Molecular regulation of myelination by Oligodendrocyte Progenitor cells

by

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Abstract

Oligodendrocytes (OL) are the myelinating cells of the central nervous system (CNS). A series of complex cell signaling events in the CNS ensures successful myelination. Various molecular cues including growth factors, transcription factors and cytokines regulate myelination by inducing OL migration, proliferation and differentiation. Platelet-derived growth factor A (PDGF-A) and fibroblast growth factor 2 (FGF2) are two of the most well characterized regulators of OP migration. The current study hypothesizes that PDGF-A and FGF2 regulate the migration of OP through transient activation of the extracellular signal-regulated protein kinase (ERK) signaling pathway. The results show that activation of ERK is required for OP migration. It also demonstrates the significance of threshold levels of growth factors and temporal regulation for OP migration. Furthermore, the chemokine CXCL1 has been shown to play a critical role in regulating the dispersal of OP during development, although the mechanisms underlying this regulation are unknown. Previous studies have shown that calcium flux is required for OP migration. CXCL1 induces calcium flux in cells; therefore we hypothesized that CXCL1 inhibition of OP migration was regulated via changes in intracellular calcium flux. However, our results show that CXCL1 inhibition of OP migration is independent of calcium signaling. In addition, we show that CXCL1 inhibition of OP migration is specific to PDGF-A induced migration. Lastly, the current study identifies a transcriptional regulator, methyl-CpG-binding protein 2 (MeCP2) as regulating the expression of myelin specific genes in a transgenic mouse. Interestingly, gene expression of myelin associated proteins myelin basic protein (MBP), myelin associated glycoprotein (MAG)

and proteolipid protein (PLP), which play an important role in regulation of OL differentiation and subsequent formation of myelin of the myelin sheath, where found to be dysregulated. Overall, these findings reveal previously unknown roles of various intrinsic factors in successive phases of OL development. It aims to provide a better understanding of complexity to myelin development, function and disease.

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List of Abbreviations

AEP	Anterior entopeduncular
ALD	Adrenoleukodystrophies
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic
BBB	Blood-brain barrier
BDNF	Brain derived neurotrophic factor
bHLH	basic helix-loop-helix
BSA	Bovine serum albumin
CNPase	Cyclic nucleotide 3’ – phosphodiesterase
CNS	Central nervous system
CNTF	Ciliary neuronotrophic factor
cPLC	cytoplasmic Phospholipase C
DAG	Diacylglycerol
DLX	Distal-less homeobox
DMEM	Dulbecco's modified eagle's medium
EGR2	Early growth factor 2
ERK	Extracellular regulating kinase
FBS	Fetal bovine serum
FGF2	Fibroblast-derived growth factor 2
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLD	Globoid cell leukodystrophies

Gro	Growth regulatory oncogene
HDAC	Histone deacetylases
IGF1	Insulin-like growth factor-1
IP3	Inositol 1,4,5-triphosphate
kDa	kilo Dalton
MAG	Myelin associated glycoprotein
MAPK	Mitogen activated kinases
MBD	Methyl-CpG binding domain
MBP	Myelin basic protein
MeCP2	Methyl CpG binding protein 2
MLD	Metachromatic leukodystrophies
MOG	Myelin oligodendritic glycoprotein
MS	Multiple Sclerosis
NT-3	Neurotrophin-3
OL	Oligodendrocyte
OP	Oligodendrocyte progenitor cell
PCD	Programmed cell death
PDGF-A	Platelet-derived growth factor A
PDGFRα	PDGF receptor-alpha
PDL	Poly-D-lysine
PI_3 K	Phosphoinositide-3 kinase
PLCγ	Phospholipase C gamma
PLP	Proteolipid protein

PMD	Pelizaeus-Merzbacher's disease
PML	Progressive multifocal leukoencephalopathy
pMN	Motor neuron progenitor
RDV	Relative densitometry value
RTK	Receptor tyrosine kinase
RTT	Rett Syndrome
Shh	Sonic hedgehog
SVZ	Subventricular zone
TF	Transcription Factor
TGF-β	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
TRD	Transcriptional repression domain
TRITC	Tetramethyl rhodamine isothiocyanate
UTR	Untranslated Region
VVZ	Ventral ventricular zone
XCI	X-chromosome inactivation

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Chapter 1: General Introduction

1.1 Development of the Brain

The human brain is arguably the most complex organ of the whole body. Knowledge of the molecular events modulating brain development has advanced dramatically over the last two decades because of the technological advancement and discovery of a range of molecules, including transcription factors, growth factors and extracellular signaling proteins. The 'brain' we know now is an end product of an intricate sequence of dynamic and adaptive developmental neurological events that are regulated by an extremely sophisticated, genetically organized network of signaling molecules.

Brain development begins in the third gestational week in humans (O'Rahilly and Gardner, 1979; O'Rahilly *et al.*, 1986), and continues throughout late adolescence. The formation of the neural tube marks the start of the brain development and is the basis of all further nervous system development. The neural tube differentiates clearly into brain and spinal cord by the end of 12 weeks, which coincides with the birth of neurons (Bayer *et al.*, 1993; Gazzaniga and Bizzi, 1995). During the weeks 12 to 20, the neural progenitor cells proliferate and migrate from their origins to destinations in the cerebral cortex of the brain (Rakic, 1990). The cell bodies of these neurons primarily form the grey matter, whereas their myelinated axons form the white matter of the brain. Brain development continues extensively in the postnatal period. For example, the whole

brain volume grows exponentially by 25% between early childhood (19–33 months) and adolescence (12–15 years), when the developmental maximum volume is reached (Courchesne *et al.*, 2000).

1.2 Brain Cells

The brain is composed of different types of cells, which mainly include the neurons and glial cells. There are about 100 billion neurons in the mature human brain (Pakkenberg and Gundersen, 1997). A complex set of connections made by neurons results in the formation of an intricate information processing network that is responsible for all our voluntary and involuntary movements. These connections are formed with the help of electrical impulses (called “action potentials”) that are sent and received between neurons. This impulse consists of a brief, reversible polarization that propagates along the axon. The neurons involved in the electrical propagation of nerve impulses can be classified into three categories according to their function. First, are the pseudo-unipolar sensory neurons that receive sensory signals from the peripheral sensory organs and propagate it to the Central Nervous System (CNS). Secondly, the multipolar motor neurons conduct commands from the cortex to the spinal cord or from the spinal cord to the muscles. Lastly, the third predominant class of neurons are the interneurons, which function to connect various neurons within the brain and the spinal cord.

Glial cells, named after the Greek word for glue, were originally thought to provide only support and protection for the neurons they surrounded. However, controversy surrounding their exact functional role in the nervous system remains up for debate.

Glia cells represent a significant portion of the nervous system suggesting a functional role in the nervous system that extends to well beyond the provision of neuronal support. Specifically, glia constitute 25% of total cells in the *Drosophila*, 65% in rodents, and ~90% in the human brain (Pfrieger and Barres, 1995). At present, the role of glial cells has expanded to include involvement in axon- myelin formation (Bunge, 1987; Ranscht *et al.*, 1987), midline axon guidance (Klambt and Goodman, 1991), neuronal migration, immunity (Frohman *et al.*, 1989; Stitt *et al.*, 1991) and neuron–glia interaction (Vernadakis, 1988). Thus, we now know that glial cells play a critical role in the formation and function of the mammalian brain.

Based on their structure and function, glial cells can be further divided into four types: microglia, astrocytes, Schwann cells and oligodendrocytes (OL).

Microglia are the resident macrophages of the CNS that are functionally important during normal growth and development, as well as during injury to the CNS (Kreutzberg, 1996; Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005). They reside in all parts of the brain and are characterized by a small soma and numerous very thin and highly branched processes. During normal development of the CNS they function as phagocytes, playing a key role in the removal of apoptotic cell bodies (Ferrer *et al.*, 1990; Egensperger *et al.*, 1996). Furthermore, microglia are activated and proliferate extensively in response to CNS damage, during trauma (Jensen *et al.*, 1994), stroke (Lehrmann *et al.*, 1997), inflammation (Gonzalez-Scarano and Baltuch, 1999) or autoimmune attack (Ponomarev *et al.*, 2005).

Astrocytes, the most abundant cell type in the mammalian brain, constitute about half of all human brain cells. 'Astrocytes', which literally means 'star-like cells', have long, thin projections giving them a starry look. Astrocytes have a very divergent role in the nervous system in maintaining the metabolic and ion homeostasis of neurons by controlling the exchange of materials between blood vessels and neurons. They ensheath the synapses and are thus implicated in synapse formation, function and stability (Pfrieger and Barres, 1997; Ventura and Harris, 1999; Ullian *et al.*, 2001). Having a unique anatomical position, the astrocytes regulate brain blood flow by linking the cerebral microvasculature with synapses (Simard *et al.*, 2003; Zonta *et al.*, 2003). Astrocytes are in addition shown to have a bidirectional conversation with the neuronal elements (Parpura *et al.*, 1994; Fellin *et al.*, 2006). In response to a CNS insult like trauma, ischemia or any neurological disease, astrocytes respond by a process known as reactive astrogliosis, which involves changes in their molecular expression, and in severe cases, scar formation (Eddleston and Mucke, 1993; Pekny and Nilsson, 2005; Maragakis and Rothstein, 2006).

Oligodendrocytes are glial cells with few processes, hence the prefix 'oligo'. They are the myelinating cells of the CNS. The oligodendrocyte (OL) contacts the axon, wrapping its lipid rich membrane around the axon to form a multi-layered spiral wrap called the myelin sheath (Bunge *et al.*, 1962; Bunge, 1968). Unlike Schwann cells of the PNS, a single OL can myelinate up to 40 different axons (Pfeiffer *et al.*, 1993). The most important function of OL is to form the myelin sheath. However, OLs also form synaptic

connections with neurons, and contribute actively and directly to neural signaling (Lin and Bergles, 2004). In addition, the myelin sheath is critical for the normal health of the axon, regulating the homeostasis of the periaxonal space (Dyer, 2002).

