slow axonal degeneration over time. Our data demonstrates that stretch-injuring axons lead to myelin breakdown (Figure 28), exposing the axons and leaving them vulnerable to further degeneration.

In chapter one, we showed that growth factor-induced demyelination is mediated by the Erk1/2 pathway (Figures 7 and 8). Interestingly, our results indicated that stretch-injury-induced oligodendrocyte demyelination might also be mediated through the Erk1/2 pathway (Figure 29). However, inhibiting Erk1/2 resulted only in partial protection from demyelination, suggesting that other pathways, such as p38 MAPK, may be involved along with Erk1/2 in mediating stretch-induced oligodendrocyte demyelination. Co-treatment with a p38 MAPK inhibitor may result in enhanced protection of myelin. Since the myelinated axons are injured using 90% strain, there could be an axonal loss after the injury. Therefore, demyelination could be due to the secondary effect of axonal loss and not from the direct effect of oligodendrocyte damage. If the myelinated axons were injured with 60% strain, at which the axons are better preserved, Erk1/2 may have a stronger protective effect on the myelin.

How stretch injury leads to Erk1/2 activation remains unclear. One possible trigger for Erk1/2 activation is the calcium influx into the oligodendrocytes following stretch injury. Previous studies from our lab demonstrated that calcium influx occurs in Schwann cells in response to injury. Furthermore, another study observed MAPK activation in response to calcium influx in PC12 cells, and this effect was mediated through Ras, an upstream activator of MKK1 (Rosen et al., 1994). These studies suggest the possibility that calcium influx into myelinating oligodendrocytes might occur, thus triggering demyelination via the Erk1/2 pathway. Another possibility is that Erk1/2 activation with in the axons elicits a secondary effect on the associated oligodendrocytes resulting in demyelination. Previous studies in our lab have

observed calcium influx into DRG axons with 60% and 90% strains. At 60%, stretch-injury induced a transient calcium influx, whereas at 90% there was a second wave of calcium influx resulting in a prolong calcium increase in the axon. Whether the calcium increase in the axon is responsible for Erk1/2 activation is unknown. It is also possible that demyelination resulted from axon-degeneration induced by the prolonged calcium increase in the axons. Stretch injury studies by inhibiting the calcium influx by blocking the calcium channels could determine whether calcium influx is the source of Erk1/2 activation. Taken together, the data presented here provides a compelling support that Erk1/2 pathway is a crucial regulator in triggering injury-induced oligodendrocyte demyelination. Therefore, inhibition of Erk1/2 pathway could be a potential therapeutic strategy to prevent oligodendrocyte demyelination.

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9. Chapter Five. Conclusions and Future Directions

9.1 Conclusions and Future Directions

The most salient characteristic underlying many demyelinating diseases is oligodendrocyte demyelination, which leads to impaired nerve conduction and neurological dysfunction. Several factors are implicated in initiating oligodendrocyte demyelination, depending on the disease type or injury. Although multiple events govern the process of demyelination, the increased levels of growth factors and cytokines during these demyelinating diseases suggest that they may have role in the pathology of the disease. For example, injecting FGF-2 into rat brains induced myelin loss (Goddard et al., 2001). Additionally, FGFs and their receptors have been found to be increased in the CSF and in demyelinated lesions, suggest that FGF-2 may play a role in disease pathology (Liu et al., 1998; Logan et al., 1992; Messersmith et al., 2000; Sarchielli et al., 2008). Also various cytokines such as IL-6, IL-17, and interferon-γ are also found to be upregulated in the brains of MS patients (Lock et al., 2002; Matusevicius et al., 1999). These studies implicate that growth factors and cytokine signals may be involved in the pathology of the demyelinating diseases.

Growth factors and cytokines were shown to activate several downstream molecules in regulating diverse cellular processes in various cell types. For example, FGF-2 regulates oligodendrocyte development by multiple intracellular signaling pathways, including Erk1/2 and p38 MAPK (Baron et al., 2000). Cytokines, such as IL-l7, were also known to activate MAPKs (Song and Qian, 2013). Because growth factors and cytokines can trigger multiple common pathways such as Erk1/2 and p38 MAPK, we hypothesized that common downstream effectors induced by these extracellular stimuli may have an effect on mature myelin and further influence

oligodendrocyte biology. Currently, the molecular mechanisms that regulate mature myelin homeostasis are unknown. Therefore, studying the molecular mechanisms that regulate the process of myelin break down in oligodendrocytes will provide insights to understanding the progression of disease pathology.

Our study investigated the role of MAPKs that are commonly activated by various growth factors and cytokines with regard to various aspects of demyelinating diseases. Several studies have shown the importance of MAPKs in oligodendrocyte development. In contrast, much less is known about their role in disease pathology, specifically in demyelination studies. Understanding the molecular mechanisms that regulate oligodendrocyte demyelination and further effects on oligodendrocyte biology will not only elucidate their importance, but also provide insights towards the development of more effective therapeutic strategies. The studies described here characterized the roles of MAPKs in regulating various aspects of demyelinating diseases such as: 1) in regulating oligodendrocyte demyelination, 2) whether mature oligodendrocytes de-differentiate and contribute to remyelination and role of MAPKs in changing their phenotype and 3) how they mediated injury associated demyelination.

The results from our studies are summarized as follows: In chapter two, we showed that growth factors such as FGF-2 can trigger oligodendrocyte demyelination as well as inhibit myelination. We demonstrated that FGF-2-induced oligodendrocyte demyelination is mediated through both Erk1/2 and p38 MAPK pathways, whereas the JNK1/2 and PI-3 kinase pathways are not involved. Furthermore, we also showed that ectopic activation of Erk1/2 or p38 MAPK is sufficient to trigger oligodendrocyte

demyelination. The results in this chapter provide evidence that MAPKs play a crucial role in triggering oligodendrocyte demyelination. Our data also suggested that oligodendrocytes remain viable after myelin loss, suggesting that demyelinated oligodendrocytes may potentially de-differentiate into competent, myelin forming oligodendrocytes.

Therefore, in chapter three, we investigated the ability of demyelinated oligodendrocytes to re-enter the cell cycle and de-differentiate into precursor cells. We began by characterizing various phenotypic changes of mature oligodendrocytes upon ectopic activation of Erk1/2 or p38 MAPK. These studies will contribute towards answering some of the important questions in regard to remyelination: 1) whether surviving oligodendrocytes after the myelin loss retain the plasticity to remyelinate axons and 2) what are the underlying mechanisms that regulate de-differentiation of the mature oligodendrocytes. These questions were addressed by previous studies that suggested that mature oligodendrocytes might have limited plasticity by demonstrating their ability to re-enter the cell cycle, upon FGF-2 treatment (Bansal and Pfeiffer, 1997a; Fressinaud et al., 1993; Grinspan et al., 1996). These studies suggest that growth factor signals regulate the plasticity of mature oligodendrocytes. However, the mechanisms underlying this process are not well understood. Our results in this chapter showed that ectopic activation of Erk1/2 or p38 MAPK in mature oligodendrocytes induces phenotypic changes such as the loss of myelin protein such as MBP and a drastic reduction in membranous network complexity (Figure 25). Interestingly, we also found that oligodendrocytes maintain viability after the Erk1/2 or p38 MAPK induction and Erk1/2 activity is upstream of

p38 MAPK (Figure 20 and 25). Furthermore, Erk1/2 but not p38 MAPK activation leads to reentry into cell cycle. Taken together, these studies suggest that mature oligodendrocytes have retained a limited plasticity to enter into a proliferating phenotype, and this ability is regulated by the Erk1/2 pathway (Figure 26).

In chapter four, we investigated whether stretch-injury induces oligodendrocyte demyelination and asked whether the demyelination induced by mechanical stretch is mediated through the Erk1/2 pathway. Our results demonstrated that stretch-injury induced oligodendrocyte demyelination and implicated a role for Erk1/2 in oligodendrocyte demyelination induced by stretch-injury.

Our studies elucidated the role of the Erk1/2 pathway in inducing oligodendrocyte demyelination and as well as changing the phenotype of the mature oligodendrocytes. Therefore, our study lead to the following key questions: 1) Is Erk1/2 a crucial downstream effector that regulates oligodendrocyte demyelination that can be activated by multiple upstream signals 2) what are the downstream effectors of Erk1/2; and 3) determine the relevance of gaining a proliferative phenotype by mature oligodendrocytes in terms of remyelination.

Molecular mechanisms regulating oligodendrocyte demyelination

We have demonstrated the importance of the Erk1/2 pathway in regulating oligodendrocyte demyelination and further causing mature oligodendrocytes to enter the cell cycle. Our studies suggest that multiple upstream signals can potentially activate Erk1/2 pathway. In our studies, we demonstrated that demyelination induced by various extracellular stimuli such as growth factors (FGF-2) and mechanical stretch was mediated through Erk1/2. These studies imply multiple extracellular

stimuli (growth factors and possibly calcium signaling) are potential activators of Erk1/2 pathway, further leading to the myelin loss in oligodendrocytes. These results also suggest that Erk1/2 plays a crucial role in integrating diverse stimuli in regulating oligodendrocyte demyelination. In fact, levels of growth factors and cytokines are increased during demyelinating diseases, implying that these extracellular stimuli may contribute to Erk1/2 activation. Although the mechanisms are not well understood, studies from our injury model suggest that injury-induced calcium signaling might be another potential signal that leads to Erk1/2 activation.

We demonstrated that p38 MAPK is involved in mediating oligodendrocyte demyelination. Furthermore, we have shown that p38 MAPK acts downstream of Erk1/2 or MKK1 in mediating oligodendrocyte demyelination, which explains the similar effects (down regulation of MBP, change in morphology) observed upon both Erk1/2 and p38 MAPK activation in mature oligodendrocytes. However, these pathways diverge in mediating further effects, where Erk1/2, but not p38 MAPK, induced mature oligodendrocytes to enter the cell cycle. These studies suggest that Erk1/2 effects are mediated through p38 MAPK, however the Erk1/2 pathway can further revert mature oligodendrocytes into a proliferating phenotype.

The downstream effectors of Erk1/2 activation in inducing oligodendrocyte demyelination remain unclear. However, demyelination could occur either by induction of the downstream effectors that can cause myelin breakdown or by disrupting the factors that are essential for the maintenance of myelin. An example for the former possibility is the function of c-jun in Schwann cells, which can be activated by p38 MAPK to induce Schwann cell demyelination and de-differentiation

(Yang et al., 2012). However, there are also transcription factors that are important for maintaining myelin homeostasis. One such factor is myelin regulatory factor (MRF), which is important for the maintenance of CNS myelin. In fact, disruption of MRF in myelinating oligodendrocytes resulted in severe demyelination in mice (Koenning et al., 2012). It would be interesting to determine whether ectopic activation of Erk1/2 in mature oligodendrocytes is associated with MRF disruption, thus causing demyelination. However, to more thoroughly understand the effectors that are downstream of Erk1/2, a more complete analysis is required. A potential approach to identify downstream signaling effectors of Erk1/2 can involve a microarray analysis of the gene profiles of CA-MKK1 mature oligodendrocytes with and without the transgene activation.

Implications of Erk1/2 induced cell cycle entry of mature oligodendrocytes in remyelination

In demyelinating diseases, the most challenging event after the insult is to regain complete remyelination. This is important because the naked axons are otherwise susceptible to degeneration resulting in functional impairment. Therefore, it is important to enhance the remyelination potential of the oligodendrocytes after the insult. Understanding the sources and mechanisms involved in contributing to remyelination will allow us to develop therapeutic strategies to enhance remyelination. Several studies suggested that adult OPCs are the main source for remyelination after the insult, however whether damaged oligodendrocytes could regenerate and contribute to remyelination remains unclear. Previous studies have addressed this issue, and their results suggested that oligodendrocytes retain a certain

amount of plasticity after myelin loss (Bansal and Pfeiffer, 1997a; Fressinaud et al., 1993; Grinspan et al., 1996). These studies are in accordance with the idea that oligodendrocytes that survived after the injury may contribute to remyelination. However, in these studies, the mechanisms underlying this process are not well-elucidated. Furthermore, other studies suggest that the surviving oligodendrocytes cannot contribute towards remyelination (Keirstead and Blakemore, 1997; Targett et al., 1996), although we cannot rule out the possibility that these results might be due to discrepancies in the experimental conditions.

Our studies in chapter three show that Erk1/2 activation drives mature oligodendrocytes to enter the cell cycle, but do not show signs of de-differentiation. Although the exact mechanism of how mature oligodendrocytes attain a proliferative phenotype upon Erk1/2 activation is unknown, several potential possibilities exist that might be involved in this process. Based on the studies from oligodendrocyte development, cell cycle inhibitors (CKI) that inhibit cell cycle progression and cyclins that are required for cell cycle progression might be few potential candidates that might be involved in this process. Previous studies have shown that during oligodendrocyte development, accumulation of cell cycle inhibitory proteins, such as $p57^{kip2}$, a cdk-inhibitory proteins (CKI) in OPCs, is required for exiting the cell cycle and proceeding into the next stages of differentiation (Dugas et al., 2007) . It is possible that CKI levels remain high in mature oligodendrocytes to maintain them in the G0 phase, thus preventing them to enter the cell cycle. In this scenario of de-differentiation, Erk1/2 activation may reduce the levels of CKI and remove the cell cycle arrest. Furthermore, Erk1/2 might also upregulate the expression of cyclins in

mature oligodendrocytes further driving them to enter the cell cycle. Therefore, Erk1/2 acting on both CKI and cyclins may drive the mature oligodendrocytes to proceed into the proliferative phenotype, however, further studies are required to confirm this possibility.

Erk1/2 activation could drive mature oligodendrocytes to attain a proliferative phenotype, but these proliferative cells did not express PDGFR-∞, suggesting that Erk1/2 alone might not be sufficient to fully attain a progenitor phenotype. Another possibility is that the phenotype attained upon Erk1/2 activation might not be PDGFR-α-positive, but might have the potential to regenerate and contribute to remyelination. The latter possibility remains to be confirmed by further studies.

Future studies addressing the effects of Erk1/2 activation on myelinated oligodendrocytes are required to confirm its role *in vivo*. This can be done by inducing MKK1 expression specifically in myelinated oligodendrocytes under a specific promoter. These studies would further enable us to understand it role on mature myelin and oligodendrocytes.

The positive and negative consequences of Erk1/2 activation in disease

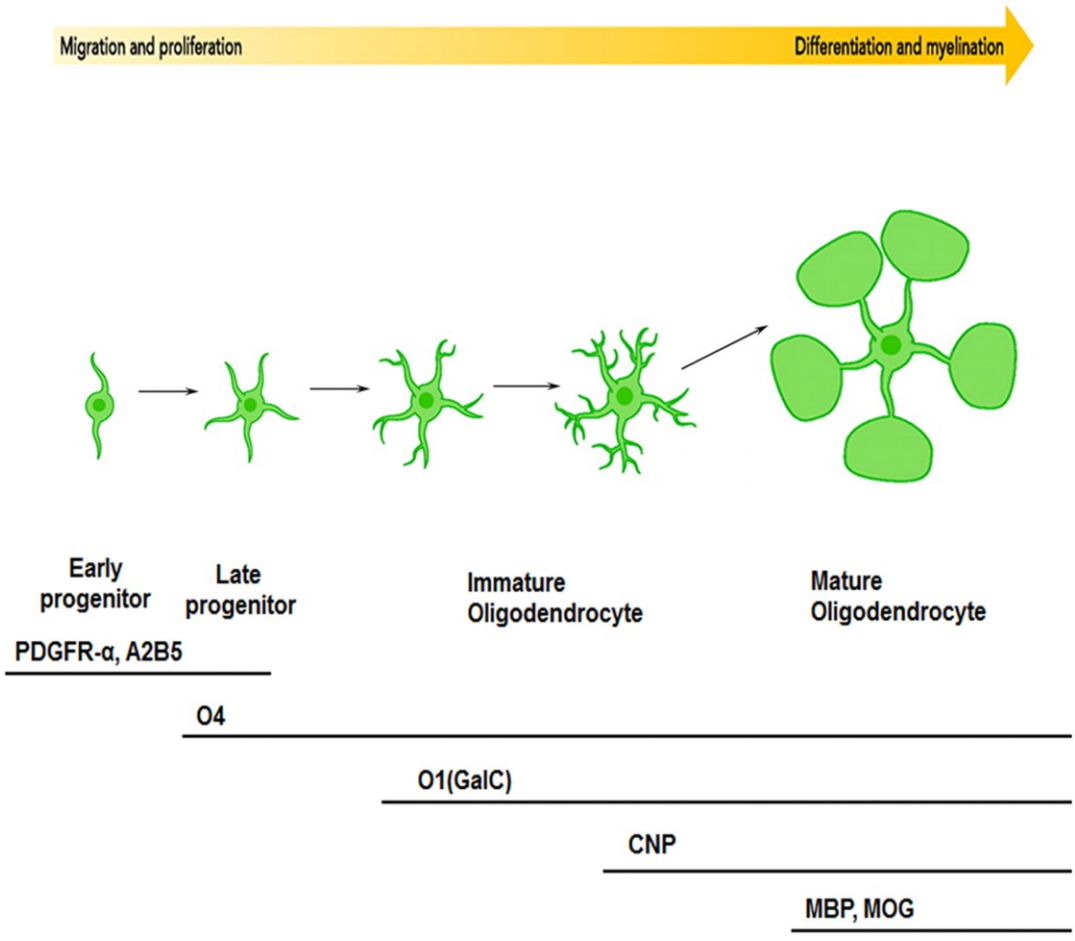
In mature oligodendrocytes, activating Erk1/2 in disease conditions may prove to be a beneficial mechanism, helping damaged oligodendrocytes to remyelinate by promoting a proliferative phenotype. Oligodendrocytes shedding their processes and attaining a simplified morphology could be a defensive mechanism to prevent further damage from other harmful factors in the disease environment. In support of this idea, studies by Butt and Dinsdale. (2005) reported that oligodendrocytes do not die but rather, simplify their morphology after demyelination. These surviving

oligodendrocytes might contribute for further remyelination once they are able to attain a proliferative phenotype. Furthermore, the correlation between enhanced remyelination and surviving oligodendrocytes in patterns I and II MS lesions, but not in patterns III and IV, supporting the concept that the surviving oligodendrocytes might contribute to the remyelination process.