1.3 OLs, the myelinating cells of CNS

The OLs originate as pre-progenitor cells in the telencephalon of the developing brain (Rakic and Zecevic, 2003). From their origin, the pre-progenitor cells migrate across the sub-pallial layer to the sub-ventricular zone (SVZ) where they proliferate and differentiate into OL progenitors (OPs) (LeVine and Goldman, 1988). The OPs then undergo further migration away from the SVZ, to populate the developing white matter tracts of the brain (Kakita and Goldman, 1999). During this migratory phase, OPs remain proliferative and only exit the cell cycle when reaching their destination. These OPs are extremely dependent on the presence of growth factor platelet derived growth factor (PDGF) for proliferation and can be identified because they express high levels of the PDGF receptor (Barres *et al.*, 1992b; Baron *et al.*, 2002a) whereas surrounding neurons secrete PDGF (Ellison *et al.*, 1996b). Eventually, the OPs reach their target regions and undergo terminal differentiation, which involves a massive increase in lipid biosynthesis and production of large amounts of myelin proteins, proteolipid protein (PLP), myelin basic protein (MBP) and myelin associated glycoprotein (MAG). These will be integrated into the myelin sheaths with which OLs wrap axons to allow rapid saltatory conductance in the vertebrate CNS. During differentiation of OPs into OLs, the

cells undergo a dramatic change of their morphology with the formation of a large network of branching processes. Their development from precursors and the initiation of myelination begins during late embryogenesis and continues for several weeks in the rodent forebrain, and for several years in the human forebrain (Minkowski, 1967; Knaap and Valk, 1995; Giedd, 2004).

1.3.1 Origin & Specification

In the embryonic brain, OLs originate ventrally as pre-progenitor OLs (OP) in the medial ganglionic eminence and the anterior entopeduncular (AEP) area in the diencephalon and then migrate dorsally as they differentiate into progenitors (Pringle and Richardson, 1993; Timsit *et al.*, 1995; Richardson *et al.*, 2000; Spassky *et al.*, 2000). However, this first population of OP cells originating from the ventricular germinal layer are completely lost and are replaced by dorsally originating OP cells (Vallstedt *et al.*, 2005). The dorsally derived OP cells arise from the lateral and/or caudal ganglionic eminences and are followed by a third wave of OP cells from the postnatal neocortex (Kessaris *et al.*, 2006).

During embryonic development of the vertebrate nervous system, gliogenesis usually follows neurogenesis. A number of cues, including transcription factors and growth factors induce the gliogenic switch to produce cells with oligodendrocytic specification in the ventricular zone. However, *Sonic hedgehog (Shh)* signaling and *Olig* gene function dominates this neuron-glial switch in the embryonic brain and spinal cord. *Shh* is

expressed during oligodendrogenesis in the ventricular and sub-ventricular zone of AEP as well as in the medial ganglionic eminence (Sussel *et al.*, 1999; Nery *et al.*, 2001; Tekki-Kessaris *et al.*, 2001). Shh signaling is known to be both required and sufficient to induce OL production in brain and spinal cord (Orentas *et al.*, 1999; Alberta *et al.*, 2001; Soula *et al.*, 2001). The *Olig* genes lie downstream of the Shh signaling and encode basic helix-loop-helix (bHLH) transcription factors that regulate many early stages of OP development (Lu *et al.*, 2000; Zhou *et al.*, 2000; Alberta, Park et al., 2001). The expression of Olig1 precedes the emergence of PDGFRα, Sox10-expressing OP cells from the medial ganglionic eminence (Lu *et al.*, 2002; Zhou and Anderson, 2002).

1.3.2 Differentiation, Migration and Proliferation

OP cells migrate long distances throughout the CNS before differentiating into mature myelinating OL after they reach their neuronal targets (Small *et al.*, 1987). During the phase of migration, the OP cells proliferate and exit the cell cycle only before terminal differentiation. As they reach their destination, OP cells differentiate by increasing the number and complexity of processes. The proliferation process produces more OLs than necessary, and the extra cells undergo programmed cell death (PCD) when they fail to myelinate (Barres *et al.*, 1992a; Raff *et al.*, 1993). On reaching their final destination, the OPs up-regulate myelin-related protein production and begin to wrap around the targeted axon. A complex, yet sophisticated set of machinery, which includes, growth factors, transcription factors and chemokines, ensure normal OL

development (Hardy and Reynolds, 1991; Barres *et al.*, 1993; Tsai *et al.*, 2002; Dziembowska *et al.*, 2005; Nicolay *et al.*, 2007).

1.3.3 Role of Growth Factors

A number of growth factors have been implicated in OP migration, proliferation and differentiation. A potent mitogen and the single most important survival factor for OP cells, PDGF-A is the best studied growth factor in relation to OL development. Please see **Chapter 3** which outlines detailed information in regards to the role PDGF-A along with another important growth factor, Fibroblast growth factor-2 (FGF2) in OP behaviour. Three other growth factors, because of their importance in the OP regulation are discussed below.

Insulin-like Growth Factor-1 (IGF-1) is known to promote proliferation, differentiation and survival of OP cells (McMorris *et al.*, 1986; McMorris and Dubois-Dalcq, 1988; Ye and D'Ercole, 1999). Furthermore, transgenic mice with targeted IGF-1 gene deletion show a decrease in the number of myelin tracts in the CNS (Beck *et al.*, 1995; Ye *et al.*, 2002). Overexpression of IGF-1 in transgenic mice leads to increased myelination by increasing the number of OP cells and myelin associated protein expression (Carson *et al.*, 1993; Ye *et al.*, 1995b; Ye *et al.*, 1995a). IGF-1 primarily uses the cell-surface receptor IGF1R (Liu *et al.*, 1993; Zeger *et al.*, 2007) for its functioning and is shown to

involve PI3-Akt kinases-GSKβ3 signaling pathway in order to mediate OP cell proliferation and survival (Vemuri and McMorris, 1996; Ness and Wood, 2002).

Neurotrophin-3 (NT-3), is another mitogen that is known to stimulate the proliferation of OP cells *in vivo* (Barres *et al.*, 1994) and helps post-mitotic OL cell survival (Kumar *et al.*, 1998). The NT-3 and TrkC (NT-3 receptor) knockout mouse has decreased OP cells in the brain and spinal cord and attenuated OL-specific markers (Kahn *et al.*, 1999).

Transforming growth factor- β (TGF-β), a pleiotrophic and multifunctional growth factor has been shown to modulate apoptosis in OLs in order to selectively eliminate the excess number of OP cells generated during the CNS development (Yu *et al.*, 2000; Schuster *et al.*, 2002).

Other factors that mediate OL development include the ciliary neuronotrophic factor (CNTF), leukemia inhibitory factor (LIF), tumor necrosis factor (TNFα), nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF)(Barres, Schmid et al., 1993; Mayer *et al.*, 1994; Gard *et al.*, 1995; Cohen *et al.*, 1996; Arnett *et al.*, 2001; Vondran *et al.*, 2010).

1.3.4 Role of Transcription Factors

A sophisticated network of transcription factors in the CNS coordinates OP-related gene expression, that, consequently results in normal functional myelin. Many of these transcription factors belong to well-known developmental regulatory families, i.e. the bHLH family, the Sox family and the homeodomain family.

The basic **Helix loop Helix (bHLH) family**, including Olig1 and Olig2 plays a significant role in early OP lineage specification in the vertebrate CNS (Lu, Yuk et al., 2000; Zhou, Wang et al., 2000; Vetter, 2001). Expression of Olig1 and Olig2 is regulated by a Shh concentration gradient in the motor neuron progenitor (pMN) domain during early embryonic development (Lu, Yuk et al., 2000). Olig2 sequentially specifies motor neuron and then OL production in the early stages of OP cell specification. Consequently, Olig2^{null} mice die at birth due to a lack of motor neurons and OLs (Lu, Sun et al., 2002). Conversely, Olig1 is involved in the final stages of myelin production. Confirmation of the importance of Olig1 and Olig2 in OP lineage specification becomes evident upon examining the various animal studies conducted in this area. For example, in the Olig1^{null} mice, the OP differentiate into mature OL, comes in contact with neuronal axons, but fail to form myelin (Xin *et al.*, 2005). An Olig1/2 double knockout mouse generated to circumvent the compensatory nature of these genes resulted in conversion of sequential production of motoneurons and OLs into sequential production of interneurons and astrocytes (Zhou and Anderson, 2002).

The Sox family represents the next transcriptional regulatory family which are implicated in myelin development. Specifically, the vertebrate **group E Sox proteins** Sox8, Sox9 and Sox10 coordinate to regulate specification and differentiation of OLs. Sox9 is widely expressed in glial precursors while Sox10 is expressed in differentiating OLs and astrocytes as the Sox9 expression weakens (Britsch *et al.*, 2001; Stolt *et al.*, 2002; Stolt *et al.*, 2003). Expressed in both OP and differentiating OLs, Sox8 acts as compensatory gene, as the *Sox8*^{null} mice display no glial defects (Stolt *et al.*, 2004). The *Sox8/Sox9* double knockout fails to develop OP cells, whereas *Sox8/Sox10* double knockout leads to severe loss of differentiating OLs (Stolt, Lommes et al., 2004; Stolt *et al.*, 2005). This suggests that OL differentiation is mediated by cooperative interactions between transcription factors of the Sox family.

In addition to the bHLH and Sox family of transcriptional regulators, the homeodomain family also contains specific transcription factors that are thought to be involved in myelin development. Specifically, the **homeodomain transcription factors** Nkx6.2/Gtx and Nkx2.2 are also implicated in the regulation of OL maturation as well as myelination in the CNS. Nkx6.2 is expressed in differentiating OLs and has multiple binding sites in the promoter regions of myelin associated proteins (Awatramani *et al.*, 1997). Surprisingly, Nkx6.2^{null} mice did not show any apparent defects in OL maturation or myelin gene expression (Cai *et al.*, 2001). However, later studies have shown small myelin defects in the paranodal loop in Nkx6.2^{null} mice with physiological deficiencies in motor coordination and CNS nerve conductance (Southwood *et al.*, 2004). Nkx2.2, another homeodomain transcription factor works in collaboration with Olig2 and is

required for normal OL development (Xu *et al.*, 2000; Qi *et al.*, 2001). A number of other transcription factors that act independently and/or synergistically in order to bring about successful OL functioning include Myt1 (Nielsen *et al.*, 2004), Sox17 (Sohn *et al.*, 2006) and Krox24/EGR1 (Sock *et al.*, 1997).