However, Erk1/2 activation in mature oligodendrocytes may not always manifest beneficial results, as it may also lead to demyelination. These opposing effects suggest that the consequences of Erk1/2 activation is highly dependent upon several factors, and it will be relevant to continue examining the role of Erk1/2 activation in disease and injury states to clarify the mechanisms of Erk1/2-dependent proliferation and myelination.

10. Figures

Figure 1



(Adopted and modified from Jackman et al., 2009, Physiology)

Figure 1. Stages of oligodendrocyte development

OPCs progress through multiple morphological and antigenic stages and differentiate into mature oligodendrocytes. Early progenitors progress into late progenitors. Late progenitors differentiate into immature oligodendrocytes. Immature oligodendrocytes further differentiate and finally become mature oligodendrocytes.

Figure 2

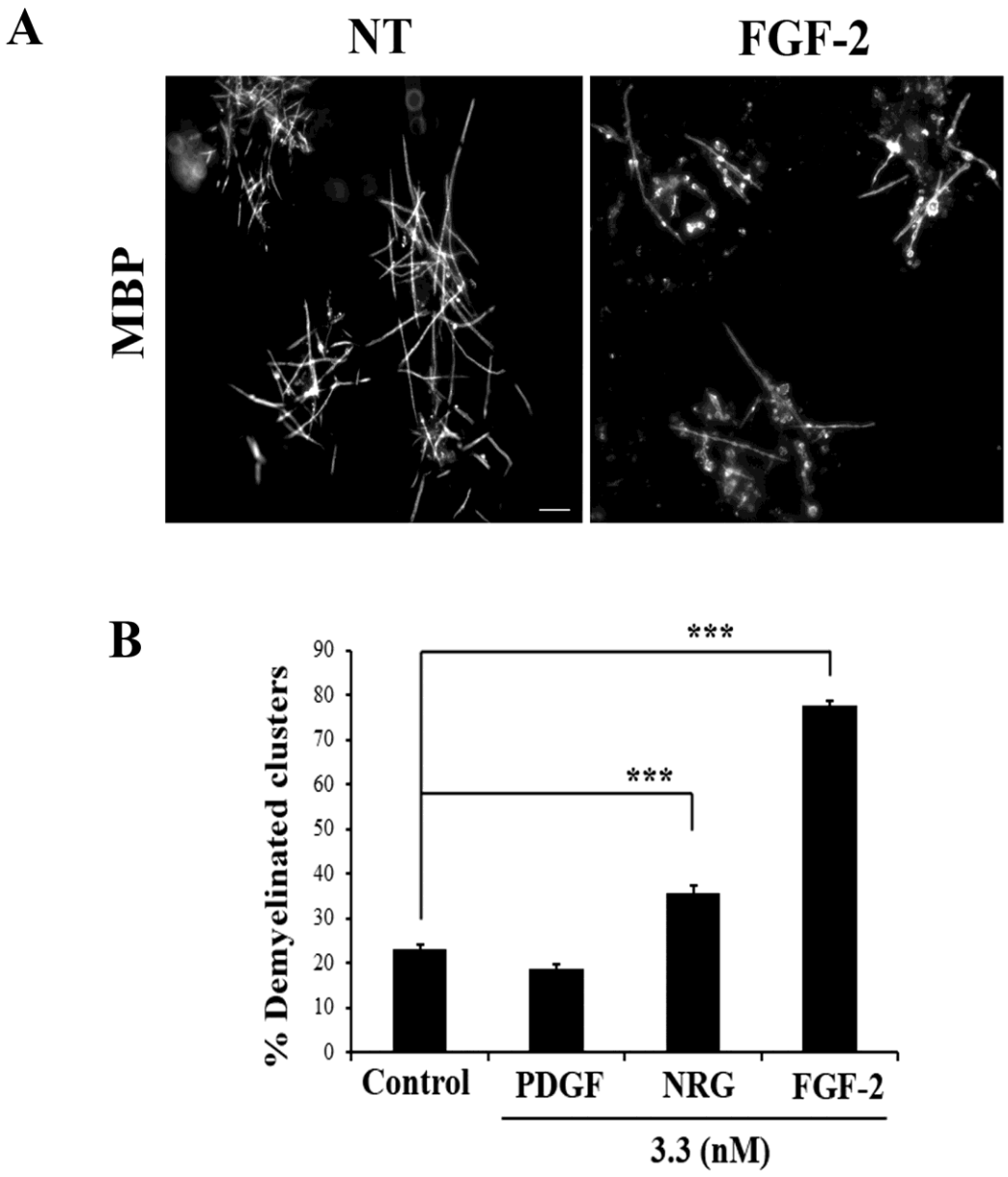

A
NT
FGF-2
MBP
B
% Demyelinated clusters
90
80
70
60
50
40
30
20
10
0
***
***
Control
PDGF
NRG
FGF-2
3.3 (nM)

Figure 2. FGF-2 induces demyelination in vitro in DRG-OPC co-cultures.

Myelinated DRG-OPC co-cultures were treated with growth factors (PDGF, NRG, and FGF-2 at 3.3nM concentration) for forty-two hours, then immunolabeled for MBP to detect demyelination. (A) MBP positive clusters in control and in FGF-2 treated co-cultures. Note the demyelination in the FGF-2 treated condition. Scale bar: 20μm. (B) The levels of demyelination determined as the percentage of MBP-positive myelin clusters showing signs of demyelination. Increased levels of demyelinated clusters were observed with FGF-2 treatment. The data represented here is from three independent experiments (3 coverslips/ experiment). One-way ANOVA followed by Tukey's post hoc analysis was done to determine the statistical difference between the treatments. Asterisk indicates significant difference from NT ***$p<0.001$ and FGF-2, NRG treated co-cultures.

Figure 3

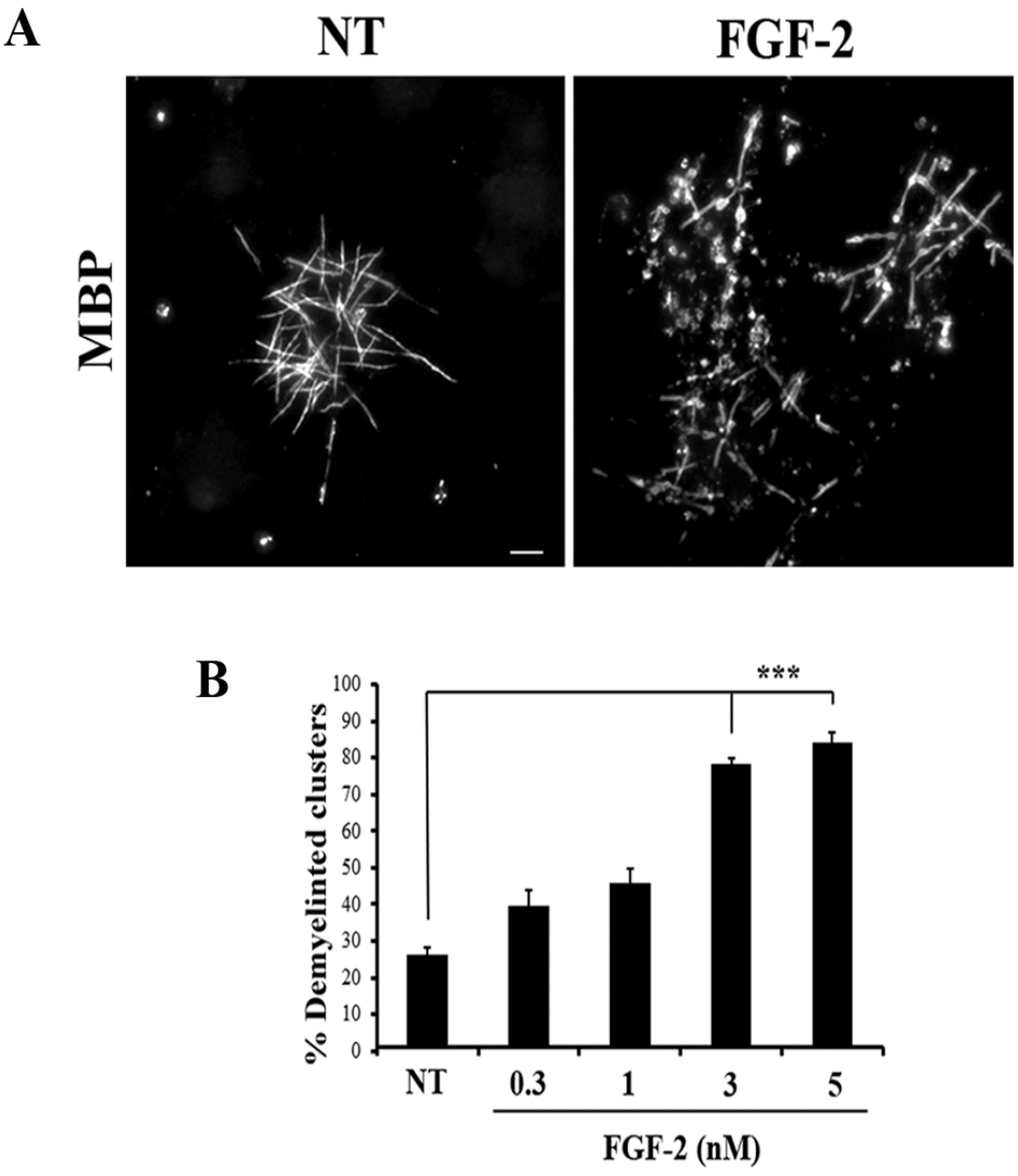

A
NT
FGF-2
MBP
B
***
% Demyelinted clusters
100
90
80
70
60
50
40
30
20
10
0
NT
0.3
1
3
5
FGF-2 (nM)

Figure 3. FGF-2 induced demyelination in a dose dependent manner

Myelinated DRG-OPC co-cultures were treated with various concentrations (0.3nM, 1nM, 3nM, 5nM) of FGF-2. Forty-two hours later, demyelination was assessed by immunostaining for MBP. (A) Images of the demyelinated clusters in FGF-2 treated cultures. Scale bar: 20μm. (B) The levels of demyelination, determined as the percentage of MBP-positive myelin clusters showing signs of demyelination. FGF-2 induced demyelination in a dose dependent manner. The data represented here is from three independent experiments (3 coverslips/ experiment). One-way ANOVA followed by Tukey's post hoc analysis was done to determine the statistical difference between the treatments. Asterisk indicates significant difference from NT ***$p<0.001$ and co-cultures treated with FGF-2 at concentrations of 3nM and 5nM.

Figure 4

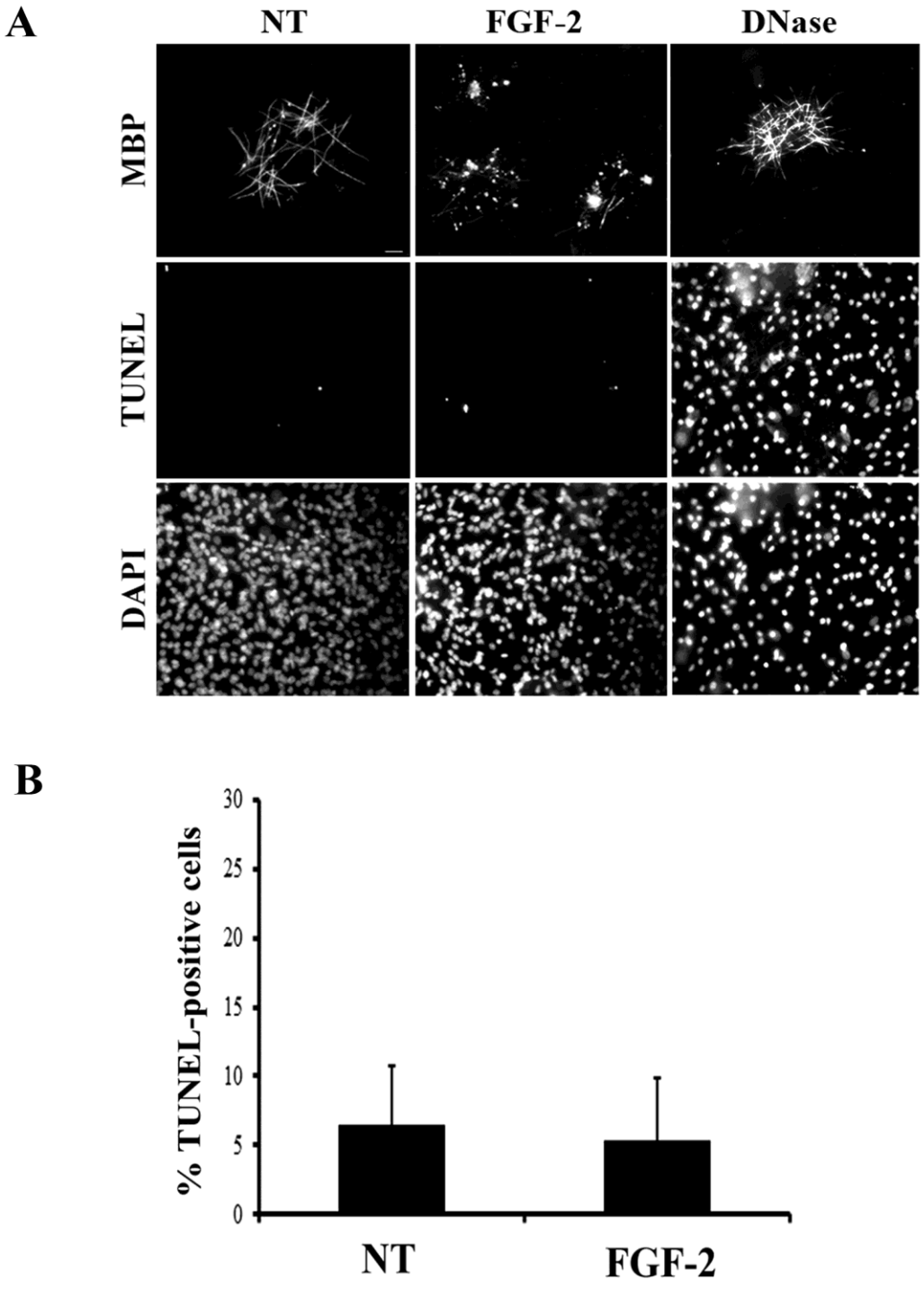

A
NT
FGF-2
DNase
MBP
TUNEL
DAPI
B
% TUNEL-positive cells
30
25
20
15
10
5
0
NT
FGF-2

Figure 4. FGF-2 induced demyelination is not due to cell death

(A) Myelinated DRG-OPC co-cultures were treated with FGF-2 (3.3nM) for 42 hours, and then immunolabel1ed for MBP, followed by TUNEL assay. Scale bar: 20µm. (B) There was no significance increase in cell death following FGF-2 treatment. The data represented here is from three independent experiments (3 coverslips/ experiment).

Figure 5

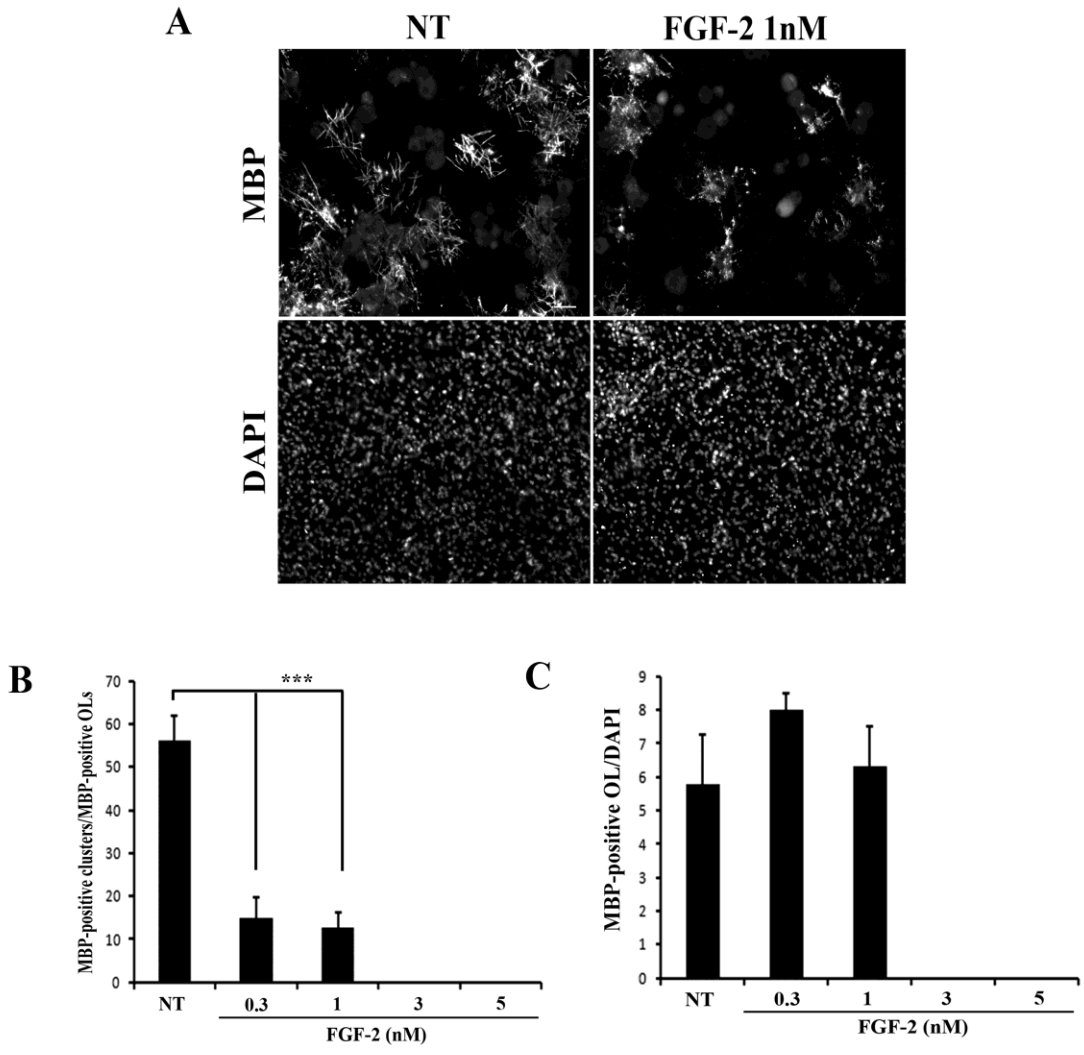

A
NT
FGF-2 1nM
MBP
DAPI
B
MBP-positive clusters/MBP-positive OLs
***
NT
0.3
1
3
5
FGF-2 (nM)
C
MBP-positive OL/DAPI
NT
0.3
1
3
5
FGF-2 (nM)

Figure 5. FGF-2 at low doses inhibits oligodendrocyte myelination

Treatment with increasing doses (0.3nM, 1nM, 3nM and 5nM) of FGF-2 was added to the co-cultures, seven days after plating the OPCs to ensure that differentiation was not affected by FGF-2 treatment. Co-cultures were supplemented with FGF-2 treatments every 48 hours. After 18 days, myelination was assessed by immunostaining for MBP. (A) Images of co-cultures with reduced number of myelin clusters in 1nM FGF-2 treated conditions. Scale bar: 50 μm. (B) Quantification of the results represented as percentage of myelin clusters/MBP-positive oligodendrocytes. Percentage of myelin clusters was reduced in 0.3nM and 1nM FGF-2 treated conditions. (C) Quantification represented as MBP-positive oligodendrocytes/DAPI. Percentage of MBP-positive oligodendrocytes in 0.3nM and 1nM FGF-2 treated conditions was not significantly different from control conditions.