1.3.5 Role of Chemokines

Chemokines are a group of more than 50 related small sized (8-14 kDa) proteins, which are divided into four major classes: CXC (or α), CC (or ß), CX_3C, and C. The proteins are named according to the number and spacing of conserved cytokine residues in their amino acid sequence (Hesselgesser and Horuk, 1999; Laing and Secombes, 2004). Chemokines selectively and non-selectively bind to G protein-coupled receptors in order to exert their biological effects. One chemokine molecule can bind more than one receptor, and a given receptor can interact with many different chemokine molecules. In rodents, OLs are shown to express at least four chemokine receptors, CXCR1, CXCR2, CXCR3 and CXCR4 (Nguyen and Stangel, 2001; Omari *et al.*, 2005). The chemokine, CXCL1 (formerly known as growth regulatory oncogene (Gro) α) binds to CXCR2, and has been shown to induce OP proliferation (Robinson *et al.*, 1998b) and inhibit migration (Tsai, Frost et al., 2002) [discussed further in **Chapter 4**]. Furthermore, CXCR4, which utilizes CXCL12 for ligand activation, is shown to promote OP cell differentiation and remyelination in the injured adult CNS (Patel *et al.*, 2010).

1.4 Myelin

Most of the larger axons in the mammalian nervous system are surrounded by a lipid-rich multi-layered structure known as the myelin sheath (Bunge, Bunge et al., 1962). Named by Virchow (Virchow, 1846), 'myelin' consists of spiralling layers of specialized plasma membranes that are supplied by OLs in the CNS and Schwann cells in the PNS. In humans, myelination starts during the second half of gestation and continues until at least 20 years of age (Minkowski, 1967; Knaap and Valk, 1995; Giedd, 2004). Interestingly, myelination occurs caudal to rostral in the brain and rostral to caudal in spinal cord (Brody *et al.*, 1987; Kinney *et al.*, 1988; Baumann and Pham-Dinh, 2001). Proximal pathways tend to myelinate before distal, sensory before motor, and projection before association pathways (Volpe, 2000). An essential constituent of the white matter, myelin encompasses ~40-50% of the CNS on the dry weight basis (O'Brien and Sampson, 1965). The structural and molecular composition of myelin is unique. A lipid-rich membrane, myelin consists of 70% lipids (highly enriched in glycosphingolipids and cholesterol) and 30% proteins (Morell *et al.*, 1994). The lipophylic nature of it combined with the low water content accounts for the insulating properties of myelin sheath.

1.5 Structure and Functions of Myelin

1.5.1 Structure of Myelin

Several structural features characterize myelin. Data from electron microscope studies visualize myelin as a periodic structure with a series of alternating concentric dense and light layers (Kirschner and Hollingshead, 1980). The major dense line (appearing dark) is formed by the close apposition of the cytoplasmic surfaces of the plasma membranes. The intraperiod line (appearing light) results from the closely apposed outer leaflets of plasma membranes. The periodicity of the lamellae depends on the species and neuron types. A myelinated axon appears as myelinated membrane segments called internodes, separated by regions of unsheathed axon called the 'node of Ranvier'. The nodes of Ranvier play a major role in nerve impulse conduction. In the late 1940s it was shown that myelin enables saltatory impulse propagation in axons (Huxley and Stampfli, 1949; Geren and Raskind, 1953). It is at the node of Ranvier the impulse is generated because of the dense clustering of sodium channels (Shrager, 1989). **(Fig. 1)**

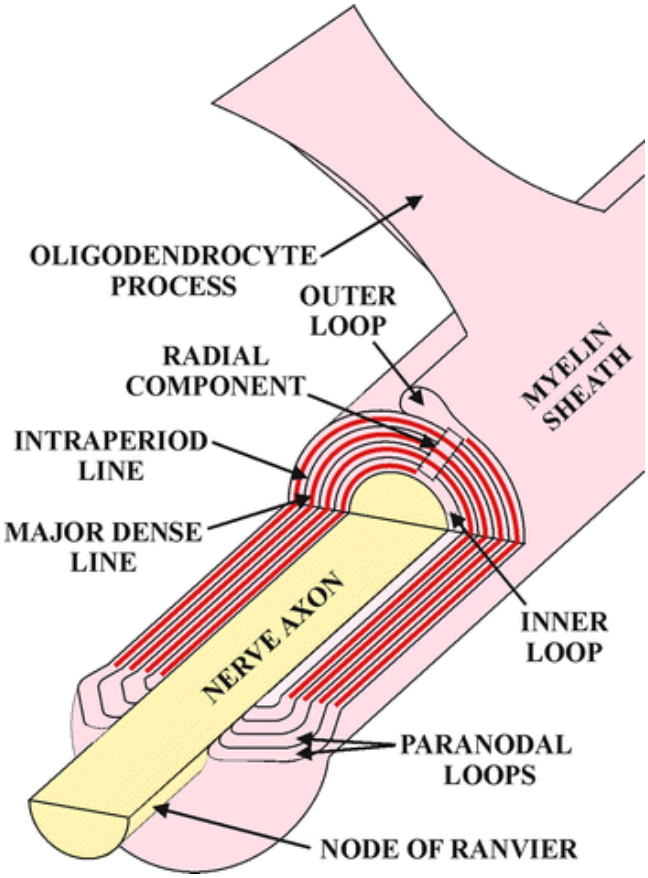


Figure 1: Structure of Myelin. (Debruin and Harauz, 2007)

The myelin membrane with its unique cytoarchitecture and many different functional microdomains.

Additionally, myelin thickness is proportional to the axonal diameter and the internodal segment (Friede and Bischhausen, 1982; Friede, 1986). Traditionally, the ratio of inner axonal diameter to the total outer diameter, called the 'g-ratio' is used to assess axonal myelination. Rushton was the first to calculate the optimal theoretical g-ratio of 0.6 based on the speed of fibre conductance (Rushton, 1951).

1.5.2 Function of Myelin

The presence of high resistance and low capacitance in the myelin sheath makes the impulse jump from node to node, thus forming the propagation termed as 'saltatory conductance' (Tasaki, 1955; Shepherd, 1988). In general, myelination increases (>10-fold) the signal transmission speed of action potentials (Waxman, 1977) and decreases the refractory time (time needed to repolarize again) by as much as 34-fold (Felts *et al.*, 1997; Sinha *et al.*, 2006).

More recent studies reveal additional functions associated with myelin. Mutation in the genes related to myelin formation leads to axonal degeneration, indicating the need of myelin to maintain axonal integrity (Griffiths *et al.*, 1998; Lappe-Siefke *et al.*, 2003). The myelin-related proteins are also shown to interact with the axon and inhibit their

outgrowth (Schwab and Caroni, 1988; McGee *et al.*, 2005). Thus, myelin also plays an important role in neuronal development and maintenance.

1.6 Myelin-related Proteins

Myelin assembly around the neuronal axons involves distribution of particular proteins in a temporally and spatially regulated manner. These proteins are synthesized in several subcellular localizations in the OLs and are then transported to its myelin forming membrane. Some of these proteins play important roles during various stages of OL development. The myelin associated proteins that are expressed by OL at different stages of development in the CNS are discussed in more detail below.

1.6.1.1 Proteoglycan NG2

NG2 is a cell-surface protein that is found as an integral membrane chondroitin sulphate proteoglycan. It is one of the widely used markers for OL progenitor in CNS (Nishiyama *et al.*, 1996; Keirstead *et al.*, 1998; Dawson *et al.*, 2000). NG2 is thought to contribute to OL proliferation by acting as a co-receptor alongside PDGFRα, the receptor for the key growth factor PDGF-A (Nishiyama, Lin et al., 1996; Murtie *et al.*, 2005c). The $NG2^{null}$ mouse shows delayed OP proliferation with subsequent delay in OL maturation (Kucharova and Stallcup, 2010). NG2 is also shown to inhibit neurite outgrowth from embryonic and neonatal neurons *in vitro* (Dou and Levine, 1994; Chen *et al.*, 2002). Furthermore, it dramatically increases at the time of CNS injuries (Keirstead, Levine et

al., 1998; McTigue *et al.*, 2001; Jones *et al.*, 2002) and is thought to inhibit axon regeneration and remyelination (Tan *et al.*, 2005; Tan *et al.*, 2006; Massey *et al.*, 2008).

1.6.1.2 Platelet-derived Growth Factor receptor α (PDGFRα)

PGDFRα is expressed during the early stages of differentiation of the myelinating OLs. It is the only known PDGF receptor expressed in OLs, and is shown to undergo activation by PDGF-A ligand binding (Pringle *et al.*, 1989; McKinnon *et al.*, 1990). PDGF signaling via PDGFRα is crucial for normal embryogenesis (Calver *et al.*, 1998; van Heyningen *et al.*, 2001). It results in activation of numerous signaling pathways, including phosphoinositide-3 kinase (PI_3 K), mitogen activated kinases (MAPK) and phospholipase C gamma (PLCγ) (Claesson-Welsh, 1994a; Ebner *et al.*, 2000; Schlessinger, 2000; Haines *et al.*, 2008; Vora *et al.*, 2011). The OL progenitor cells in the PDGFRα-deficient mice undergo early maturation and also exhibit abnormal migration and proliferation, before death *in utero* (McKinnon *et al.*, 2005). This supports the theory that PDGFRα plays an important role in OL development (see more detailed discussion in **Chapter 3)**.

1.6.1.3 Proteolipid Protein (PLP)

PLP is the most abundant protein in the vertebrate CNS and accounts for nearly 50% of total protein found in adult CNS myelin (Eng *et al.*, 1968; Norton and Poduslo, 1973; Jahn *et al.*, 2009). PLP is required for the fusion of extracellular membranes of myelin

lamellae in order to form the intraperiod line (Kettenmann and Ransom, 2005) **(Fig. 2)**. The *PLP* gene encodes alternative transcripts, which result in two transmembrane proteins: proteolipid protein (PLP) (30kDa) and DM20 (26kDa). The expression levels of DM20 are at its highest during embryonic development, whereas *PLP* expression increases markedly during the postnatal period (LeVine *et al.*, 1990). PLP is a cholesterol binding protein located in lipid rafts of the OL plasma membrane and it plays an important role in myelin compaction. Mice that are PLP/DM20 deficient have myelin that appears grossly normal but is less compact than wild-type myelin (Griffiths, Klugmann et al., 1998; Simons *et al.*, 2000). PLP overexpressing mice exhibit impaired neuro-glia interaction and show significant loss of the speed of impulse conduction in brain neurons (Tanaka *et al.*, 2009). Mutation in the PLP gene in humans leads to a progressive degenerative neurological disease known as Pelizaeus-Merzbacher disease, characterized by deterioration in motor functions, coordination and intellectual functions (Willard and Riordan, 1985; Gencic *et al.*, 1989; Hudson *et al.*, 1989).