Figure 6

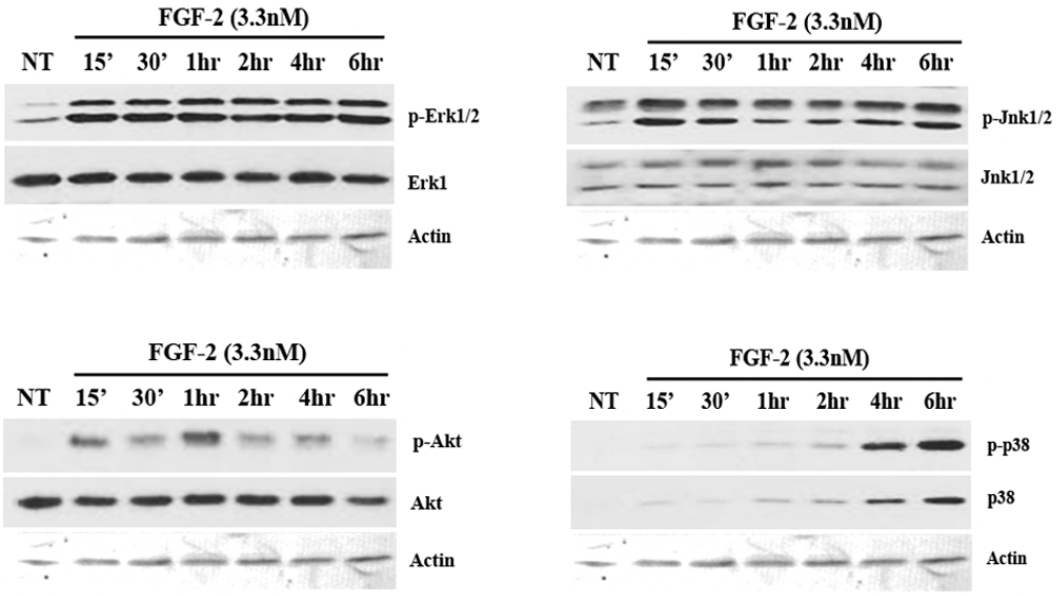

FGF-2 (3.3nM)
NT
15'
30'
1hr
2hr
4hr
6hr
p-Erk1/2
Erk1
Actin
FGF-2 (3.3nM)
NT
15'
30'
1hr
2hr
4hr
6hr
p-Jnk1/2
Jnk1/2
Actin
FGF-2 (3.3nM)
NT
15'
30'
1hr
2hr
4hr
6hr
p-Akt
Akt
Actin
FGF-2 (3.3nM)
NT
15'
30'
1hr
2hr
4hr
6hr
p-p38
p38
Actin

Figure 6. Activation of downstream molecules after FGF-2 treatment on myelinated DRG-OPC co-cultures.

Myelinated co-cultures were treated with FGF-2 (3.3nM) for indicated time points (15', 30', 1hr, 2hr, 4hr, and 6hr). Lysates were collected and analyzed by Western blot for active Erk1/2, JNK1/2, Akt and p38 MAPK. Activation kinetics for Erk1/2 and JNK1/2 pathways were similar, both persisting for 6 hours. Activation of Akt peaked at 1 hour and diminished slowly. p38 MAPK activation was not observed at earlier time points, but was activated at later time points (4 hr and 6 hr) after FGF-2 treatment.

Figure 7

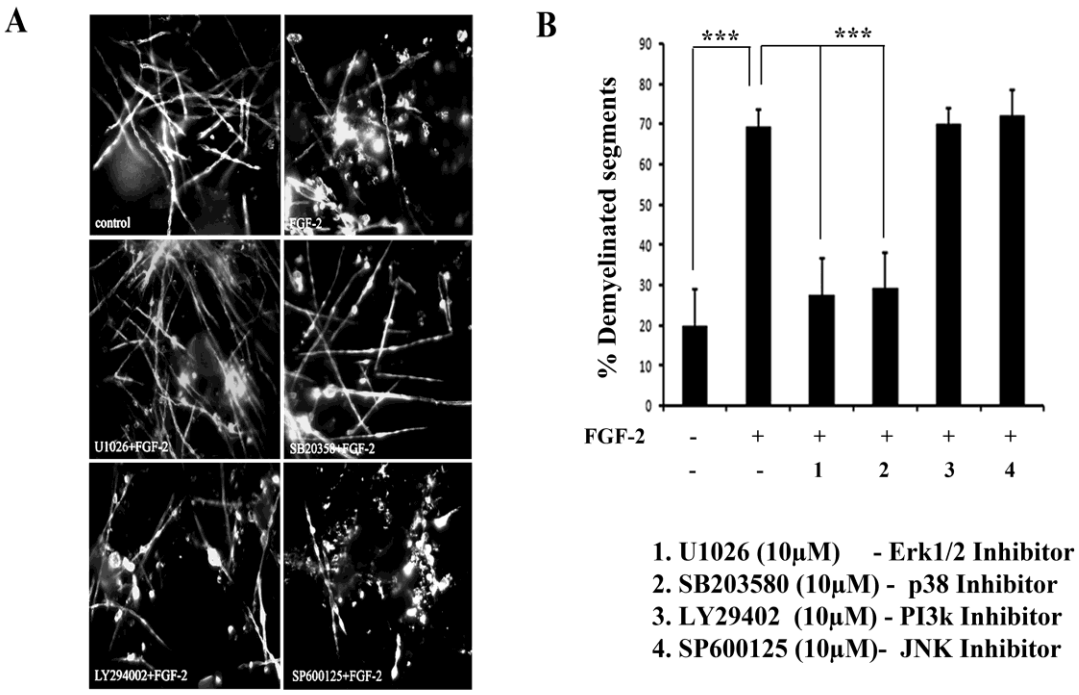

A
control
FGF-2
U1026+FGF-2
SB203580+FGF-2
LY294002+FGF-2
SP600125+FGF-2
B
% Demyelinated segments
***
***
0
10
20
30
40
50
60
70
80
90
FGF-2 - + + + + +
- - 1 2 3 4
1. U1026 (10µM) - Erk1/2 Inhibitor
2. SB203580 (10µM) - p38 Inhibitor
3. LY29402 (10µM) - PI3k Inhibitor
4. SP600125 (10µM)- JNK Inhibitor

Figure 7. Inhibition of Erk1/2 or p38 MAPK activity blocks FGF-2 induced demyelination in DRG-OPC myelinated co-cultures.

Myelinated DRG-OPC co-cultures were pretreated with U1026 (10μM), SB203580 (10μM), LY29402 (10μM), SP600125 (10μM) for 1 hour, and then FGF-2 (3.3nM) was added. Forty-two hours later, demyelination was assessed by immunostaining for MBP. (A) Images of myelin clusters with reduced demyelinating symptoms were observed in U1026+FGF-2 and SB203580+FGF-2 treated co-cultures, whereas extensive demyelination was observed in FGF-2 treated conditions and in cultures treated with LY29402, SP600125. (B) Quantification of the results represented as percentage of MBP-positive myelinated segments showing signs of demyelination. Treatment with U1026 or SB203580 independently blocks FGF-2 induced demyelination in myelinated co-cultures.

Figure 8

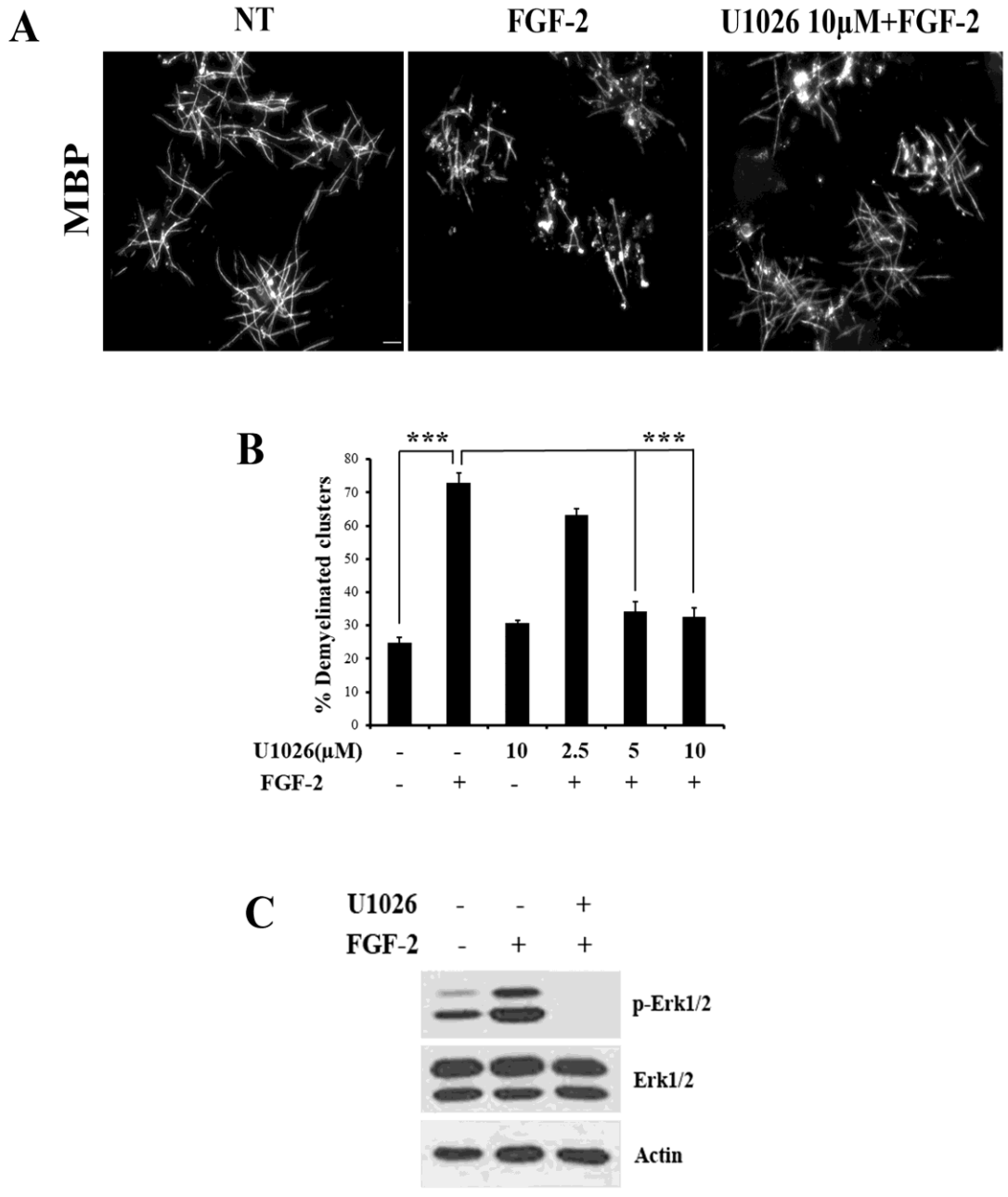

A
NT
FGF-2
U1026 10μM+FGF-2
MBP
B
***
***
% Demyelinated clusters
0
10
20
30
40
50
60
70
80
U1026(μM) - - 10 2.5 5 10
FGF-2 - + - + + +
C
U1026 - - +
FGF-2 - + +
p-Erk1/2
Erk1/2
Actin

Figure 8. Inhibition of Erk1/2 activity blocks FGF-2 induced demyelination in DRG-OPC myelinated co-cultures

Myelinated DRG-OPC co-cultures were treated with U1026 at different doses (2.5μM, 5μM and 10μM) along with FGF-2 (3.3nM). Forty-two hours later, demyelination was assessed by immunostaining for MBP. (A) Images of MBP-positive myelin clusters treated with U1026 (10μM) in presence or absence of FGF-2. Intact myelin clusters with reduced demyelinating symptoms were observed in U1026+FGF-2 treated co-cultures, whereas extensive demyelination was observed in FGF-2 treated conditions. Scale bar: 20 μm. (B) Quantification of the results represented as percentage of MBP-positive myelinated clusters showing signs of demyelination. Treatment with U1026 blocks FGF-2 induced demyelination in a dose-dependent manner. The data represented here is from three independent experiments (3 coverslips/ experiment). One-way ANOVA followed by Tukey's post hoc analysis was done to determine the statistical difference between the treatments. Asterisk indicates significant difference from NT ***$p<0.001$ and FGF-2 treated co-cultures, and also significant difference between FGF-2 ***$p<0.001$ and co-cultures treated with 5μM U1026+FGF-2 and 10μM U1026+FGF-2. (C) Erk1/2 activity was analyzed in U1026 treated co-cultures using Western blot analysis. U1026 (10μM) blocks Erk1/2 activity in DRG-OPC myelinated co-cultures.

Figure 9

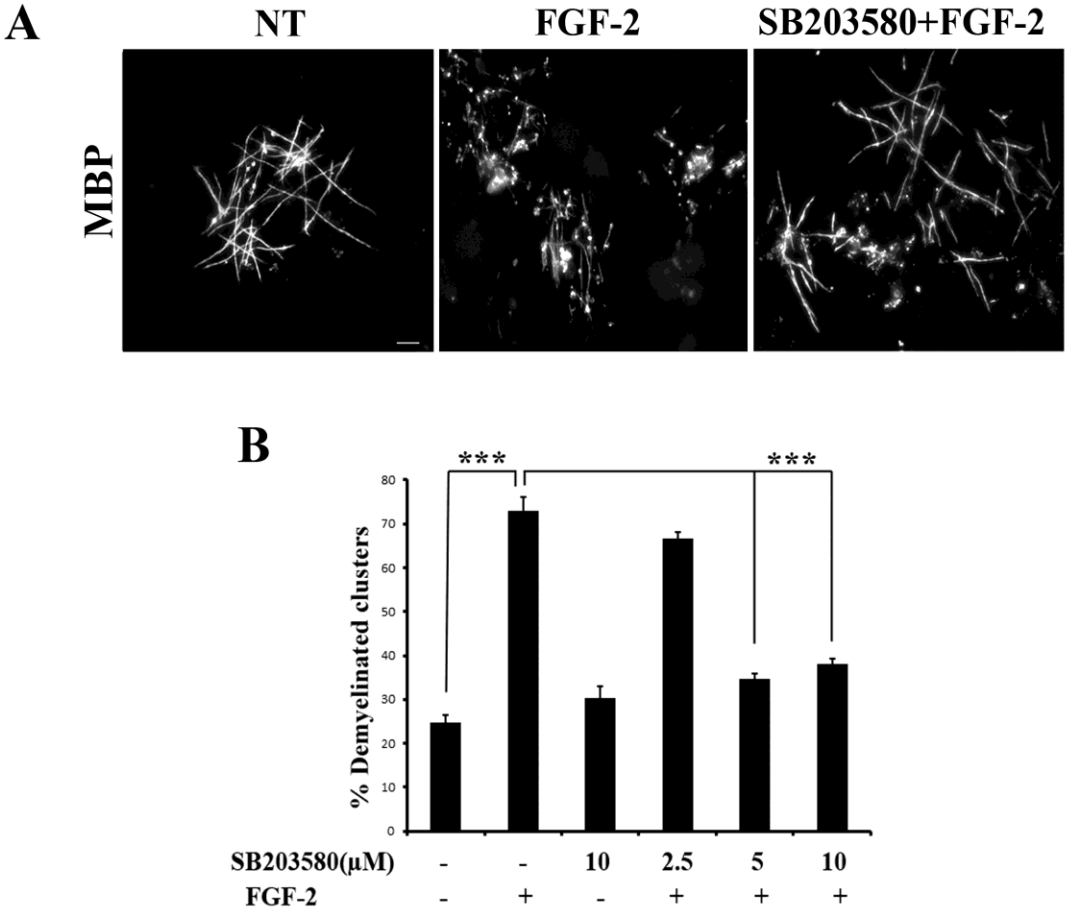

A
NT
FGF-2
SB203580+FGF-2
MBP
B
***
***
% Demyelinated clusters
0
10
20
30
40
50
60
70
80
SB203580(μM) - - 10 2.5 5 10
FGF-2 - + - + + +

Figure 9. Inhibition of p38MAPK blocks FGF-2 induced demyelination in DRG-OPC myelinated co-cultures.

Myelinated DRG-OPC co-cultures were treated with SB203580 at different doses (2.5μM, 5μM and 10μM) in the presence of FGF-2 (3.3nM). Forty-two hours later, demyelination was assessed by immunostaining for MBP. Treatment with SB203580 also decreased FGF-2 induced demyelination in a dose-dependent manner. ***$p<0.001$. (A) Images of MBP-positive myelin clusters treated with FGF-2 with or without SB203580 (10μM). Demyelinated symptoms were reduced in co-cultures treated with SB203580 (10μM) compared to FGF-2 treated co-cultures. Scale bar: 20μm. (B) Quantification of the results represented as percentage of MBP-positive myelin clusters showing signs of demyelination. Percentage of demyelinated clusters was reduced in co-cultures treated with 5μM SB203580+FGF-2 and 10μM SB203580+FGF-2 compared to FGF-2 treated co-cultures. The data represented here is from three independent experiments (3 coverslips/ experiment). ***$p<0.001$. Scale bar: 20μm.

Figure 10

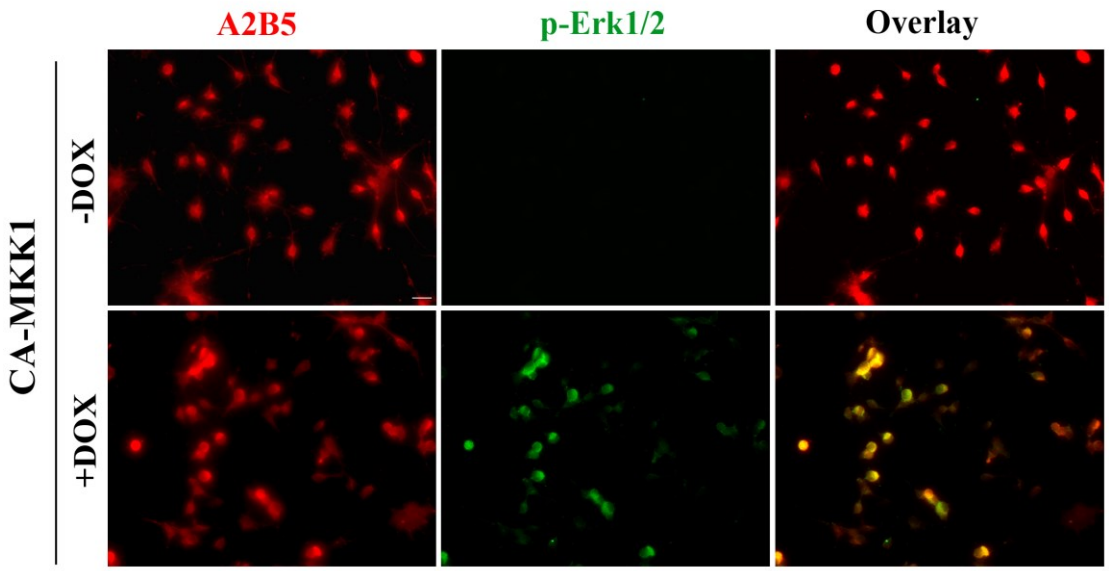


Figure 10. Ectopic activation of Erk1/2 in OPCs using inducible lentivirus system.

OPCs were infected with lentiviruses encoding CA-MKK1 mutant, and express the transgene upon treatment with doxycycline. Images of CA-MKK1 infected OPCs showing Erk1/2 activation upon doxycycline (DOX) treatment. Scale bar: 20 μm.

Figure 11

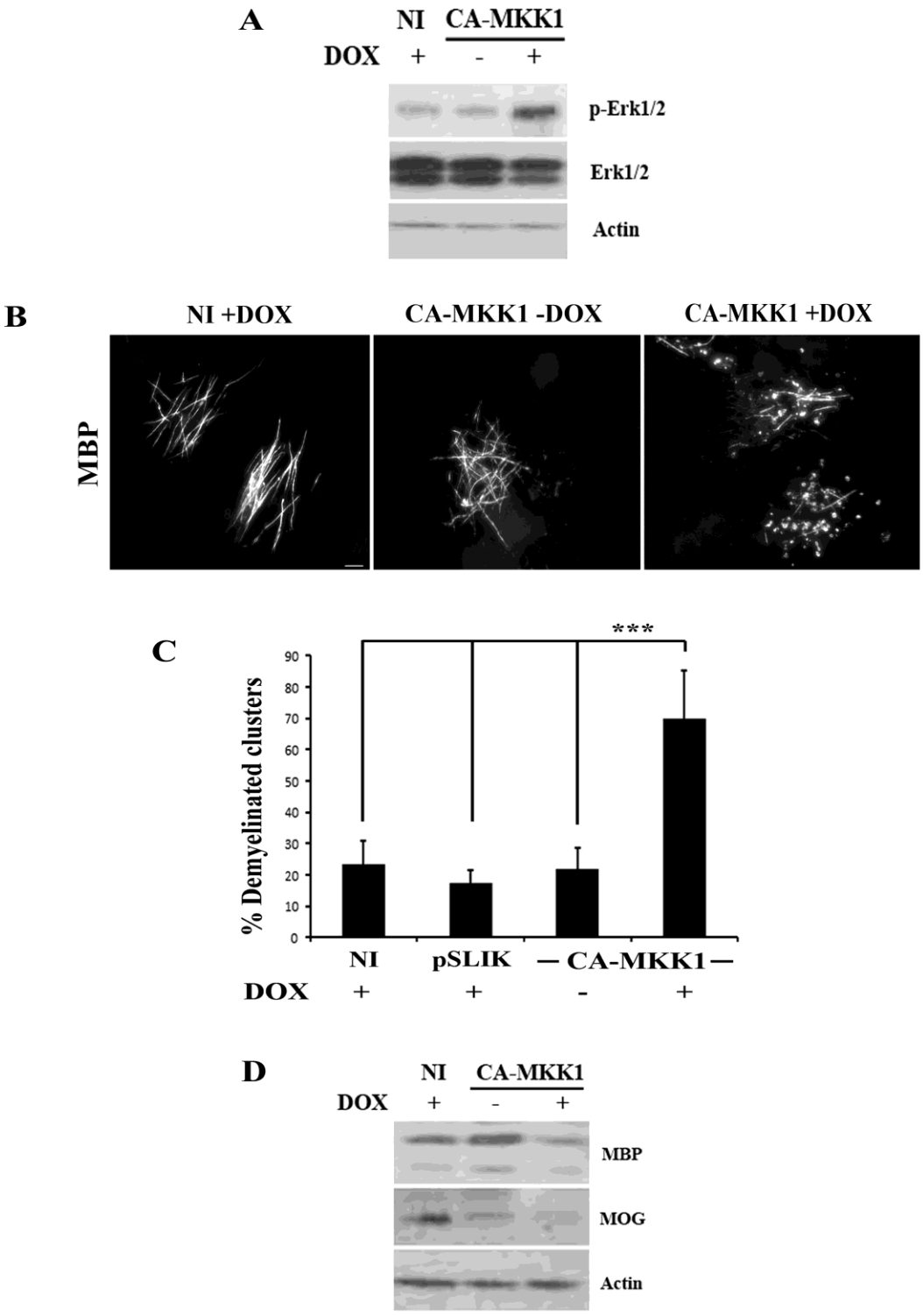

A
NI
CA-MKK1
DOX
+
-
+
p-Erk1/2
Erk1/2
Actin
B
NI +DOX
CA-MKK1 -DOX
CA-MKK1 +DOX
MBP
C
***
% Demyelinated clusters
90
80
70
60
50
40
30
20
10
0
NI
pSLIK
CA-MKK1
DOX
+
+
-
+
D
NI
CA-MKK1
DOX
+
-
+
MBP
MOG
Actin

Figure 11. Activation of MKK1 is sufficient to induce oligodendrocyte demyelination in myelinated DRG-OPC co-cultures.

OPCs infected with lentiviruses encoding CA-MKK1 mutant were co-cultured with DRG neurons for 18 days, followed by the addition of doxycycline. Forty-eight hours later, integrity of the myelin clusters was assessed. (A) Upon doxycycline (DOX) treatment, activation of Erk1/2 was observed in CA-MKK1 co-cultures by using western blot analysis. (B) Images of myelinated co-cultures with or without doxycycline treatment. Scale bar: 20μm. (C) Ectopic activation of Erk1/2 by doxycycline induced CA-MKK1 expression is sufficient to induce oligodendrocyte demyelination in myelinated DRG-OPC co-cultures. Quantification of the result is represented as percentage of demyelinated clusters. The data represented here is from three independent experiments (3 coverslips/ experiment). One-way ANOVA followed by Tukey's post hoc analysis was done to determine the statistical difference between the treatments. ***$p<0.001$. (D) Immunoblot showing that doxycycline treatment reduces MBP and MOG expression.

Figure 12

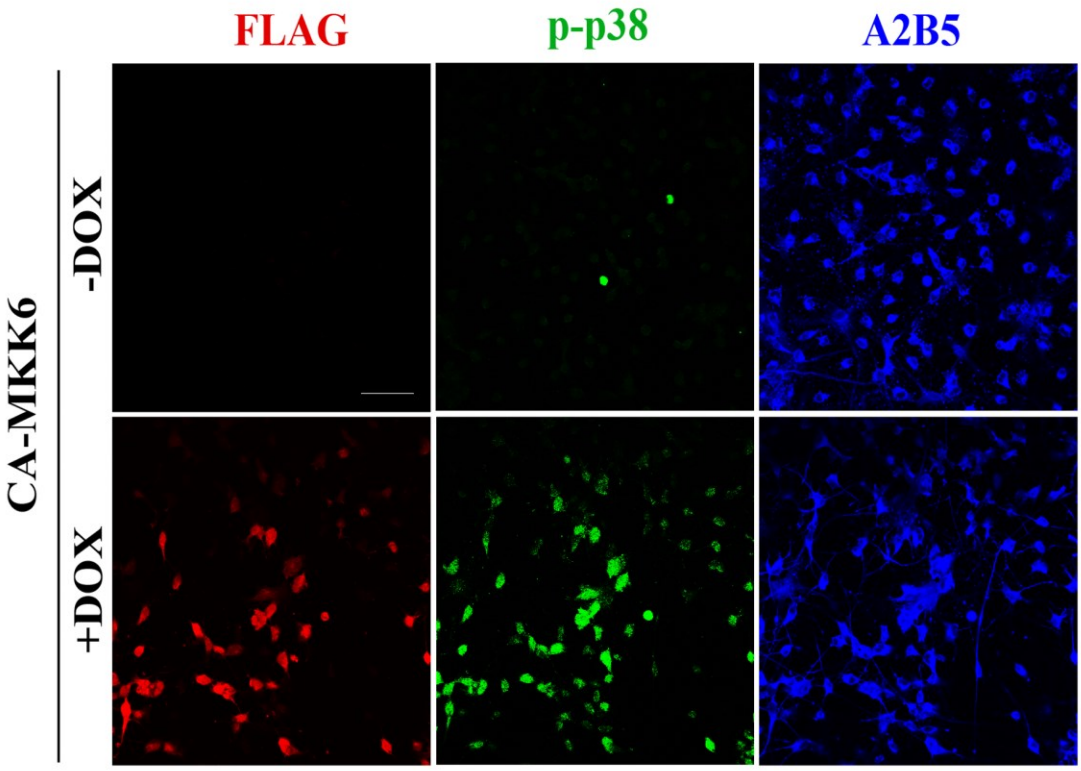
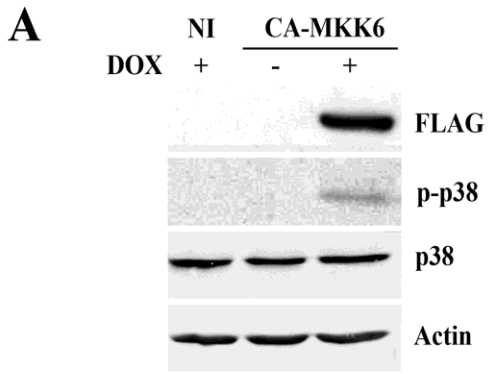
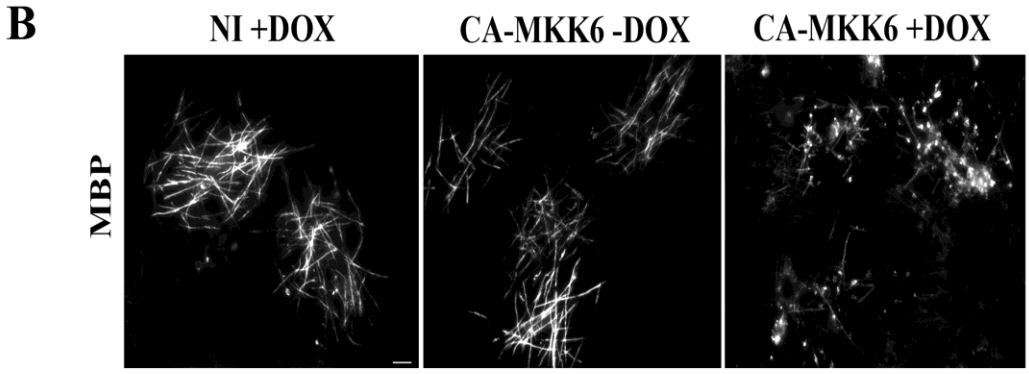


Figure 12. Ectopic activation of p38 MAPK in OPCs using inducible lentivirus system.

OPCs were infected with lentiviruses encoding CA-MKK6 mutant, and underwent doxycycline (DOX) treatment to induce transgene expression. Images of CA-MKK6 infected OPCs showing p38 MAPK activation upon doxycycline treatment. Scale bar:50 μm.

Figure 13


A
NI
CA-MKK6
DOX + - +
FLAG
p-p38
p38
Actin


B
NI +DOX
CA-MKK6 -DOX
CA-MKK6 +DOX
MBP

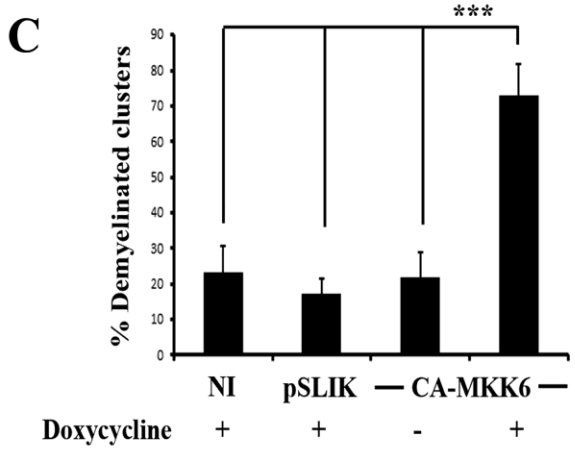

C
***
% Demyelinated clusters
0
10
20
30
40
50
60
70
80
90
NI
pSLIK
CA-MKK6
Doxycycline + + - +

Figure 13. Activation of MKK6 is sufficient to induce oligodendrocyte demyelination in myelinated DRG-OPC co-cultures.

OPCs infected with lentiviruses encoding CA-MKK6 mutant were co-cultured with DRG neurons for 18 days, followed by the addition of doxycycline. Forty-eight hours later, demyelination was assessed. (A) Treatment with doxycycline (DOX) induced CA-MKK6 (FLAG+) expression and was sufficient to activate p38 MAPK in infected co-cultures. (B) Images of the CA-MKK6 myelinated co-cultures treated with and without doxycycline. Demyelinated clusters were observed in doxycycline treated CA-MKK6 infected co-cultures. Scale bar: 20μm. (C) Ectopic activation of p38 MAPK by doxycycline induced CA-MKK1 expression is sufficient to induce oligodendrocyte demyelination in myelinated DRG-OPC co-cultures. Quantification of the result is represented as percentage of MBP-positive clusters showing signs of demyelination. The data represented here is from three independent experiments (3 coverslips/ experiment). One-way ANOVA followed by Tukey's post hoc analysis was done to determine the statistical difference between the treatments. ***$p<0.001$.