1.6.1.4 Myelin Basic Protein (MBP)

The *MBP* gene encodes two families of proteins: classic MBPs and golli MBPs (Campagnoni *et al.*, 1993; Pribyl *et al.*, 1993). The classic MBPs are important myelin constituent proteins in the CNS & PNS. Associated with the myelin membrane, the isoforms 14- and 18.5 kDa classic MBPs are predominantly present in adult human myelin and regulate myelin compaction, while the 17- and 21.5 kDa isoforms are dispersedly present in cytoplasm and accumulated in the nucleus of OLs (Allinquant *et*

al., 1991; Hardy *et al.*, 1996; Pedraza, 1997). Together, they constitute about 30% of the total myelin protein in CNS (Morell, 1984). A peripheral transmembrane protein, MBP acts as an intracellular adhesion molecule, fusing and stabilizing the myelin sheath (Kettenmann and Ransom, 2005) **(Fig. 2)**. The classic MBPs are shown to interact with cytoskeletal proteins, including actin and tubulin (Dyer and Benjamins, 1988; Gillespie *et al.*, 1989; Wilson and Brophy, 1989; Boggs *et al.*, 2006; Galiano *et al.*, 2006). In OLs lacking MBP, actin bundles do not form, and are not co-localized with microtubules (Dyer *et al.*, 1997). Such an interaction of membrane-bound MBP with cytoskeletal components helps actin to bind to the cytosolic surface of OLs. This interaction also helps in membrane sheet extension as well as stabilization. Apparently, mice lacking functional MBP (Shiverer) exhibit severe ataxia associated with CNS hypomyelination and have only one or two layers of loosely compacted myelin sheath. (Rosenbluth, 1980; Barbarese *et al.*, 1983; Wolf and Billings-Gagliardi, 1984; Roach *et al.*, 1985) The myelin defects seen in the Shiverer mouse are rescued by MBP-producing OL progenitors (Windrem *et al.*, 2004). Thus, any little dysregulation in the expression levels of MBP results in significant changes in myelin adhesion and/or stability, thus affecting myelin function (for example (Min *et al.*, 2009).

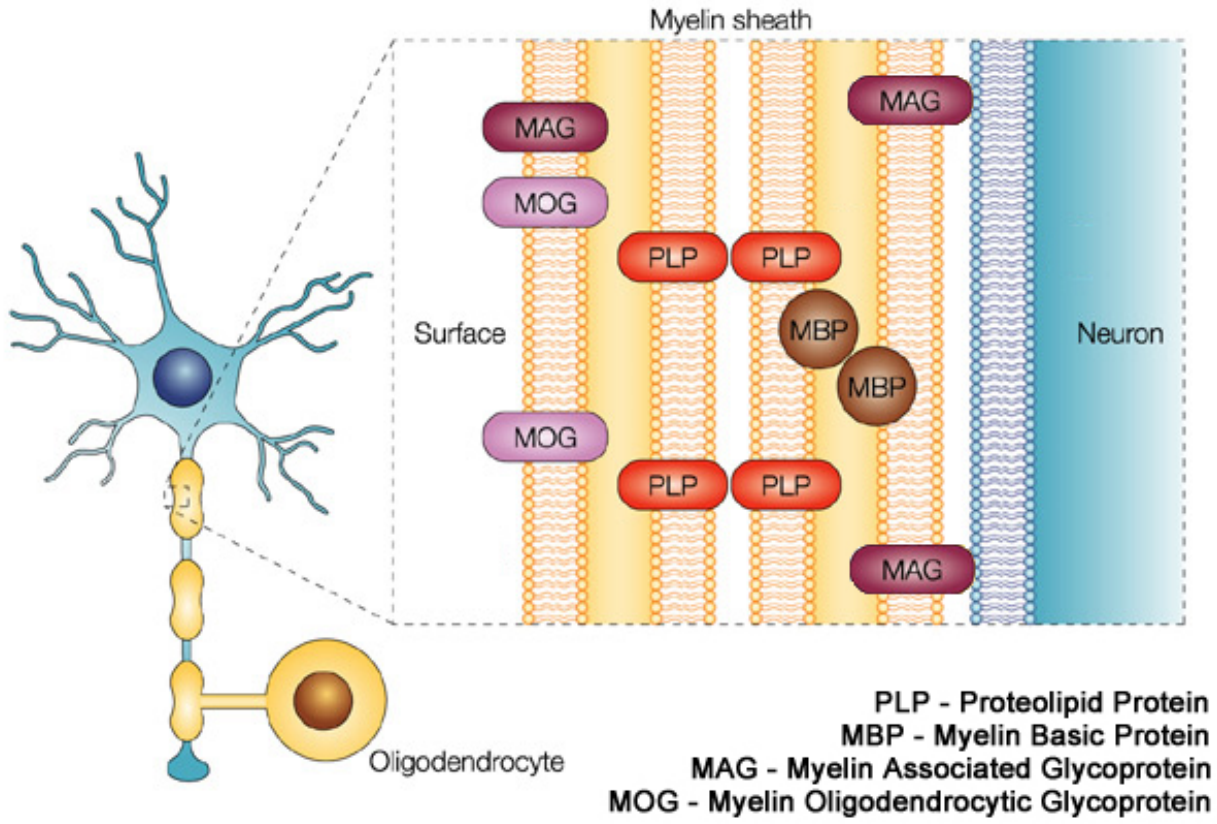


Figure 2: The structural components of myelin sheath. (Hemmer, Archelos et al., 2002)

The main structural components of myelin include: MAG, myelin-associated glycoprotein; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein. Reprinted by permission from Macmillan Publishers Ltd: Nature Publishing Group (Hemmer, Archelos et al., 2002), copyright (2002) http://www.nature.com/nrn/journal/v3/n4/full/nrn784.html

1.6.1.5 Myelin Associated Glycoprotein (MAG)

MAG, a relatively minor component of myelin, is critical for membrane wrapping and paranodal organization on the neuronal axon (Schachner and Bartsch, 2000) **(Fig. 2)**. It is spliced in two major forms, known as large (L-) and small (S-) MAG (Schachner and Bartsch, 2000). MAG isoforms are generated through alternative exon splicing (Tropak *et al.*, 1988). L-MAG (72kD) is dominant during early development until the start of myelinogenesis; after which levels decline and S-MAG (67kD) becomes more increasingly expressed in the adult brain (Lai *et al.*, 1987; Mikoshiba *et al.*, 1991). Early expression of MAG suggests its role in the initial interaction of OL processes with axons before myelination. However, relatively high levels of MAG in the postnatal brain may play an important role in maintenance of myelin and myelinated axons. Acting as a bi-functional cue, MAG is known to promote embryonic and neonatal neurite outgrowth. It also acts as a major inhibitor of postnatal neurite outgrowth (Johnson *et al.*, 1989; McKerracher *et al.*, 1994; DeBellard *et al.*, 1996; Turnley and Bartlett, 1998). MAG^{null} mice show significant neurological deficits (Li *et al.*, 1994; Montag *et al.*, 1994; Yin *et al.*, 1998; Pan *et al.*, 2005). It displays structural abnormalities in the periaxonal areas of the myelin sheath, more specifically in the periaxonal cytoplasmic collar. The myelin compaction seems to be relatively normal, but there is a significant delay in the onset of CNS myelination. Such observations suggest the presence of compensatory mechanisms by molecules similar to MAG in the CNS, which at least partially, makes up for the absence of MAG in the MAG-deficient mice (Montag, Giese et al., 1994).

1.6.1.6 Myelin Oligodendritic Glycoprotein (MOG)

MOG is a minor glycoprotein important for myelination in CNS. It was first identified as an antigen responsible for the demyelination in animals injected with CNS homogenate (Birling *et al.*, 1993). Only present in mammalian species, the functionality of MOG is relatively unknown. It is a transmembrane protein expressed on the surface of OLs and on the outermost lamellae of myelin sheaths (Brunner *et al.*, 1989) **(Fig. 2)**. MOG is expressed by OLs in their later stage of differentiation, and is widely used as a surface marker of OL maturation (Scolding *et al.*, 1989; Coffey and McDermott, 1997). MOG is speculated to serve as a necessary "adhesion molecule" to provide structural integrity to myelin (Johns and Bernard, 1999).