Figure 14

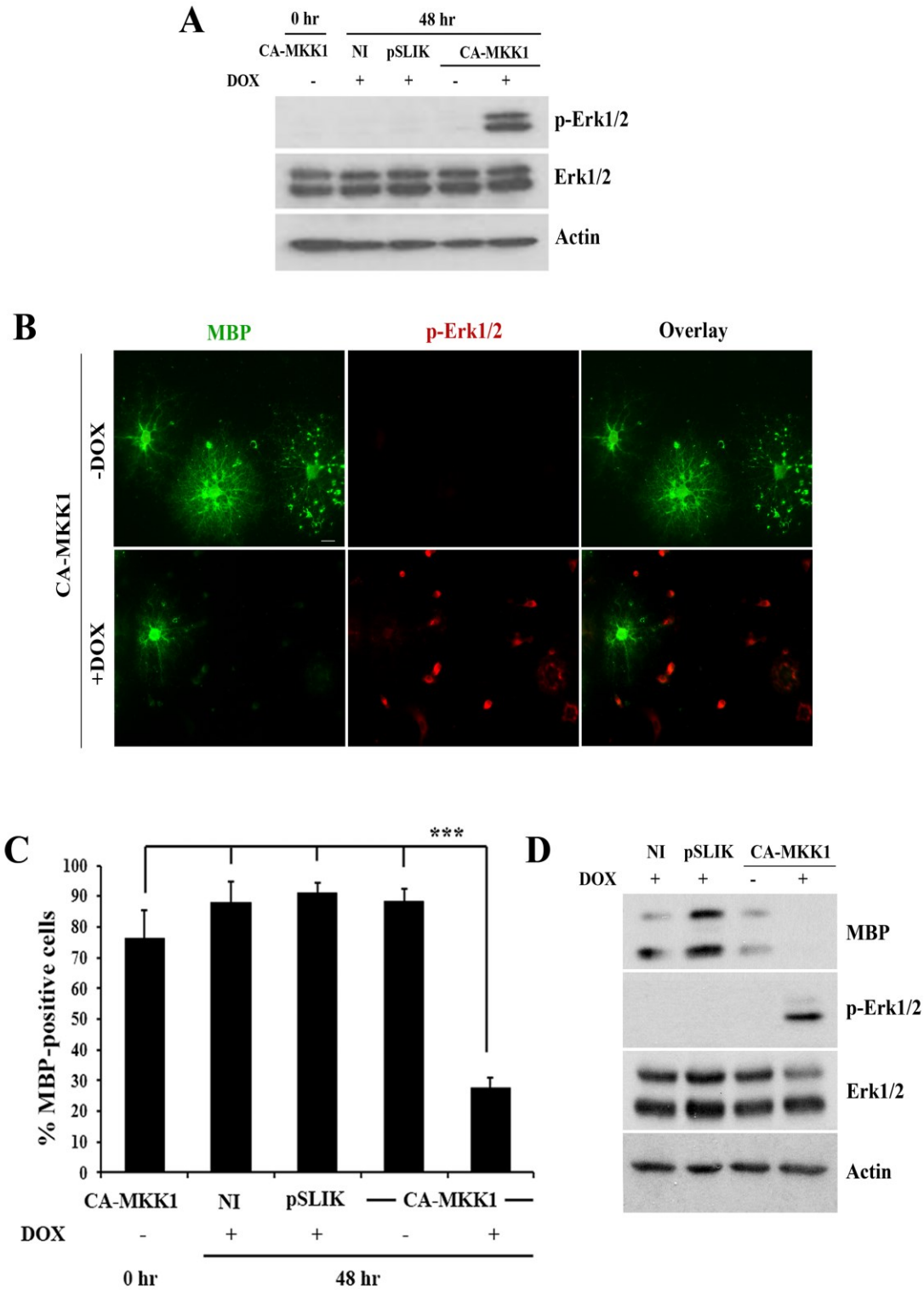

A
0 hr
48 hr
CA-MKK1
NI
pSLIK
CA-MKK1
DOX
- + + - +
p-Erk1/2
Erk1/2
Actin
B
MBP
p-Erk1/2
Overlay
CA-MKK1
-DOX
+DOX
C
***
% MBP-positive cells
100
90
80
70
60
50
40
30
20
10
0
CA-MKK1
NI
pSLIK
CA-MKK1
DOX
- + + - +
0 hr
48 hr
D
NI
pSLIK
CA-MKK1
DOX
+ + - +
MBP
p-Erk1/2
Erk1/2
Actin

Figure 14. Ectopic activation of Erk1/2 in mature oligodendrocytes down regulates MBP expression.

OPCs infected with lentiviruses encoding CA-MKK1 mutant were differentiated into mature oligodendrocytes in presence of T3 for 5-6 days, followed by the addition of doxycycline (DOX) for 48 hours. (A) Activation of Erk1/2 was detected only in doxycycline-treated CA-MKK1 infected oligodendrocytes, using Western blot analysis. (B) Images of CA-MKK1infected oligodendrocytes showing loss of MBP expression upon Erk1/2 activation, after 48 hours of doxycycline treatment. Scale bar: 20 μm. (C) Quantification of the results represented as percentage of MBP-positive cells. A drastic reduction in percentage of MBP-positive cells is observed in doxycycline-treated CA-MKK1 infected oligodendrocytes compared to non-infected (NI) doxycycline-treated or pSLIK alone infected doxycycline-treated or CA-MKK1 infected without doxycycline or CA-MKK1infected before doxycycline treatment (0 hr) oligodendrocytes. (D) Immunoblot showing reduction of MBP expression upon ectopic activation of Erk1/2 in mature oligodendrocytes.

Figure 15

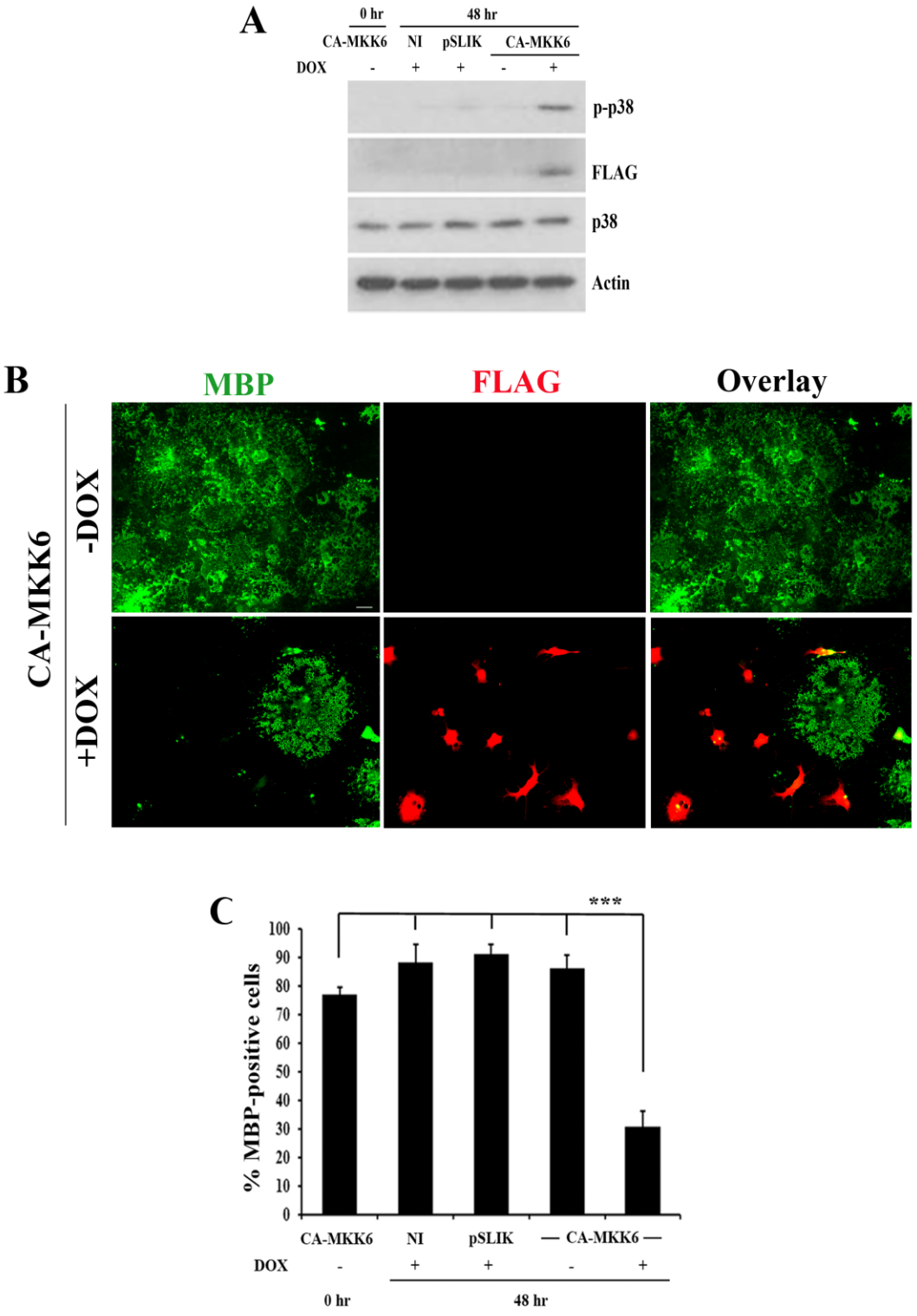
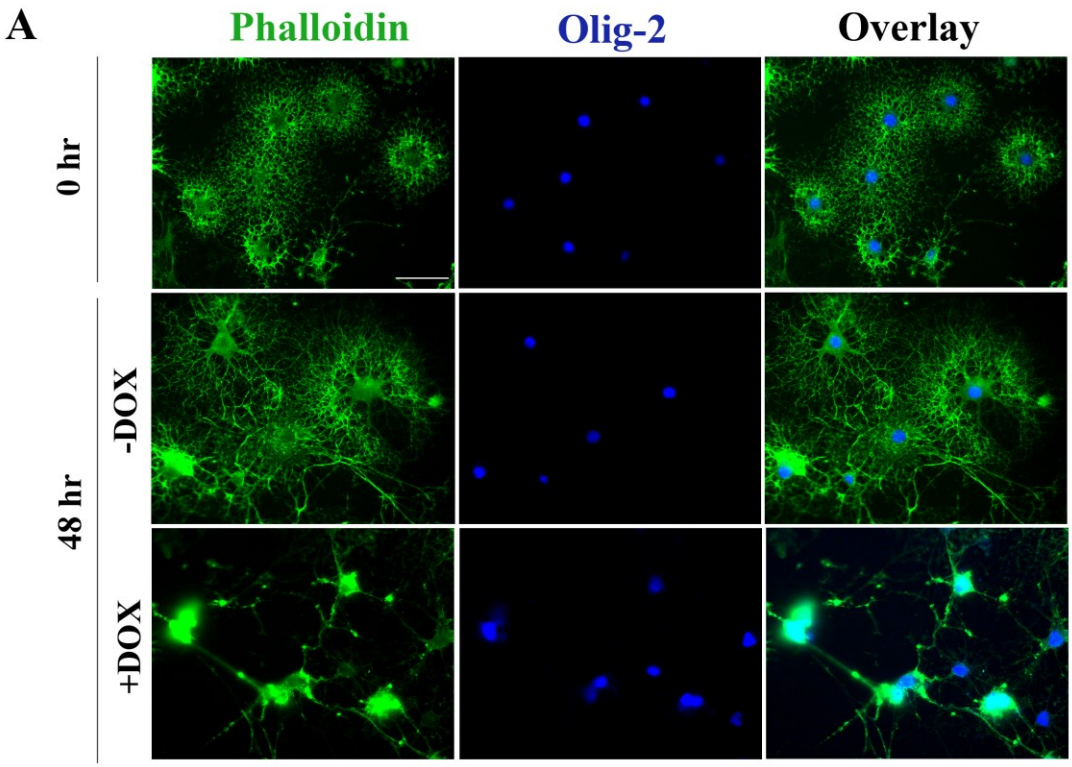

A
0 hr
48 hr
CA-MKK6
NI
pSLIK
CA-MKK6
DOX
- + + - +
p-p38
FLAG
p38
Actin
B
MBP
FLAG
Overlay
CA-MKK6
-DOX
+DOX
C
***
% MBP-positive cells
100
90
80
70
60
50
40
30
20
10
0
CA-MKK6
NI
pSLIK
CA-MKK6
DOX
- + + - +
0 hr
48 hr

Figure 15. Ectopic activation of p38 MAPK in mature oligodendrocytes down regulates MBP expression.

OPCs infected with lentiviruses encoding CA-MKK6 mutant were differentiated into mature oligodendrocytes in presence of T3 for 5-6 days, followed by the addition of doxycycline (DOX) for 48 hours. (A) Upon doxycycline treatment, CA-MKK-6 (FLAG) expression was sufficient to activate p38 MAPK in infected oligodendrocytes. (B) After 48 hours of doxycycline treatment, loss of MBP expression was observed in FLAG-positive oligodendrocytes. Scale bar: 20 μm. (C) Quantification of the results shown as percentage of MBP-positive oligodendrocytes. The percentage of MBP-positive cells was reduced in doxycycline-treated CA-MKK6 infected cells compared non-infected doxycycline-treated or pSLIK alone infected doxycycline-treated or CA-MKK6 infected without doxycycline or CA-MKK6 infected before doxycycline treatment (0 hr) oligodendrocytes.

Figure 16


A
Phalloidin
Olig-2
Overlay
0 hr
48 hr
-DOX
+DOX

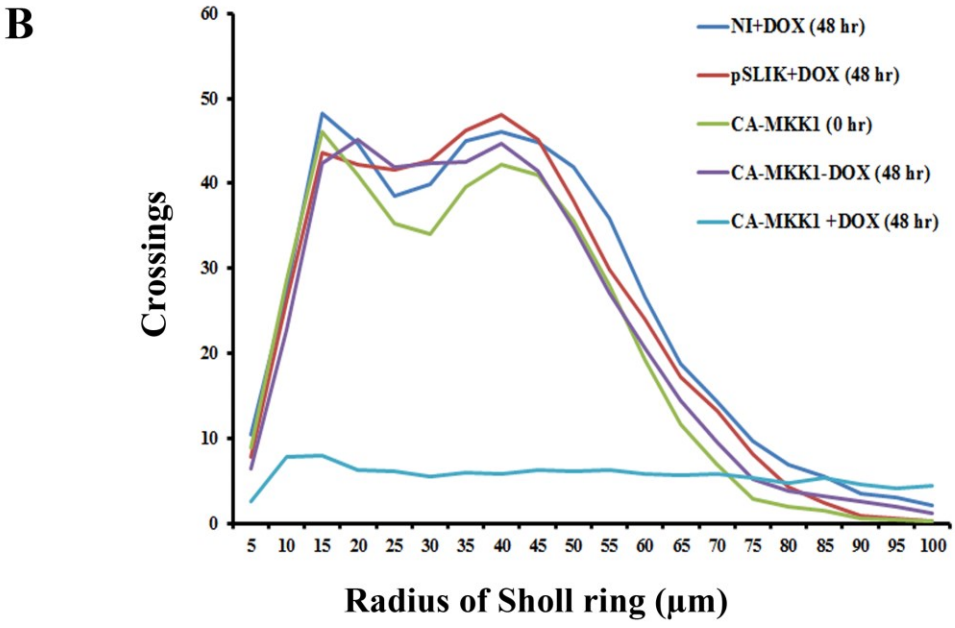

B
NI+DOX (48 hr)
pSLIK+DOX (48 hr)
CA-MKK1 (0 hr)
CA-MKK1-DOX (48 hr)
CA-MKK1 +DOX (48 hr)
Crossings
Radius of Sholl ring (µm)
0
10
20
30
40
50
60
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100

Figure 16. Ectopic activation of Erk1/2 in mature oligodendrocytes changed oligodendrocytes morphology.

OPCs infected with lentiviruses encoding CA-MKK1 mutant were differentiated into mature oligodendrocytes in presence of T3 for 5-6 days, followed by the addition of doxycycline for 48 hours. (A) Representative images of CA-MKK1 expressing mature oligodendrocytes with or without doxycycline for 48 hours. Morphological changes were observed in doxycycline-treated CA-MKK1 infected oligodendrocytes compared to CA-MKK1 infected oligodendrocytes without doxycycline. Scale bar: 10μm. (B) Morphological changes in CA-MKK1 oligodendrocytes in the presence or absence of doxycycline are represented quantitatively by plotting the average number of intersections per Sholl ring against the radius of the ring (Sholl profile). Sholl profiles are generated using Image J software analysis.

Figure 17

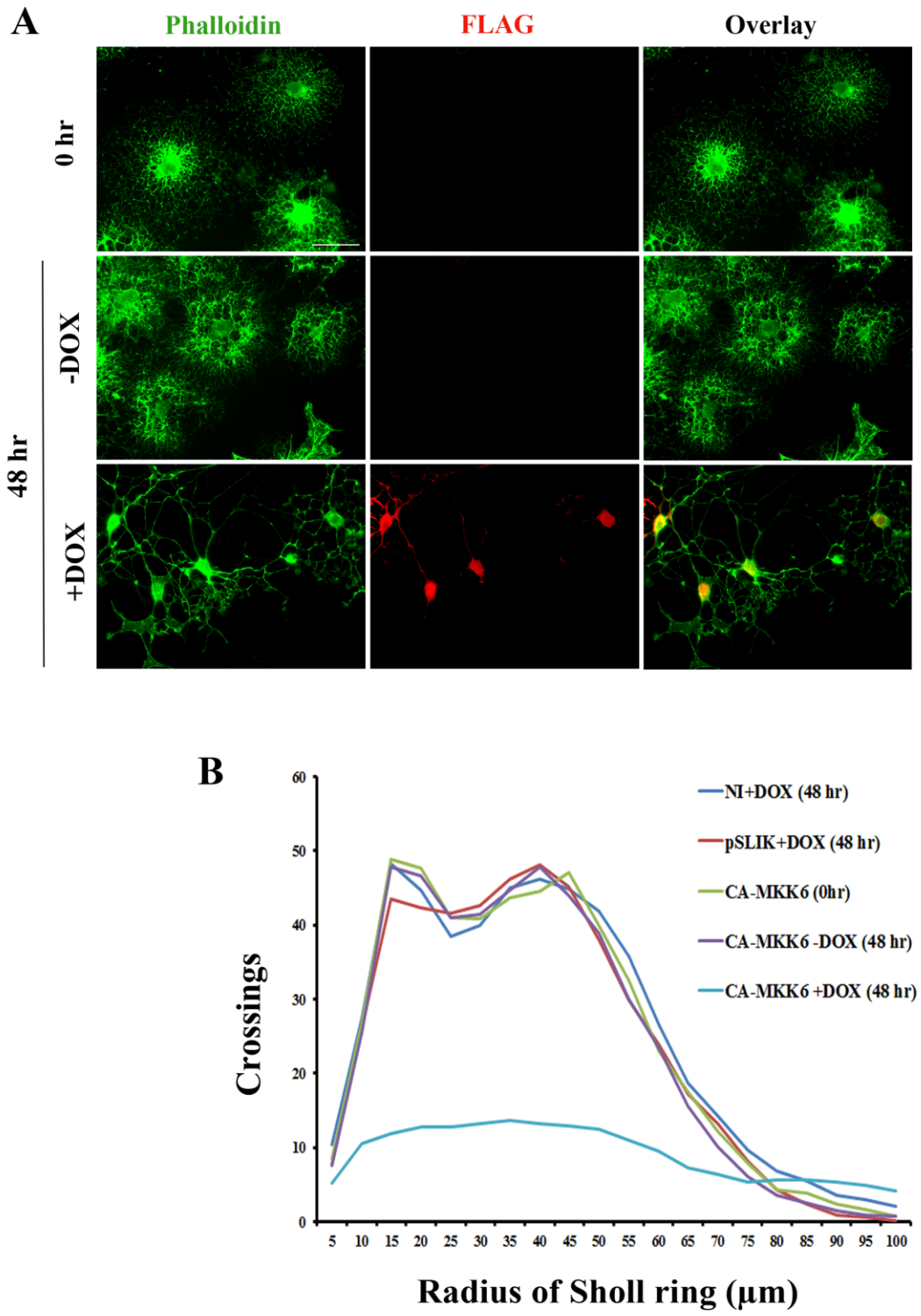

A
Phalloidin
FLAG
Overlay
0 hr
48 hr
-DOX
+DOX
B
NI+DOX (48 hr)
pSLIK+DOX (48 hr)
CA-MKK6 (0hr)
CA-MKK6 -DOX (48 hr)
CA-MKK6 +DOX (48 hr)
Crossings
0
10
20
30
40
50
60
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100
Radius of Sholl ring (µm)

Figure 17. Ectopic activation of p38 MAPK in mature oligodendrocytes changed oligodendrocytes morphology.