1.6.1.7 2' 3' – Cyclic Nucleotide 3' – Phosphodiesterase (CNPase)

CNPase constitutes ~4% of the total myelin protein. *In vitro*, this enzyme hydrolyzes 2', 3' cyclic nucleotide to produce a 2' nucleotide derivative. However, 2',3' nucleotides have not been detected in the brain (Vogel and Thompson, 1988) and hence the role of this protein remains uncertain. CNPase may be playing some role unrelated to its enzymatic property for which no substrate has been found (Braun *et al.*, 1991). It is found in all stages of the OL lineage (Sprinkle, 1989; Yu *et al.*, 1994). The CNPase protein is mainly found in the cytoplasm of noncompacted OL sheath of the axons and

in the paranodal loops. It is absent from compacted myelin (Braun *et al.*, 1988; Trapp *et al.*, 1988). CNPase-deficient mice reveal that the absence of CNPase leads to axonal swelling and neuronal degeneration (Lappe-Siefke, Goebbels et al., 2003). Recent studies also show defective paranodal loop formation with cytoplasm filled extensions of OL on either side of the node (Rasband *et al.*, 2005). Overexpression of CNPase leads to abnormal myelin formation with aberrant OL membrane expansion (Gravel *et al.*, 1996)

1.7 Myelin Plasticity

Until recently, the term 'neuroplasticity' largely referred to changes in structural and functional ability of neurons upon exposure to different environmental conditions. However, lately, myelin forming OLs are shown to have some degree of plasticity with structural changes in the white matter after learning complex tasks (reviewed in (Fields, 2010)). For example, magnetic resonance diffusion-tensor imaging studies reveal that professional concert pianists have more myelinated fibre tracts in the posterior limb of the internal capsule, compared to the age-matched control (Bengtsson *et al.*, 2005). The same region was also shown to have extensive myelination in subjects who practiced piano in their childhood (Bengtsson, Nagy et al., 2005). Similar imaging techniques show changes in the white matter structure in the human adult brain following training involving complex visuo-motor skills (Scholz *et al.*, 2009). Furthermore, structural scans of the brains of adults that learned to read as an adult

compared to those who were illiterate shows increased white matter in their corpus callosum (Carreiras *et al.*, 2009). In addition, housing rats in a complex environment results in increased number of OLs and more extensive myelination in specific areas of the brain (Sirevaag and Greenough, 1987; Markham and Greenough, 2004). Similar results were observed upon differential rearing of rhesus monkeys (*Macaca mulatta*) (Sanchez *et al.*, 1998). On the contrary, imaging studies on humans show decreased (17%) corpus callosum in subjects who had experienced childhood neglect (Teicher *et al.*, 2004). Studies on early blind subjects (>2 years) also show a decrease in white matter in the early visuo-optic areas with an equally significant increase in myelin in the sensory-motor system (Noppeney *et al.*, 2005). Such an observation demonstrates the presence of compensatory experience-dependant white matter plasticity in brain.

1.8 Disorders of Myelin

A number of neurological disorders lead to defects in the myelin sheath resulting in abnormal functioning of the nervous system. Some of these disorders lead to demyelination, which results in destruction of the existing myelin sheath along with degeneration of axons lying beneath. Conversely, other group of myelin disorders develop from defective myelin sheath formation or maintenance and thereby referred as the dysmyelinating diseases. These disorders are mainly because of dysfunctional OL or Schwann cells. Several prominent myelin related disorders are discussed below.

1.8.1 Multiple Sclerosis (MS)

MS is the most prevalent demyelinating disorder that represents the principle cause of neurological disability in young adults (Noseworthy *et al.*, 2000). The exact mechanism, underlying the formation of the characteristic chronic inflammatory demyelinating lesions in the CNS, is still not clear. However, the most widely accepted theory is that of an autoimmune disease which leads to inflammation, followed by demyelination and OL death. The inability to repair the areas where myelin has eroded, results in astrocytic scar formation, further myelin degeneration and eventual axonal loss (Prineas and Raine, 1976; Adams *et al.*, 1989; Ransohoff, 1999; Melanson *et al.*, 2009). Induction of autoimmune responses against myelin or myelin related components in the peripheral blood, leads to activation of naive T cells to the inflammatory Th1 subtype that eventually cross the blood-brain barrier (BBB) to exert their targeted attack against CNS myelin. Within the CNS, these inflammatory TH1 cells start to proliferate and also secrete pro-inflammatory cytokines such as, but not limited to BDNF, TNFα, IFNγ etc., which results in the recruitment of B cells that ultimately damage the myelin (Zamvil and Steinman, 2003; Peterson and Fujinami, 2007). The resultant myelin damage interrupts or prevents the normal propagation of electrical impulses essential for normal physiological functioning. As a result, patients with MS initially experience, weakness in limbs, pain insensitivity, gait instability and ataxia (Roxburgh *et al.*, 2005). These clinical neurological deficits start to occur either in the form of discrete attacks (relapsing-remitting MS) that the patient fully recovers from or progress slowly accumulating more neurological disabilities over time (progressive MS) from which the patient never

recovers, thereby leaving them with a permanent disability. As the disease worsens, other physiological areas deteriorate such as bladder function, fatigue, cognitive deficits, memory loss , impaired attention and slower information processing (Hauser and Oksenberg, 2006; Melanson, Miao et al., 2009; Melanson *et al.*, 2010).

1.8.2 The Leukodystrophies

The leukodystrophies include a group of hereditary metabolic disorders with similar genetic origin and are characterized by demyelination/dysmyelination in the CNS as well as PNS. Each of these disorders is caused by a specific inherited biochemical defect in the metabolism of myelin-related proteins or lipids in the brain tissue. The major leukodystrophic conditions are listed below:

1.8.2.1 Adrenoleukodystrophies (ALD)

ALD is an X-linked genetic disorder characterized by mutation in the ATP-binding cassette, sub-family D (ALD), member 1(*ABCD1*) gene (Mosser *et al.*, 1993). ALD affects 1 in 16800 individuals who are hemizygous males and heterozygous females (Bezman *et al.*, 2001). About 40% of these males, who have a mutation in their childhood, die within several years because of cerebral demyelination (Moser *et al.*, 1980; Suzuki *et al.*, 2005). The mutation in the *ABCD1* gene results in accumulation of a very long chained fatty acid (VLCFA) in the neural tissues and adrenal glands (Bezman,

Moser et al., 2001). After exhibiting normal early development, impaired metabolism of VLCFA, which is the structural component of myelin, leads to myelin deterioration followed by loss of motor skills and regression (Moser *et al.*, 1999).

1.8.2.2 Metachromatic leukodystrophies (MLD)

MLD is an autosomal recessive disorder caused by mutation in lysosomal hydrolase arylsulfatase A (ARSA) gene (Gieselmann *et al.*, 1991). This leads to accumulation of ARSA substrate galactosylceramide I^3 –sulfate (sulfatide), a major sphingolipid of myelin. It results in myelin deterioration in the CNS and PNS and accumulation of sulfatide in glial and neuronal cells. The characteristic symptoms of MLD are progressive neurological dysfunction, including seizures and ataxia, leading to deformed cerebral cortex and death in infancy (Hagberg, 1971)

1.8.2.3 Globoid Cell leukodystrophies (GLD)

GLD, also known as Krabbe's disease, is a lysosomal storage disorder caused by mutation in the lysosomal enzymes cerebroside β-galactocerebrosidase (GALC), which catabolizes galactocerebrosides (Suzuki and Suzuki, 1970; Wenger *et al.*, 1997). This manifestation leads to accumulation of galactocerebrosides and psychosine in OL and Schwann cells and ultimately results in demyelination and cell death (Miyatake and Suzuki, 1972). Children with Krabbe's disease display hyperirritability, fever followed by

seizures, and also cognitive decline and developmental arrest (Kolodny *et al.*, 1991; Wenger, Rafi et al., 1997; Suzuki, 2003).

1.8.2.4 Pelizaeus-Merzbacher's disease (PMD)

PMD is a recessive X-linked dysmyelinating disorder of the CNS. Mutation in the *Proteolipid Protein 1 (PLP1)* gene, a major structural component of myelin, located on the chromosome Xq22.3 (Mattei *et al.*, 1986) is the causative factor of PMD (Gencic, Abuelo et al., 1989; Hudson, Puckett et al., 1989). Patients with PMD have reduced white matter because of the inability of OLs to myelinate axons. Involuntary eye movement or nystagmus is noted in early infancy, with poor motor control of the head. A progressive disease, PMD patients exhibit abnormally slow & scanning speech, ataxia and cognitive impairment later in life (reviewed in (Koeppen, 2005; Garbern, 2007)).

1.8.2.5 Progressive multifocal leukoencephalopathy (PML)

First described in 1958, Progressive Multifocal leukoencephalopathy (PML) is a demyelinating disorder of the brain caused by a destructive infection of OLs by polyomavirus JC (Astrom *et al.*, 1958; Berger and Major, 1999). It is an opportunistic infection which occurs predominantly in immunosuppressed patients with lymphoid malignancies, HIV infection and individuals with cell transplantation (Richardson, 1988; Major *et al.*, 1992; Berger and Houff, 2009). The JC virus which can replicate only in OLs, presents with progressive demyelinating lesion in white matter, or near grey-white

matter junctions. Infected OLs are characterized by enlarged hyperchromatic nuclei. The demyelination in the CNS is secondary to the death of these virus-infected OLs (Hair *et al.*, 1992; Weber *et al.*, 2001). PML patients develop neurological symptoms including dementia, apraxia, visual deficits and motor problems within weeks of infection. Without immunomodulatory therapies, the disease progresses very fast and can lead to death of the patient within months (Krupp *et al.*, 1985; Berger and Major, 1999).

1.8.3 Other Diseases

The importance of myelination to normal brain function is evident from the pathology of various disorders of myelin as discussed above. However, the significance of myelination has been more apparent recently from the pathology of numerous disorders of the brain, which were previously thought to have no myelin defects. For example, in Down's syndrome and Alzheimer's Disease, the expression levels of CNPase is significantly decreased (Vlkolinsky *et al.*, 2001). Furthermore, patients suffering from schizophrenia show decreased density of OLs (Tkachev *et al.*, 2003; Uranova *et al.*, 2007; Vostrikov *et al.*, 2007), down-regulation of myelin genes (Hakak *et al.*, 2001; Haroutunian *et al.*, 2007) and decreased white matter volume (Sigmundsson *et al.*, 2001). In addition, myelin defects also characterize several disorders of later life, e.g. age related cognitive decline (Hinman and Abraham, 2007), major depression and bipolar disorder (Tkachev, Mimmack et al., 2003; Fields, 2005). Disorders like autism

(Hendry *et al.*, 2006; Ke *et al.*, 2009) and cerebral palsy (Ancel *et al.*, 2006; Juliet *et al.*, 2009) are also known to show myelin irregularities

Chapter 2: "Differential Regulation of OL Progenitor (OP) migration by Extracellular Regulated Kinase (ERK) 1/2 in the developing brain"

2.1 Introduction

2.1.1 Importance of OP migration

Oligodendrogenesis requires careful orchestration of migration, differentiation, and proliferation of OP. The first evidence of OP migration came from the *in vitro* studies of optic nerve, where the neuronal axons were myelinated by OL that originated in the brain (Small, Riddle et al., 1987). In the brain, OLs originate as pre-progenitors in the embryonic telencephalon (Rakic and Zecevic, 2003). The pre-progenitors subsequently migrate to the germinal matrix of the subventricular zone (SVZ) via the ganglionic eminences (Rakic and Zecevic, 2003). The OPs migrate away from the SVZ to populate the white matter tracts of the brain, including the corpus callosum and the cortex (Levison and Goldman, 1993). Thus, OP migration is a pre-requisite for myelination in the brain. Aberrant migration patterns can result in delayed onset or complete absence of myelination (Back et al., 2001; Volpe, 2001). Unusual OP migration can be a result of lack, or failure, of transiently and temporally regulated functional regulatory signals (Fruttiger *et al.*, 1999; Spassky *et al.*, 2002; Tsai, Frost et

al., 2002; Tsai *et al.*, 2003; Finzsch *et al.*, 2008). In addition, a large number of locally and transiently expressed environmental cues have been reported to be involved in regulating OP migration, as well as modulating phenotypic plasticity of differentiated OL. These signals include soluble signaling proteins and extracellular matrix proteins (Canoll *et al.*, 1996; Ellison *et al.*, 1996a; Frost *et al.*, 1996; Milner *et al.*, 1997; Tsai, Frost et al., 2002).

2.1.2 Platelet-derived growth factor

One such signaling molecule, platelet derived growth factor A (PDGF-A), is known to be essential for the development of an intact myelin system (Richardson *et al.*, 1988; Calver, Hall et al., 1998; Fruttiger, Karlsson et al., 1999). Transgenic animal models show that ablation of PDGF expression leads to significant developmental defects and development of tremor phenotype (Soriano, 1997; Fruttiger, Karlsson et al., 1999). Moreover, the mice show reduced number of OPs in the CNS with the most severe deficiency of these cells at the sites more distal to the origin of the precursors near the periventricular region. Thus, lack of PDGF-A leads to insufficient OL expansion and migration rather than a failure to specify and develop OPs. A mouse model that is heterozygous for PDGF receptor-alpha (PDGFRα) also shows significantly reduced numbers of OP in the developing CNS (Smith *et al.*, 1991; Li *et al.*, 1996). Further, over-expression of PDGF-A leads to a significantly increased numbers of OPs in the developing CNS (Soriano, 1997). PDGF-A is known to modulate several different OP

behaviours, including proliferation (Baron *et al.*, 2000; Ebner, Dunbar et al., 2000; Baron *et al.*, 2002b; Frost *et al.*, 2003), migration (Armstrong *et al.*, 1991; Frost, Kiernan et al., 1996; Frost *et al.*, 2009; Vora, Pillai et al., 2011), and survival (Barres, Hart et al., 1992a; Ebner, Dunbar et al., 2000).

2.1.3 Fibroblast-derived growth factor

At least 22 different FGFs are expressed in a temporally and spatially regulated manner in the brain (Ford-Perriss *et al.*, 2001). FGF2 is known to play a significant role in the regulation of OP behaviour, including migration (Milner, Anderson et al., 1997; Fortin *et al.*, 2005), proliferation (Bogler *et al.*, 1990; Wolswijk and Noble, 1992; Frost, Nielsen et al., 2003), differentiation (Bansal *et al.*, 1996; Baron, Metz et al., 2000; Fortin, Rom et al., 2005), and survival (Fortin, Rom et al., 2005; Murtie *et al.*, 2005a). Like PDGF receptors, FGF receptors are also RTKs. Three different FGF receptors are expressed at different stages of the OL lineage, and they regulate different behavioural processes of the cell (McKinnon, Matsui et al., 1990; Bansal, Kumar et al., 1996; Fortin, Rom et al., 2005). In contrast to PDGF-A, FGF2 knockout mice are viable and fertile (Milner, Anderson et al., 1997; Fortin, Rom et al., 2005). Though the myelin of the $FGF2^{-/-}$ mouse appears to be normal (Murtie, Zhou et al., 2005a), the $FGF2^{-/-}$ brain is smaller in size and has reduced cell numbers (Vaccarino *et al.*, 1999; Raballo *et al.*, 2000). Similar to PDGF-A, FGF2 promotes OP migration (Eccleston and Silberberg, 1985;

Milner, Anderson et al., 1997), proliferation, and prevents their differentiation (Eccleston and Silberberg, 1985; Bogler, Wren et al., 1990; Gard and Pfeiffer, 1993).

2.1.4 Extracellular Regulating Kinase 1/2

Extracellular regulating kinase (ERK) signaling is required for normal brain development, specifically in processes like proliferation, differentiation and myelination (Samuels *et al.*, 2008; Satoh *et al.*, 2011). The ERK1 and ERK2 (also known as p44 and p42 MAP kinases respectively) are members of the mitogen-activated protein (MAP) kinase family and share 84% amino acid sequences (Boulton *et al.*, 1991; Yoon and Seger, 2006). Both, ERK1 and ERK2 have been shown to be activated in order to bring about proper neurogenesis (Satoh, Kobayashi et al., 2011). Predominantly involved in protective mechanisms, ERKs in the brain are activated by phosphorylation of both threonine and tyrosine residues with the help of growth factors and cytokines (Boulton, Nye et al., 1991; Hetman *et al.*, 1999; Widmann *et al.*, 1999; Pearson *et al.*, 2001). ERK1/2 is involved in a variety of cell migration (Klemke *et al.*, 1997; Cheresh *et al.*, 1999; Webb *et al.*, 2000; Olsson *et al.*, 2001; Huang *et al.*, 2004), but the functions of these isoforms in relation to OL progenitor migration remain to be determined.

2.2 Rationale and Hypothesis

PDGF-A regulates OP behaviour via the PDGFRα, a tyrosine kinase receptor (RTK) which, upon phosphorylation, activates numerous different signaling pathways.

Previous studies have shown that OP proliferation is regulated via the PI_3K pathway (Ebner, Dunbar et al., 2000), whereas differentiation is regulated via $p38^{MAPK}$ (Haines et al., 2007). Recently, it was shown that OP migration is regulated by ERK2/1 (also known as $p44/p42^{MAPK}$) signaling (Frost, Zhou et al., 2009). Our earlier study also showed that transient activation of the PDGFRα receptor, for less than 30 minutes, is sufficient to induce OP migration for at least 72 hours (Frost, Zhou et al., 2009). The receptors of both, PDGF-A and FGF2 are RTKs; however, the studies of knockout mice have shown that FGF2 is functionally redundant during developmental myelination (Armstrong *et al.*, 2002; Murtie *et al.*, 2005b). Moreover, FGF2 and PDGF-A were found to interact with each other in order to regulate the OP proliferation (McKinnon, Matsui et al., 1990; Wolswijk and Noble, 1992). Hence, in order to fully understand the role of PDGF-A and FGF2 in the regulation of OP migration in the developing brain, we hypothesized that:

1) OP migration is regulated through transient activation of ERK1/2 signaling pathway upon PDGF-A and FGF2 treatment.
2) Combinatorial treatment of OPs with growth factors, PDGF-A and FGF2 results in enhanced migration.

2.3 Methods

2.3.1 Cell isolation and culture

OP were isolated from neonatal rat pups (P0-P1), by a previously described method (Armstrong, 1998). The cells were cultured in complete medium DMEM-FBS-Glutamine

(DFG) (modified Dulbecco's Modified Eagle's Medium (DMEM)) (Sigma-Aldrich; Cat# D5546) supplemented with 5000U penicillin and streptomycin (Sigma-Aldrich; Cat# 85886), 4mM L-Glutamine (Sigma-Aldrich; Cat# 3126) and 10% FBS (Thermo Scientific; Cat# SH30070). The medium was changed every third day.

Approximately 7-10 days *in vitro* (DIV) after plating, the microglia were dislodged by shaking the flasks for 2 hours, followed by an overnight shake to collect the OP, as previously described (Frost, Zhou et al., 2009). The supernatant, containing cells shaken off the monolayer was placed in a non-tissue culture plastic petri dish (Sterilin, UK) for 25-40 minutes at 37ºC to allow the differential adhesion of microglial cells. After the "subtraction" process, the cells obtained were more than 95% OPs (Milner and Ffrench-Constant, 1994; Frost *et al.*, 1999) **(Fig. 3)**. The cell suspension was collected, concentrated and re-suspended in Sato defined medium with high insulin (Bottenstein and Sato, 1979).

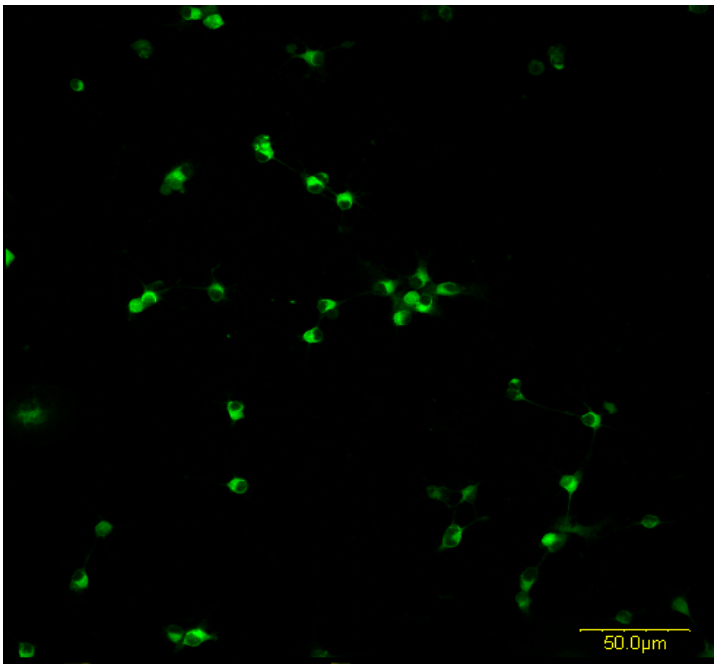


Figure 3: Fluorescein isothiocyanate (FITC) (green) staining PDGFRα +ve cells (OL progenitor cells)

2.3.2 Agarose Drop Migration Assay

Migration was assessed using the agarose drop assay (Frost et al., 2000). Briefly, 35,000-50,000 OPs, counted by a haemocytometer, were suspended in 1.5 µl 0.3% low melting point agarose (Invitrogen; Cat# 16520100) and plated on a poly-D-lysine (PDL: Sigma-Aldrich; Cat# 7405) coated 24-well plate in Sato defined medium (Bottenstein and Sato, 1979). 24 hours after plating, OPs were either exposed to growth factor at 10 ng/ml or 1 ng/ml for 10 minutes (transient), or the growth factor remained in the well for the duration of the experiment (continuous). The 10 ng/ml PDGF-A concentration was based on a number of previous studies investigating the regulation of OP behaviour by PDGF-A (Armstrong, Harvath et al., 1991; Frost, Kiernan et al., 1996; Milner, Anderson et al., 1997; Frost, Zhou et al., 2009) while 1 ng/ml is the physiological concentration of PDGF-A (Baron, Metz et al., 2000; van Heyningen, Calver et al., 2001). The pharmacological inhibitor, U0126 (10 µm) (Sigma-Aldrich; Cat# U120), was added to the well for 30 minutes prior to the addition of growth factor, and remained in the well for the duration of the experiment, as described previously (Frost, Zhou et al., 2009). U0126 is a specific pharmacological inhibitor of MEK1/2, the upstream activator of ERK1/2 (Duncia *et al.*, 1998). The PDGFRα neutralizing antibody (R&D Systems; Cat# AF1062) was added at the concentration of 5 µg/ml, 30 minutes prior to the addition of the growth factor. Migration was measured 72 hours after addition of growth factor. For transient exposure, growth factor was added to the well for the specified time, then aspirated out, the well was washed with one wash of serum free Sato, and the Sato replaced with or without inhibitor, as previously described (Frost, Zhou et al., 2009).