OPCs infected with lentiviruses encoding CA-MKK6 mutant were differentiated into mature oligodendrocytes in presence of T3 for 5-6 days, followed by the addition of doxycycline (DOX) for 48 hours. (A) Representative images of CA-MKK6 infected mature oligodendrocytes with or without doxycycline for 48 hours. Changes in morphology were observed in doxycycline-treated CA-MKK6 expressing (FLAG-positive) mature oligodendrocytes compared to CA-MKK6 infected oligodendrocytes without doxycycline. Scale bar: 10μm. (B) Morphological changes in CA-MKK6 infected oligodendrocytes were represented quantitatively by plotting the average number of intersections for each Sholl ring against its radius measurement (Sholl profile). Sholl profiles are generated using Image J software analysis.

Figure 18

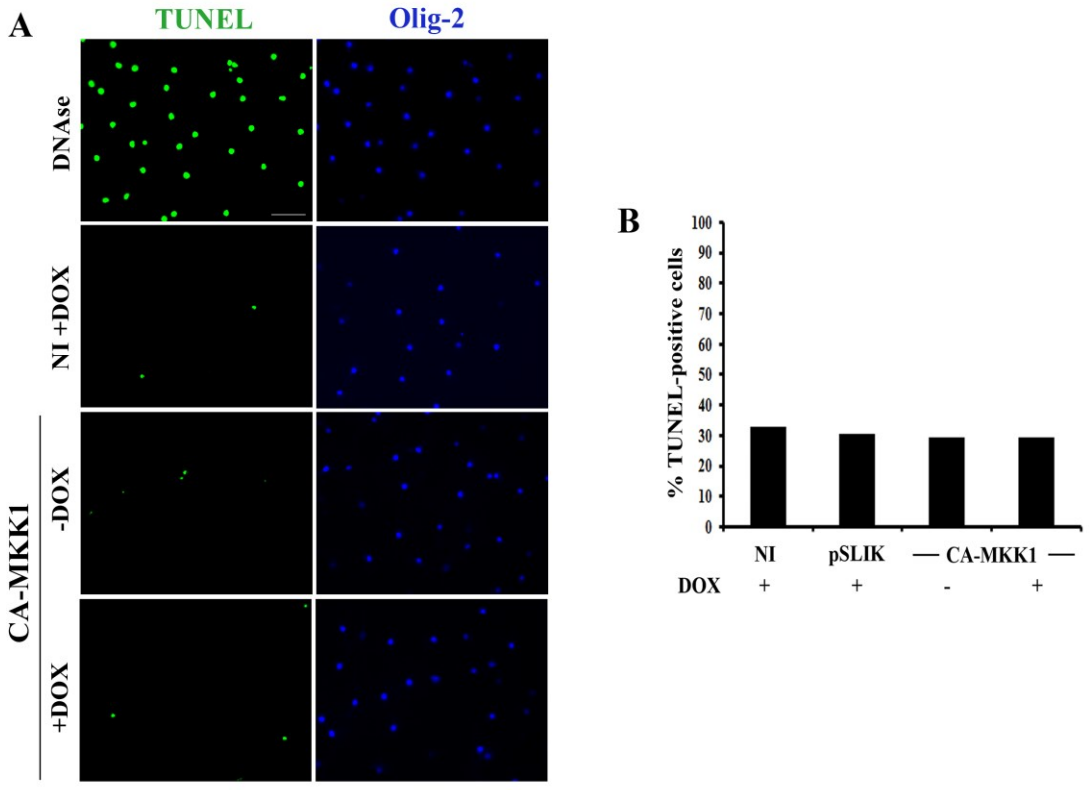

A
TUNEL
Olig-2
DNAse
NI +DOX
CA-MKK1
-DOX
+DOX
B
% TUNEL-positive cells
100
90
80
70
60
50
40
30
20
10
0
NI
pSLIK
CA-MKK1
DOX
+
+
-
+

Figure 18. Ectopic activation of CA-MKK1 in mature oligodendrocytes did not induce cell death by 48 hours.

OPCs infected with lentiviruses encoding CA-MKK1 mutant were differentiated into mature oligodendrocytes in presence of T3 for 5-6 days, followed by the addition of doxycycline (DOX) for 48 hours. (A) TUNEL assay of non-infected (NI) cells with doxycycline treatment and CA-MKK1 infected cells with or without doxycycline. Scale bar: 50μm. (B) Quantification of the results re represented as percentage of TUNEL-positive cells. There was no significant difference among the treatments.

Figure 19

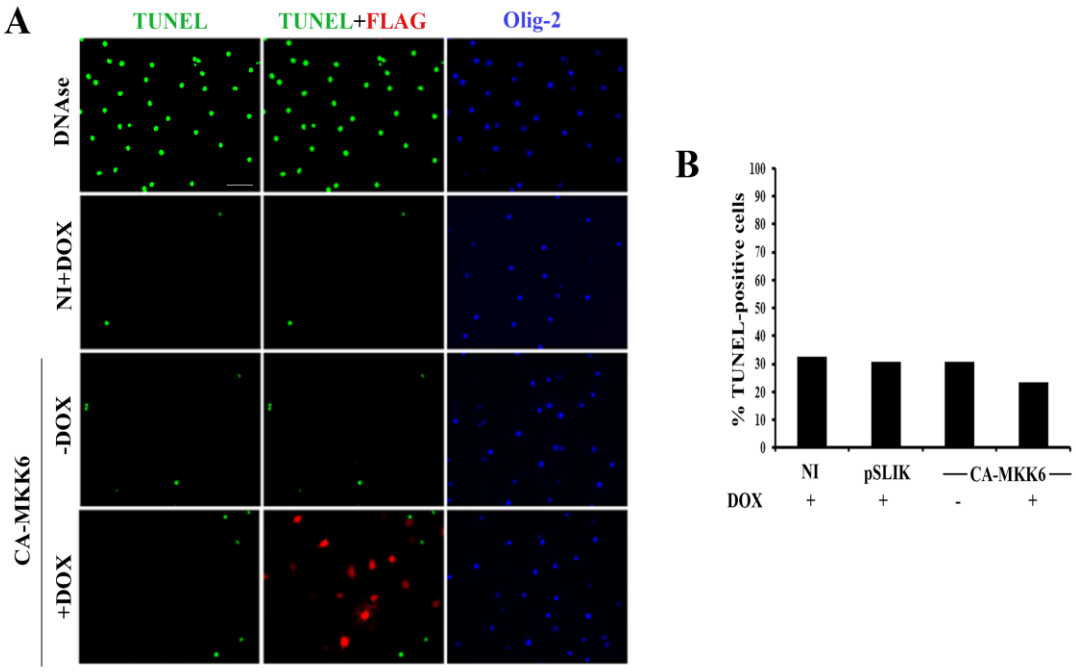

A
TUNEL
TUNEL+FLAG
Olig-2
DNAse
NI+DOX
CA-MKK6
-DOX
+DOX
B
% TUNEL-positive cells
100
90
80
70
60
50
40
30
20
10
0
NI
pSLIK
CA-MKK6
DOX
+
+
-
+

Figure 19. Ectopic activation of CA-MKK6 in mature oligodendrocytes did not induce cell death by 48 hours.

OPCs infected with lentiviruses encoding CA-MKK6 mutant were differentiated into mature oligodendrocytes in presence of T3 for 5-6 days, followed by the addition of doxycycline for 48 hours. (A) TUNEL assay of non-infected (NI) cells with doxycycline treatment and CA-MKK6 infected cells with or without doxycycline. Scale bar: 50μm. (B) Quantification of the results represented as percentage of TUNEL-positive cells. There was no significant difference among the treatments.

Figure 20

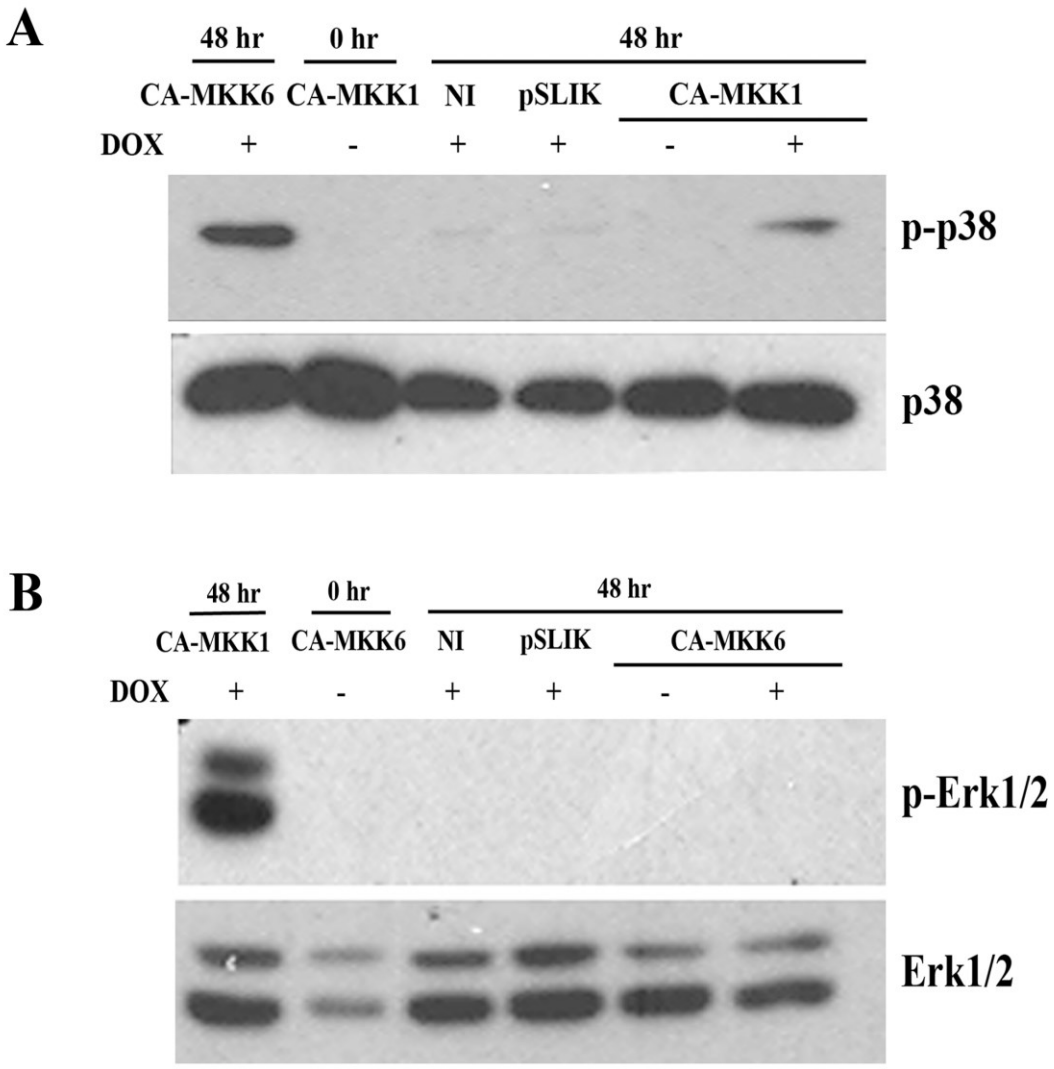
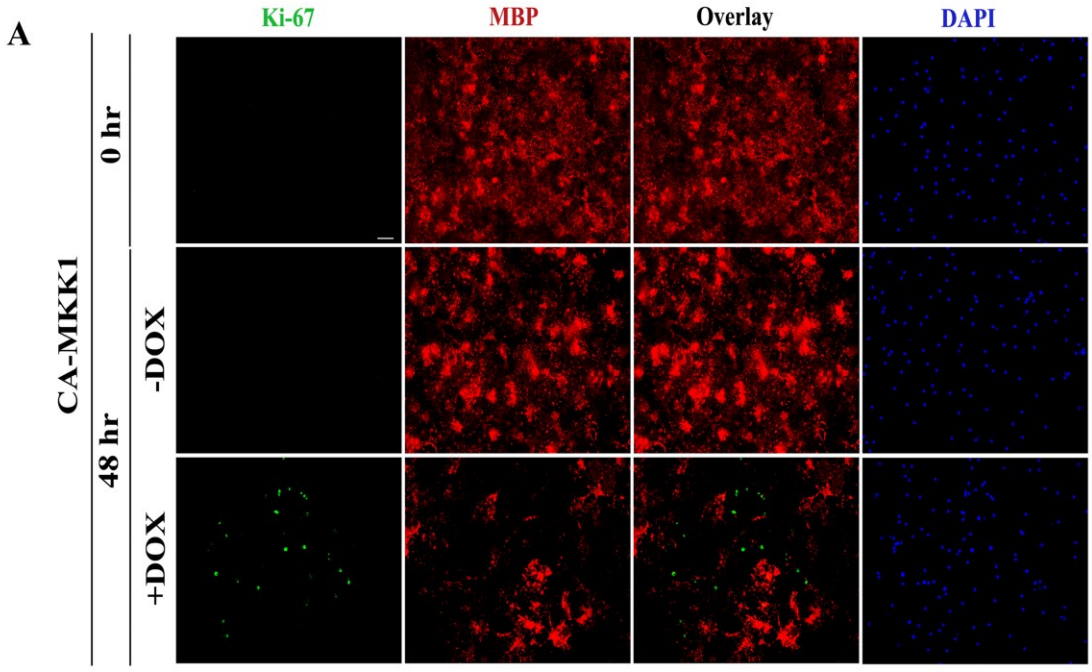

A
48 hr
0 hr
48 hr
CA-MKK6
CA-MKK1
NI
pSLIK
CA-MKK1
DOX
+
-
+
+
-
+
p-p38
p38
B
48 hr
0 hr
48 hr
CA-MKK1
CA-MKK6
NI
pSLIK
CA-MKK6
DOX
+
-
+
+
-
+
p-Erk1/2
Erk1/2

Figure 20. p38 MAPK is activated downstream of Erk1/2 or MKK1

Differentiated mature oligodendrocytes expressing CA-MKK1 or CA-MKK6 mutant were treated with doxycycline (DOX). Forty-eight hours after doxycycline treatment, lysates were collected and Erk1/2 or p38 MAPK activation was assessed by Western blot analysis. Lysates collected from non-infected (NI) oligodendrocytes, oligodendrocytes expressing vector alone (pSLIK) in presence of doxycycline, and oligodendrocytes expressing CA-MKK1 (0hr) at the time of doxycycline treatment served as controls (A) Upon doxycycline treatment, p38 MAPK activation was observed in doxycycline-treated CA-MKK1 expressing oligodendrocytes, and also in doxycycline-treated CA-MKK6 expressing oligodendrocytes. . (B) Erk1/2 activation was observed in doxycycline-treated CA-MKK1expressing oligodendrocytes but not in doxycycline-treated CA-MKK6 expressing oligodendrocytes.

Figure 21


A
Ki-67
MBP
Overlay
DAPI
CA-MKK1
0 hr
48 hr
-DOX
+DOX

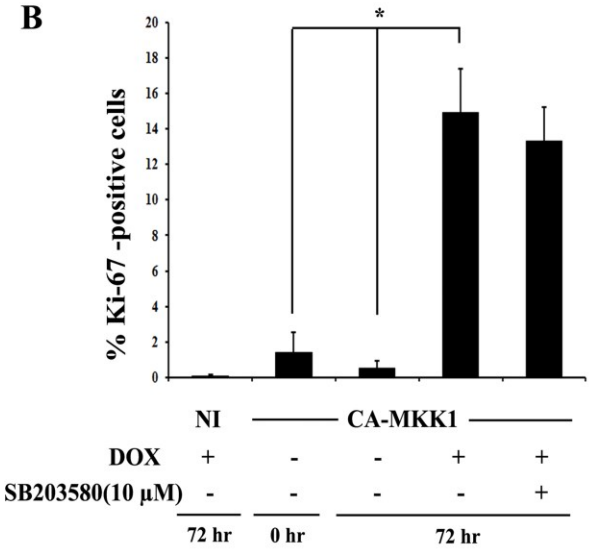

B
% Ki-67 -positive cells
*
NI
CA-MKK1
DOX + - - + +
SB203580(10 µM) - - - - +
72 hr
0 hr
72 hr

Figure 21. Ectopic activation of MKK1in mature oligodendrocytes is sufficient to induce cell cycle re-entry.