2.3.3 Protein concentration

Total protein concentrations of each sample were assayed by Bradford assay (Bradford, 1976), and diluted to give 20-30 μg in 15 μl sample buffer. Protein values were calculated from a standard curve, using a Molecular Devices - Spectra MAX 190 with SoftMax Pro v5.

2.3.4 Western blot analysis of protein expression

Western blot analysis of protein expression was performed on whole cell lysates prepared from purified OPs treated with growth factor in the presence or absence of pharmacological inhibitors. OPs were treated with the pharmacological inhibitor for 30 minutes. Growth factor was added for 5 minutes, followed by Laemmli sample buffer (Laemmli, 1970) plus protease inhibitor cocktail 1 (Sigma-Aldrich; Cat# P1860) and phosphatase inhibitor cocktail 2 (Sigma-Aldrich; Cat# P2850). The lysates were boiled for 5 minutes and stored at -80°C until analyzed.

For analysis of ERK phosphorylation after 24 hours, cells were plated onto PDL coated 6-well culture plates (Sigma-Aldrich; Cat# M8562) in Sato medium supplemented with 0.5% FBS. Two hours after plating the wells were flooded with serum-free Sato medium for a further 4 hours (reducing the FBS concentration to 0.025%). After which, the pharmacological inhibitor was added to the wells at the specified concentration.

Cells were returned to the incubator for 1-2 hours, following which the growth factor was added to the wells. After a further 30 minutes, the medium was removed from the wells, the cells were washed with serum-free Sato medium, and serum-free Sato medium containing the pharmacological inhibitor alone was added to the cells for the following 24 hours. Cells were lysed by washing with PBS followed by addition of Laemmli sample buffer. Samples were boiled for 4-5 minutes then stored at -80°C until analyzed.

Protein samples (20-30 μg total protein) were resolved by SDS-PAGE (12% gels; 100V for 2 hours) and transferred to PVDF membranes (GE Health Care Worldwide). Blots were blocked with 5% skimmed milk in Tris-buffered saline containing 0.5% Tween-20 (TBS-T) for one hour at room temperature (23C). The blot was incubated with an appropriate dilution of the primary antibody (phospho-ERK (pERK), 1:2000; total ERK (panERK), 1:40,000; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 1:1000) (R&D Systems) in 5% skimmed milk in TBS-T at 4º overnight. Membranes were incubated with HRP-conjugated secondary antibody (Jackson ImmunoResearch) in TBS with 5% Bovine serum albumin (BSA), for one hour at room temperature. A luminol-HRP-chemiluminescence reaction using the Chemilucent ECL Detection System (Chemicon, Temecula, CA, USA) was used to detect the protein bands and membranes exposed to X-ray film.

Densitometry readings were performed using a FluorChem 8900 scanner (Alpha Innotech, San Leandro, CA, USA) with Alpha Ease FC software, and relative density

value (RDV) was calculated against the total ERK1/2 band density for each sample, and then normalized to the control RDV of 1. GAPDH levels were analyzed to assess equality of sample loading. Three different cell preparations were assayed by western blot and the mean densities for each band calculated, normalized to zero and their mean value was used (Frost, Zhou et al., 2009).

2.3.5 Statistical Analysis

The data was expressed as mean ± standard error of the mean. Differences between treatment groups were analyzed using Student's *t* test or a one way analysis of variance with Bonferroni's post-test where appropriate. Statistical analysis was performed with Prism 4.03 software (GraphPad Software, Inc). $p < 0.05$ was considered significant.

2.4 Results

2.4.1 PDGF-A induces dose-dependent response on OP migration for up to 72 hours

Dose-dependent effect of PDGF-A on OP migration was studied using the agarose drop assay (Frost, Milner et al., 2000; Frost, Zhou et al., 2009). A 72 hour time point was

used to assess migration as longer times would require the media to be refreshed, which could significantly affect the outcome of the study.

Continuous exposure to 1 ng/ml PDGF-A resulted in 666.67 ± 56.8 μm migration compared to the control (75.00 ± 26.3 μm). However, there was no significant increase in OP migration when the PDGF-A concentration was further increased from 1 ng/ml up to 20 ng/ml of the growth factor **(Fig. 4)**.

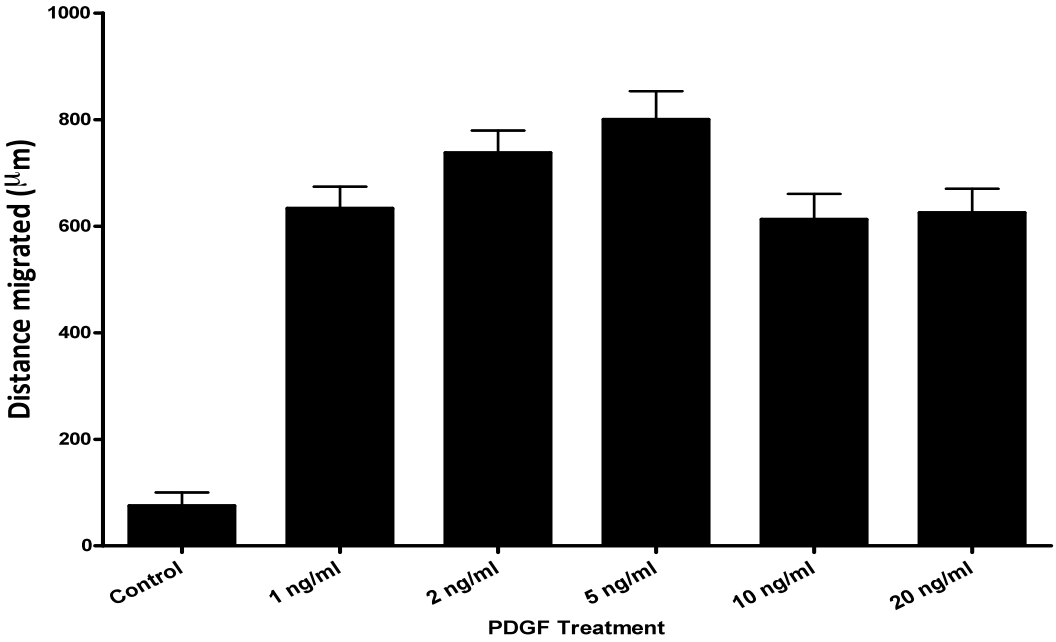
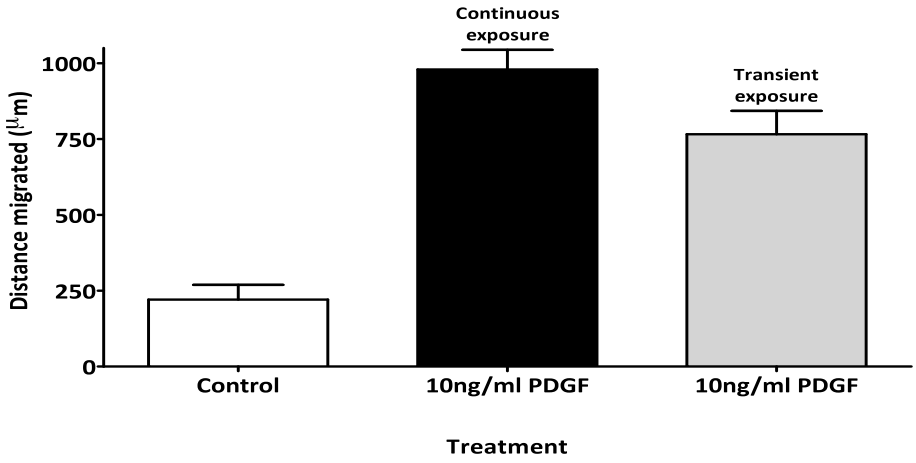


Figure 4: Dose-dependent analysis of the effect of PDGF-A on OP migration, 72 h after exposure.
Error bars represent ±SEM. (Vora, Pillai et al., 2011).

2.4.2 Transient exposure to PDGF-A induces OP migration for up to 72 hours

It was previously shown that OP exposed to 10 ng/ml PDGF-A for 30 minutes, migrate to the same extent as cells continuously exposed to PDGF-A (Frost, Zhou et al., 2009). The present study shows that transient exposure to PDGF-A (10 ng/ml) for 10 minutes resulted in almost the same extent of migration as seen in cells continuously exposed to PDGF-A, at 766.0 ± 76.8 μm and 979.25 ± 64.8 μm respectively **(Fig. 5A)**. However, OP transiently exposed to PDGF-A at 1 ng/ml, which is closer to physiological concentrations (Baron, Shattil et al., 2002b; Brunmark et al., 2002), do not migrate to the same extent as OP exposed continuously to PDGF-A, at 192.38 ± 74.9 μm and 767.0 ± 33.1 μm respectively **(Fig. 5B)**.