OPCs infected with lentiviruses encoding CA-MKK1 mutant were differentiated into mature oligodendrocytes in presence of T3 for 7 days; AraC was added in the last 48 hours. Cultures were then treated with or without doxycycline (DOX) for 72 hours, and immunostained for Ki-67 and MBP. (A) Images of CA-MKK1 infected cells prior to doxycycline-treatment and seventy-two hours after with or without doxycycline. Doxycycline treatment induced the expression of Ki-67 in CA-MKK1 oligodendrocytes. Notice the reduced MBP expression by mature oligodendrocytes, upon doxycycline treatment. Scale bar: 50μm. (B) Quantification of the results represented as percentage of Ki-67-positive cells. An increase in Ki-67-positve cells was observed in doxycycline-treated CA-MKK1 oligodendrocytes compared to CA-MKK1 oligodendrocytes prior to doxycycline treatment or without doxycycline treatment. Doxycycline-induced Ki-67 expression was not reduced upon treatment with SB203580.

Figure 22

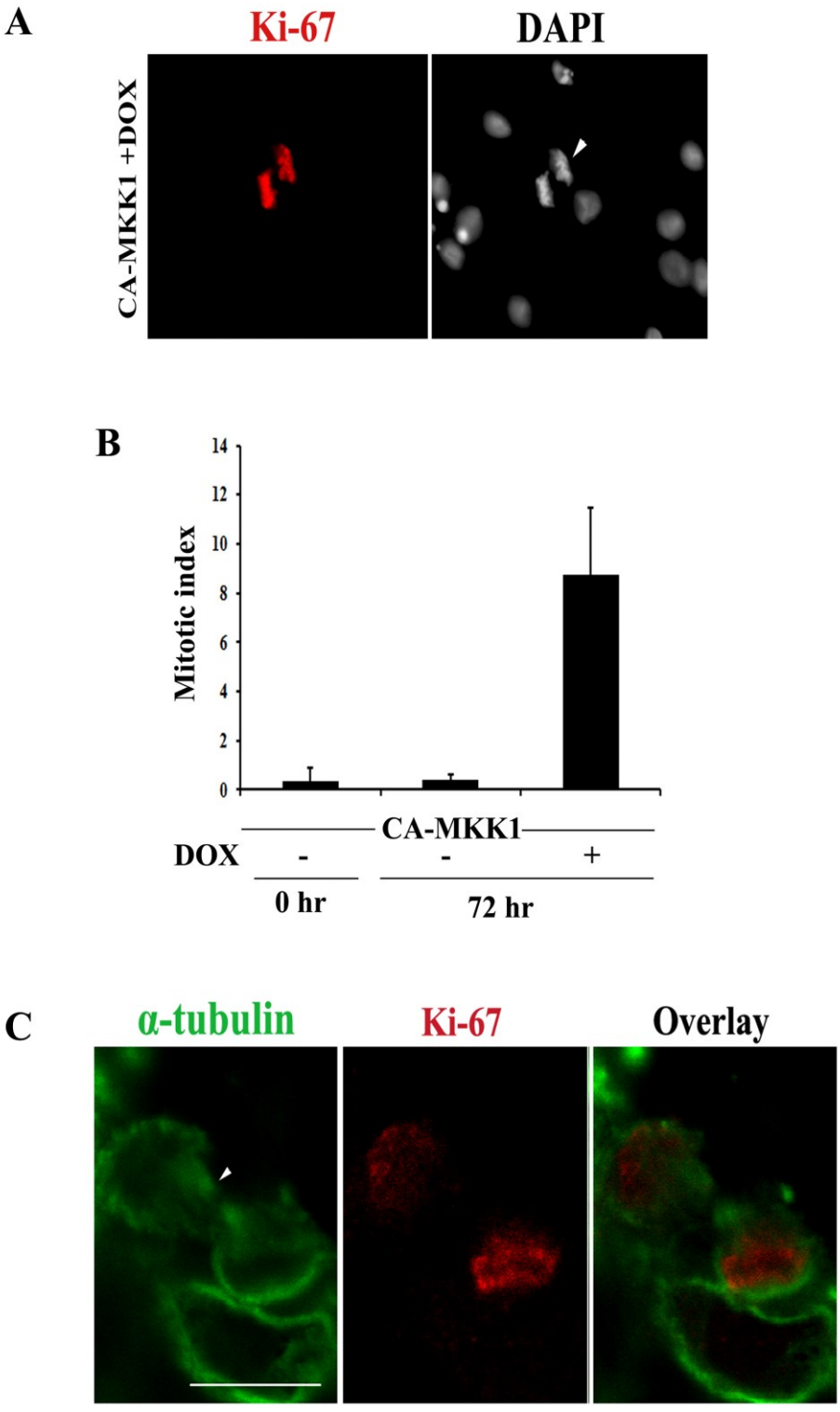

A
Ki-67
DAPI
CA-MKK1 +DOX
B
Mitotic index
14
12
10
8
6
4
2
0
CA-MKK1
DOX
-
-
+
0 hr
72 hr
C
α-tubulin
Ki-67
Overlay

Figure 22. Activation of MKK1 is sufficient to induce mature oligodendrocytes to reenter the cell cycle and complete mitosis.

(A) Images of doxycycline-treated CA-MKK1 oligodendrocytes showing the signs of mitosis. Notice the arrow head pointing to a DAPI-positive cell in M-phase. (B) Quantification of the mitotic index in CA-MKK1 infected cultures before and after doxycycline (DOX) treatment. Mitotic index is increased in CA-MKK1 cultures treated with doxycycline. (C) Images of the Ki-67-positive cells in CA-MKK1 cultures showing the signs of cytokinesis. Notice the arrow head pointing to the localized presence of α-tubulin at the center of the dividing cells. Scale bar: 10μm.

Figure 23

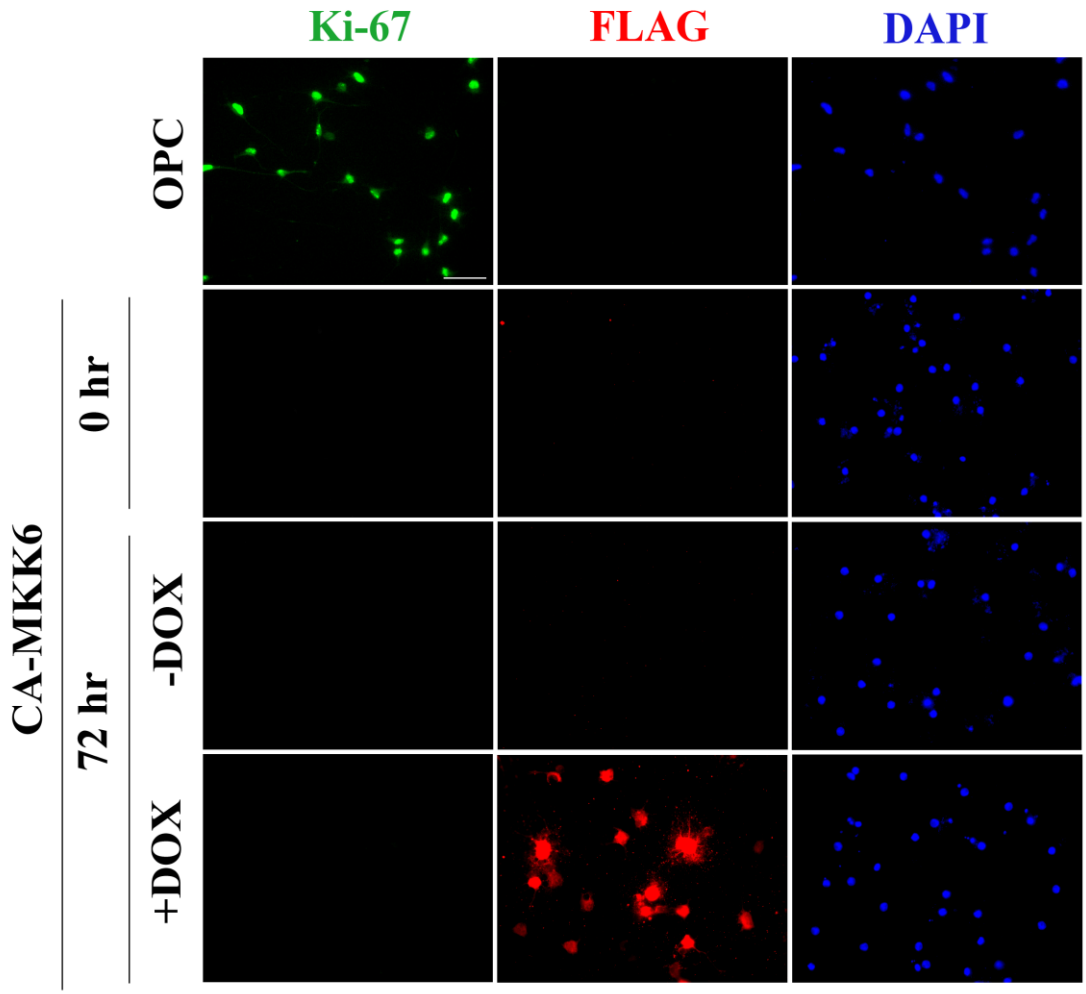


Figure 23. Ectopic activation of MKK6 in mature oligodendrocytes is not sufficient to induce cell cycle re-entry.

OPCs infected with lentiviruses encoding CA-MKK6 mutant were differentiated into mature oligodendrocytes in presence of T3 for 7 days; AraC was added in the last 48 hours. Cultures were then treated with or without doxycycline (DOX) for 72 hours, and immunostained for Ki-67 and FLAG. Scale bar: 50μm. (A) Images of CA-MKK6 infected cells prior to doxycycline treatment and seventy-two hours after with or without doxycycline, immunostained for Ki-67. MKK6 expressing mature oligodendrocytes (FLAG-positive) fail to re-enter the cell cycle.

Figure 24

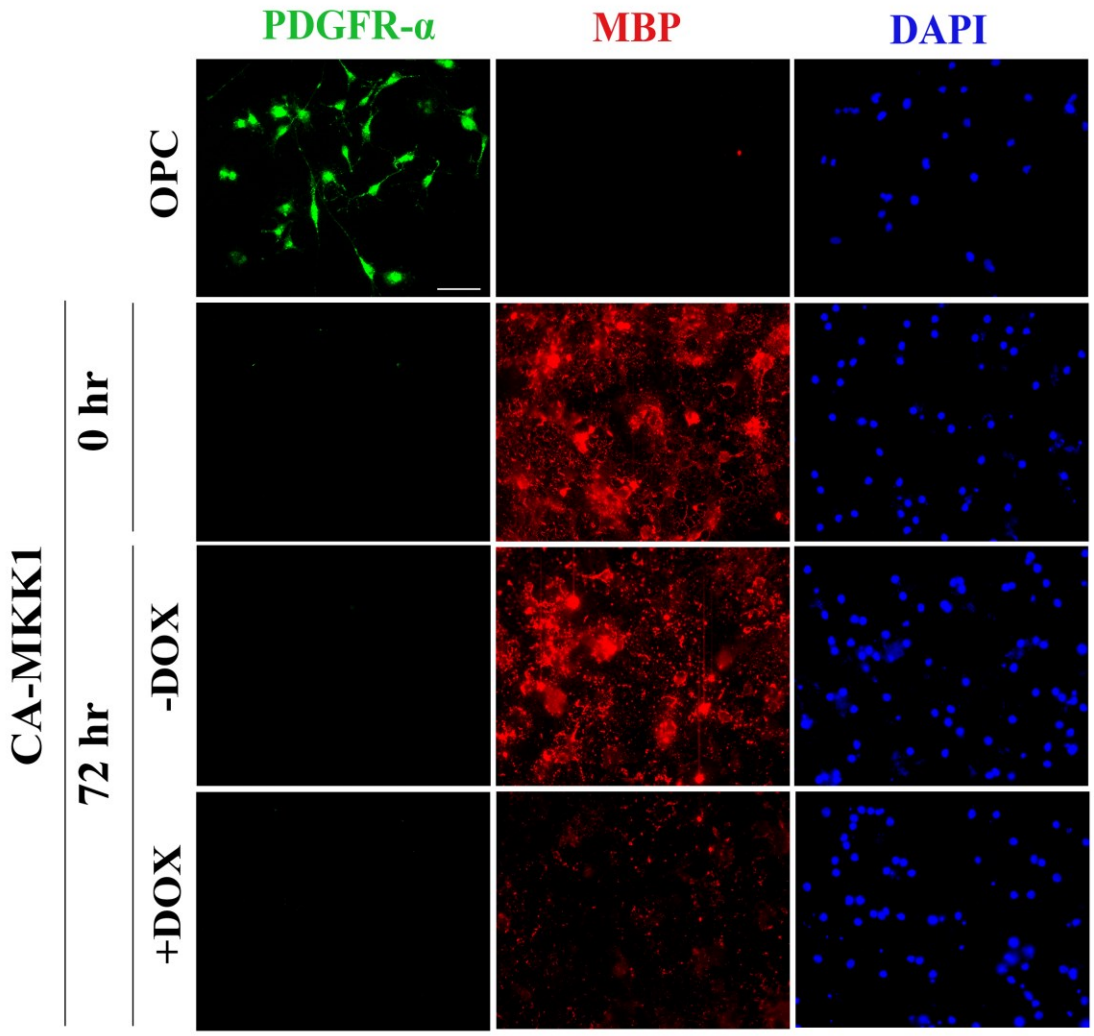


Figure 24. Ectopic activation of MKK1 in mature oligodendrocytes is not sufficient to induce PDGFR-α expression

Differentiated mature oligodendrocytes expressing CA-MKK1 were treated with doxycycline (DOX). Seventy-two hours later, the cells were fixed and immunostained for PDGFR-α. Mature oligodendrocytes did not re-express PDGFR-α after doxycycline treatment, indicating that Erk1/2 is not sufficient to induce de-differentiation of mature oligodendrocytes into PDGFR-α –positive progenitors. Scale bar: 50 μm.

Figure 25

A

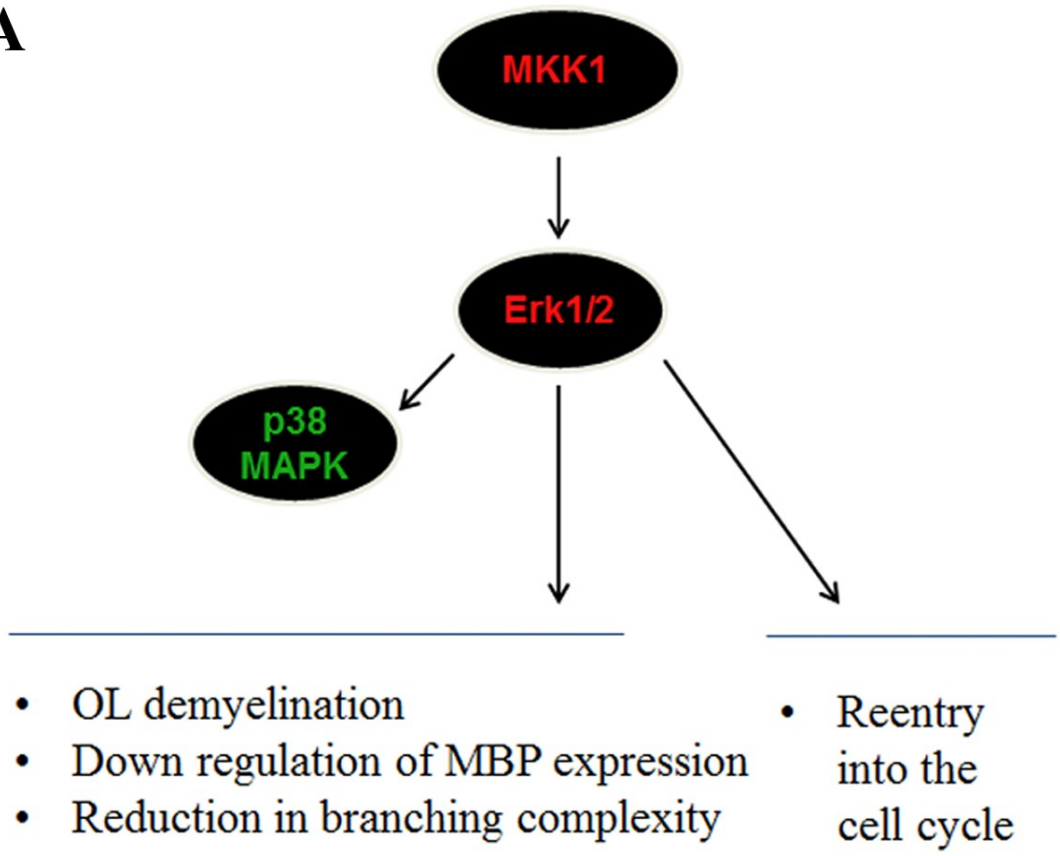
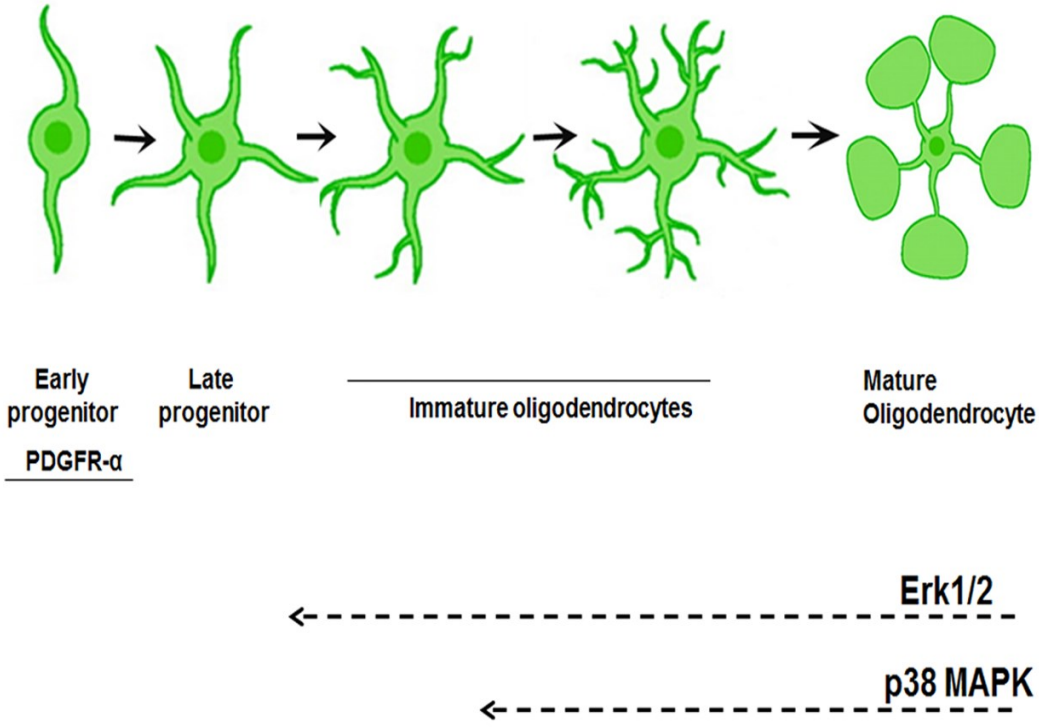

MKK1
Erk1/2
p38
MAPK
• OL demyelination
• Down regulation of MBP expression
• Reduction in branching complexity
• Reentry into the cell cycle

Figure 25. Summary of the responses induced by MAPKs in mature oligodendrocytes

Erk1/2 or p38 MAPK activation in mature oligodendrocytes induces phenotypic changes such as loss of MBP expression and reduces the branching complexity. Erk1/2 activity but not p38 MAPK further leads them to reenter the cell cycle. Erk1/2 activation is upstream of p38 MAPK in inducing these phenotypic changes in mature oligodendrocytes.

Figure 26

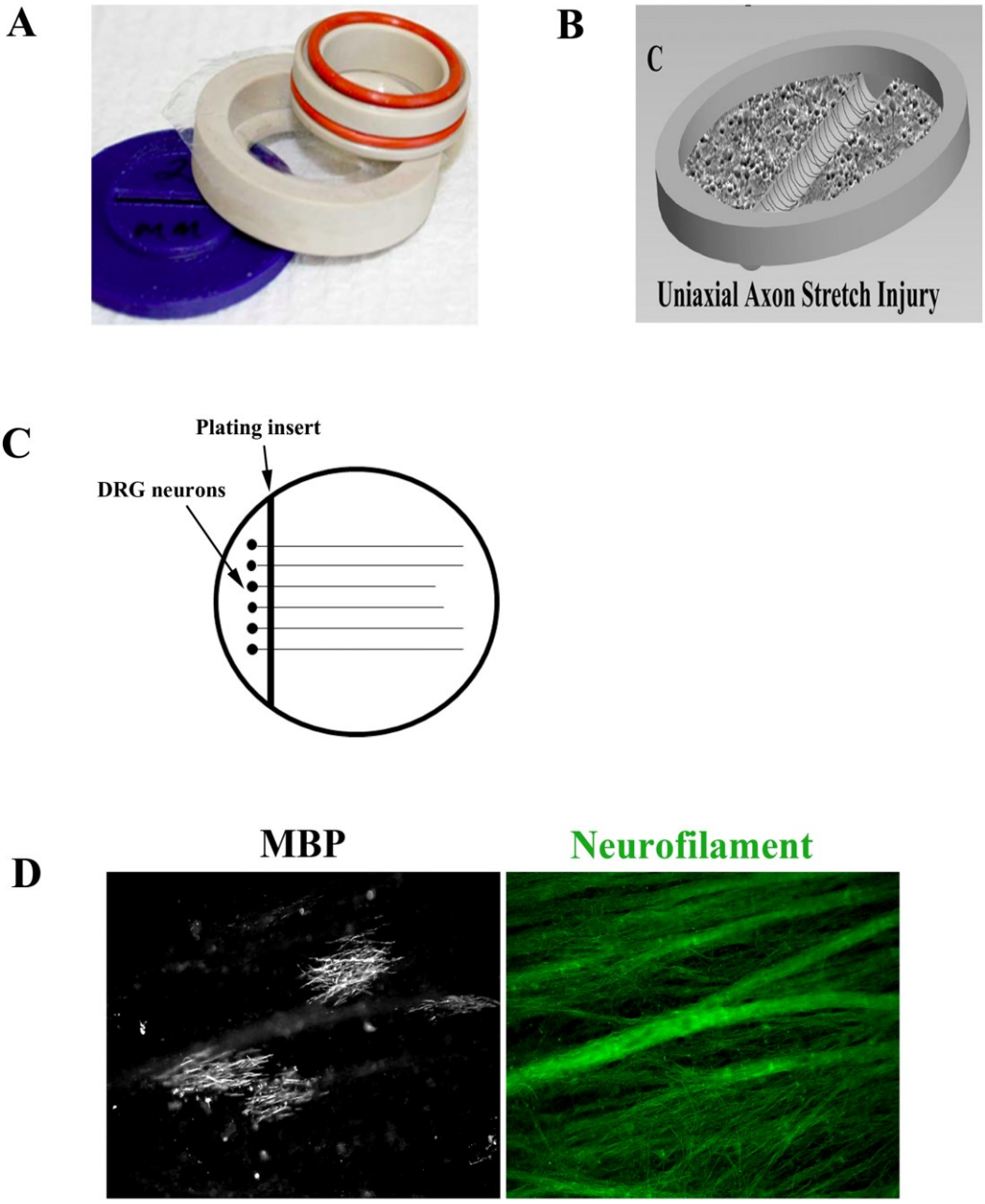


(Adopted and modified from Jackman et al., 2009, Physiology)

Figure 26. Proposed model of MAPK pathways involved in regulating dedifferentiation of mature oligodendrocytes

Erk1/2 or p38 MAPK activation may induce dedifferentiation of mature oligodendrocytes. p38 MAPK might revert mature oligodendrocytes into an immature phenotype whereas Erk1/2 might have the ability to dedifferentiate further into proliferative stage.

Figure 27



(Figure 27B: *Adopted from Magou et al., 2011, Journal of Neurotrauma)*

Figure 27. Components of the *In vitro* DAI model.

(A) Images of the injury well components, with the two PEEK rings that were used to insert the silicone membrane to make the well. Image of the deformation mask (Blue) with a 2mm gap in the center. (B) Image showing the deformation of the substrate using a pressure-pulse, and the deformation is restricted to the area in the center. (C) Schematic representation of the co-culture system used in the *in vitro* DAI model. DRG neurons were plated on one side of the plating insert, and 24 hours later the insert is removed allowing the DRG neurons to extend their axons. (D) Images of the myelinated axons on the silicone membrane. Notice the semi-unidirectional axonal growth of the axons (neurofilament, green).

Figure 28

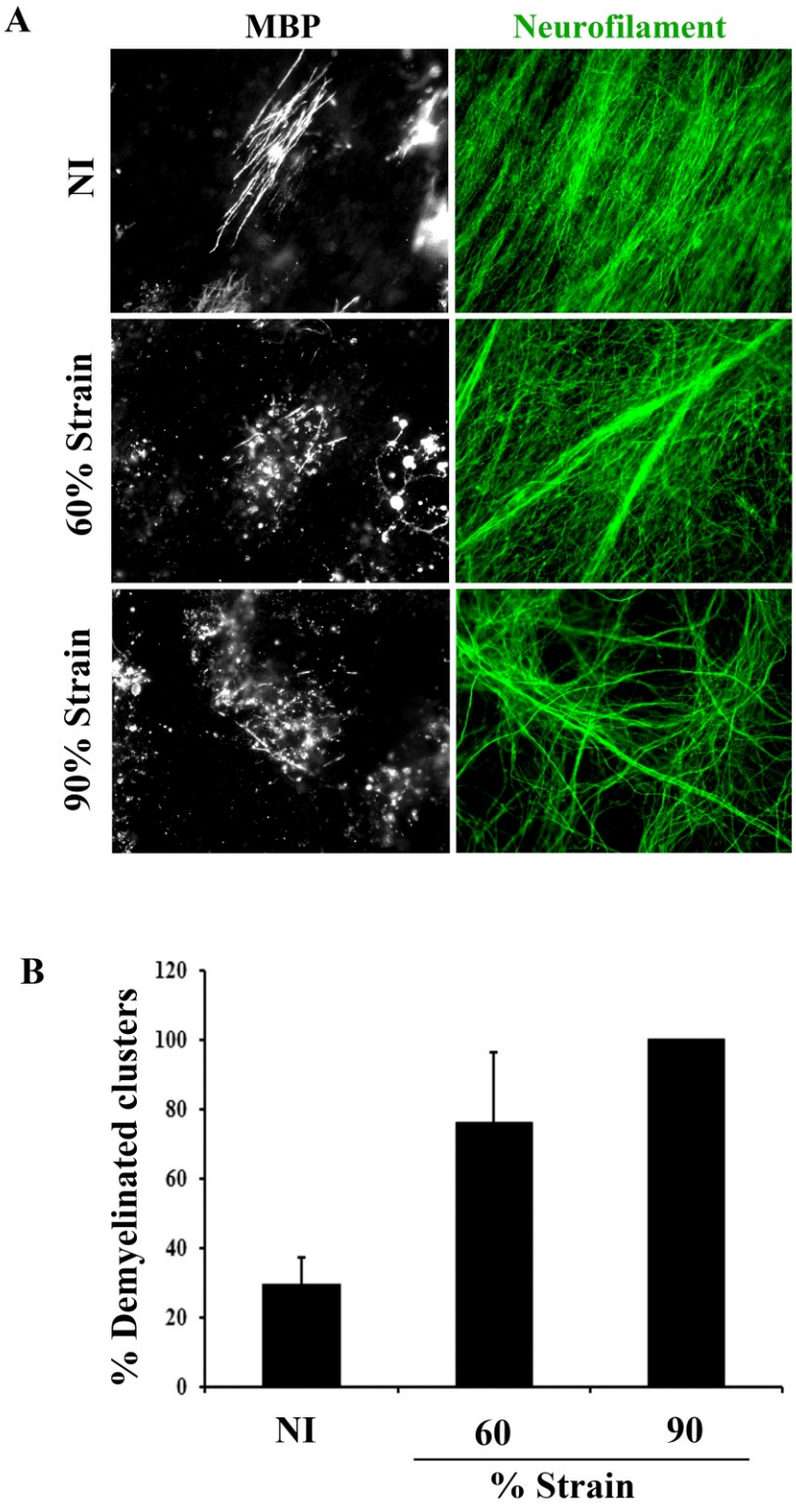

A
MBP
Neurofilament
NI
60% Strain
90% Strain
B
% Demyelinated clusters
120
100
80
60
40
20
0
NI
60
90
% Strain

Figure 28. Stretch-injury induces oligodendrocyte demyelination in DRG-OPC co-cultures.

Myelinated axons were stretch-injured using 60% and 90% strain, and were fixed 24 hours later and immunostained for MBP and neurofilament. (A) Images of MBP-positive myelin clusters at the site of injury. MBP-positive clusters with fragmented myelin segments were observed in both injury conditions (60% and 90% strain) compared to non-injured (NI) conditions. Notice that the axons (neurofilament, green) were not disconnected after the stretch-injury. (B) Quantification represented as percentage of demyelinated clusters. Both conditions (60% and 90% strain) induced oligodendrocyte demyelination after stretch-injury.

Figure 29

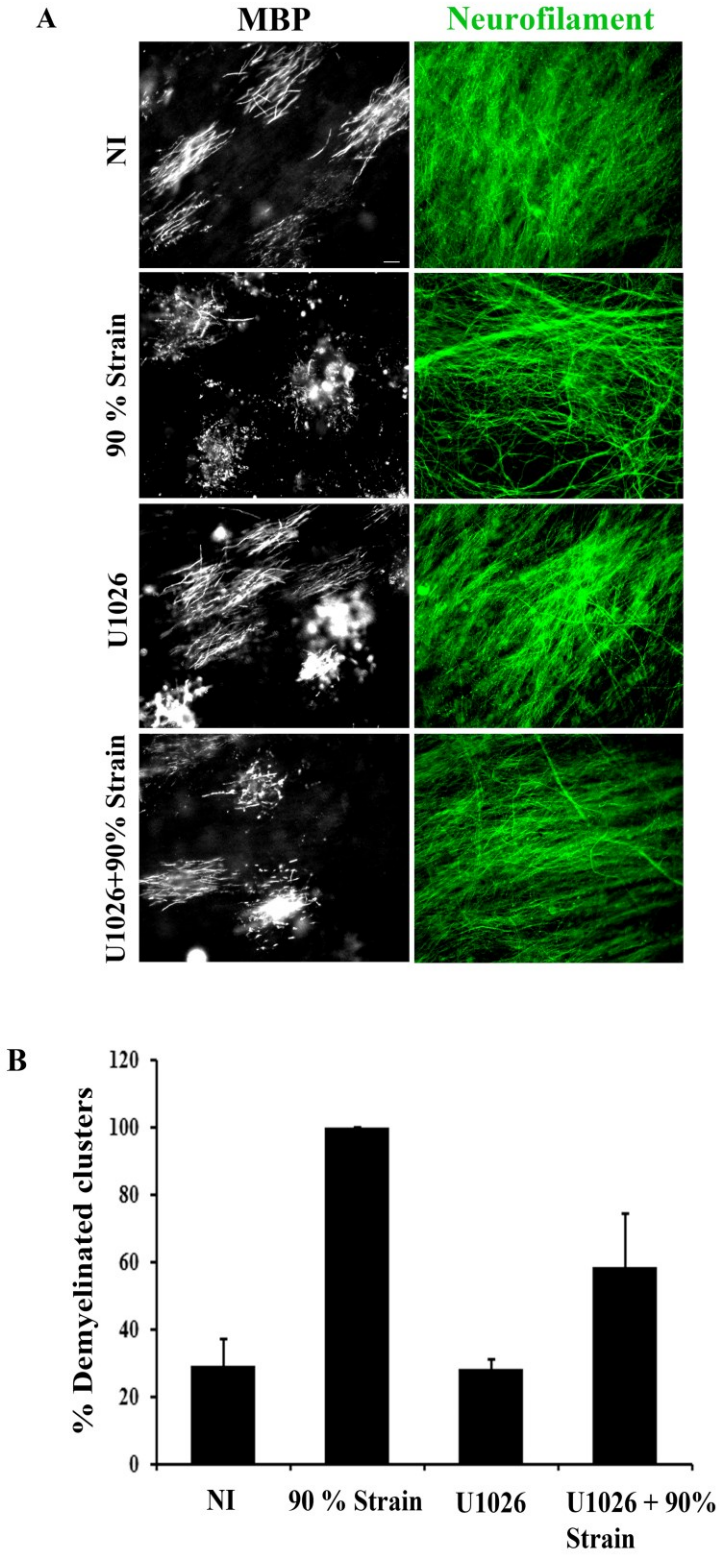

A
MBP
Neurofilament
NI
90 % Strain
U1026
U1026+90% Strain
B
% Demyelinated clusters
120
100
80
60
40
20
0
NI
90 % Strain
U1026
U1026 + 90% Strain

Figure 29. Inhibition of Erk1/2 activity blocks oligodendrocyte demyelination induced by stretch-injury in DRG-OPC co-cultures

Myelinated axons were stretch-injured and 24 hours later, immunostained for MBP and neurofilament (green). (A) Images of MBP-positive myelin clusters at the site of stretch injury.U1026-treated cultures showed reduced symptoms of demyelination after the stretch injury compared injured cultures. Scale bar: 20μm. (B) Quantification of the results are represented as percentage of demyelinated clusters. Treatment with U1026 (10μM) decreased stretch-injury induced oligodendrocyte demyelination.

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GENERAL INFORMATION

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1. The role of MAPKs in CNS demyelination.
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TEACHING AND SUPERVISION

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PUBLICATIONS

Original Articles:

1. Tyler WA, Gangoli N, Gokina P, Kim HA, Covey M, Levison SW, Wood TL. Activation of the mammalian target of rapamycin (mTOR) is essential for oligodendrocyte differentiation. *J Neurosci.* 2009 May 13;29(19):6367-78
2. Cragnolini AB, Huang Y, Gokina P, Friedman WJ. Nerve growth factor attenuates proliferation of astrocytes via the p75 neurotrophin receptor. *Glia*. 2009 Oct;57(13):1386-92.
3. Crawford AT, Desai D, Gokina P, Basak S, Kim HA. E-cadherin expression in postnatal Schwann cells is regulated by the cAMP-dependent protein kinase a pathway. *Glia.* 2008 Nov 15;56(15):1637-47.

Poster presentations

1. Gokina P and Kim H.A. p38 MAPK activation promotes oligodendrocyte demyelination and down-regulates myelin gene expression. *Society for Neuroscience*, 2012
2. Gokina P and Kim H.A. FGF-2 triggers Oligodendrocyte demyelination in myelinate co-cultures: The role of the MAPK pathways. *American Soceity for Neurochemistry* 2011.
3. Desai D.J, Crawford A.T, Gokina P, Basak S, Kim H.A. E-cadherin expression in postnatal Schwann Cells is regulated by the cAMP-dependent protein kinase A pathway. *Society for Neuroscience*, 2008