(A)



(B)

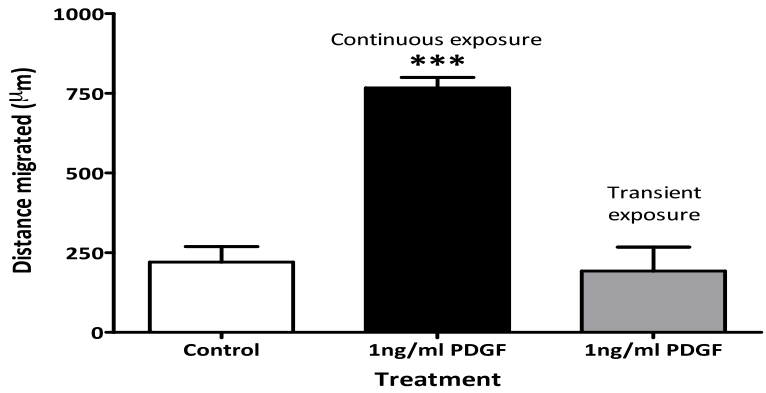
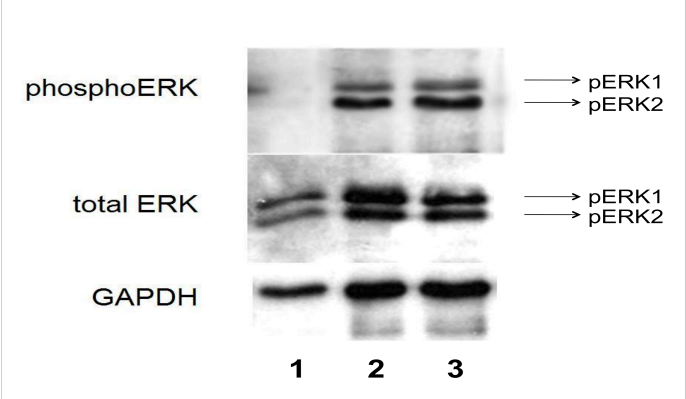


Figure 5: OP migration in response to PDGF-A treatment.
Migration of OP cells in suspension (40,000 cells per well) for both continuous and transient exposure to (A) 10 ng/ml PDGF-A and (B) 1 ng/ml PDGF-A treatment. Migration measured 72 h after the addition of growth factor. N= 5; 4–6 replicates each N. Error bars represent±SEM. ***p < 0.001. (Vora, Pillai et al., 2011)

2.4.3 Transient exposure to PDGF-A is sufficient to activate ERK

Western blot analysis was used to assess levels of ERK phosphorylation resulting from transient exposure to PDGF-A. PDGF-A concentrations of 1 ng/ml and 10 ng/ml both induced phosphorylation of ERK following 10 minutes of exposure time **(Fig. 6A)**. All densitometry values were normalized to the control (in the absence of PDGF-A), which was given a relative densitometry value (RDV) of one (Frost, Zhou et al., 2009) **(Fig. 6B)**. Densitometry analysis shows that the levels of pERK1 following 1 ng/ml PDGF-A and 10 ng/ml PDGF-A are not significantly different from control (untreated cells). However, the levels of pERK2 were significantly increased as compared to control (untreated) levels after 10 minutes exposure to either 1 ng/ml or 10 ng/ml PDGF-A **(Fig. 6B)**.

(A)



(B)

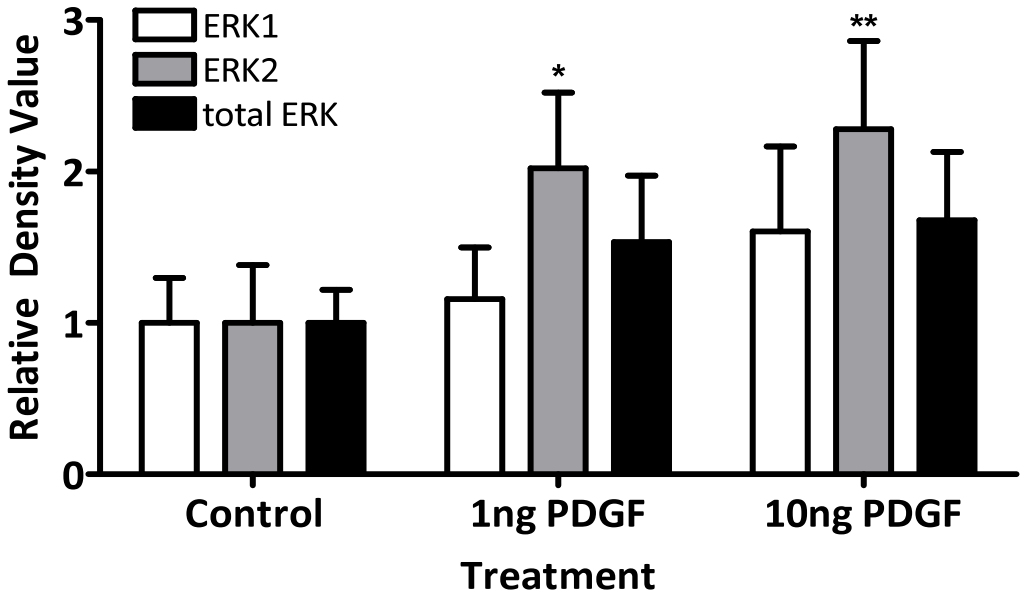


Figure 6: Effect of PDGF-A on ERK phosphorylation.
(A) Representative Western blot for phosphorylated and total ERK. Lane 1 – Growth factor naïve OP; Lane 2 – OP treated with 1 ng/ml PDGF-A for 10 min; Lane 3 – OP treated with 10 ng/ml PDGF-A for 10 min.
(B) Relative density values represent band densities (pERK1/2) relative to the GAPDH loading control and then normalized to a control (no PDGF-A treatment) value of 1. Three different cell preparations were analyzed three times and the mean RDV value was used. N= 3; 3 replicates each N. Error bars represent±SEM. ***p < 0.001 and **p < 0.01. (Vora, Pillai et al., 2011)

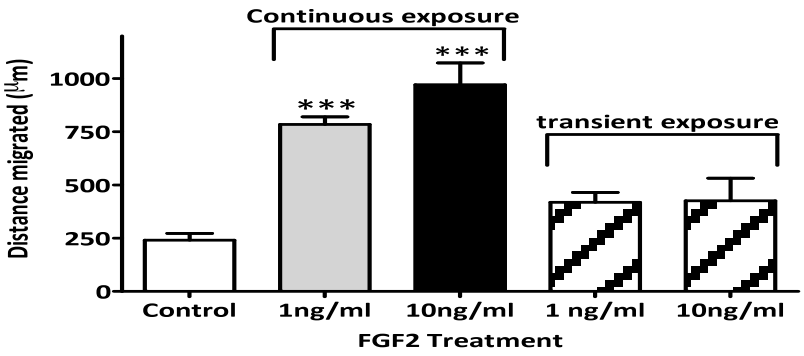


Figure 7: OP migration in response to FGF2 treatment.
Migration of OP cells in suspension (40,000 cells per well) for both continuous and transient exposure to 10 ng/ml FGF2 and 1 ng/ml FGF2 treatment. Migration measured 72 h after the addition of growth factor. N= 5; 4–6 replicates each N. Error bars represent±SEM. ***p < 0.001. (Vora, Pillai et al., 2011)

2.4.4 Transient FGF2 is not sufficient to induce OP migration

To investigate the role of FGF2 in the regulation of OP migration, cells were exposed to FGF2 transiently (10 minutes) and continuously (72 hours). In contrast to the effect of transient PDGF-A exposure, transient exposure to 1 ng/ml and 10 ng/ml FGF2 did not result in any significant change in OP migration (425.98 ± 105.75 μm and 418.64 ± 46.45 μm) from control migration as compared to 970.75 ± 102.35 μm when exposed to the growth factor continuously (66% decrease) **(Fig. 7)** . When exposed to FGF2 continuously throughout the migration assay, OPs migrate to the same extent as when exposed to PDGF-A. Further, transient exposure to 1 ng/ml FGF2 results in only 418.64 ± 46.45 μm migration as compared to 784.66 ± 35.58 μm when the cells are continuously exposed to FGF2 (57% decrease) **(Fig. 7)**.

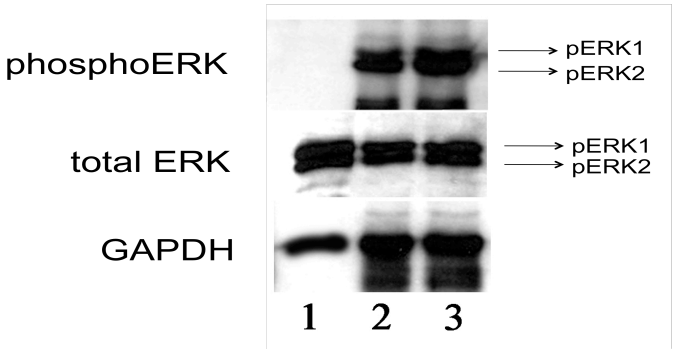
2.4.5 Transient exposure to FGF2 is sufficient to activate ERK

Western blot analysis was performed to assess changes in the levels of ERK as a result of transient exposure to FGF2 at 1 ng/ml and 10 ng/ml concentration. Results revealed significant levels of pERK after only 10 minutes exposure of OP to FGF2 at both concentrations **(Fig. 8A)**. There was no significant difference between FGF2 induced pERK1 at either concentration (1 ng/ml FGF2 RDV 1.17 ± 0.32 and 10 ng/ml FGF2 1.15 ± 0.29) **(Fig. 8B)**. In addition, there was no significant difference between FGF2 induced pERK2 at either concentration (1 ng/ml FGF2 RDV 1.98 ± 0.41 and 10 ng/ml

FGF2 1.87 ± 0.46) **(Fig. 8B)**. Furthermore, there was no significant difference between the levels of pERK1 or pERK2 induced by PDGF-A and FGF at either 1 ng/ml or 10 ng/ml concentrations.

Additionally, we show inhibition of PDGF-A induced OP migration in presence of PDGFRα neutralizing antibody (5 µg/ml concentration)**, however** FGF2 induced OP migration remained unaffected when the receptor for PDGF-A was blocked **(Fig. 9).**

(A)



(B)

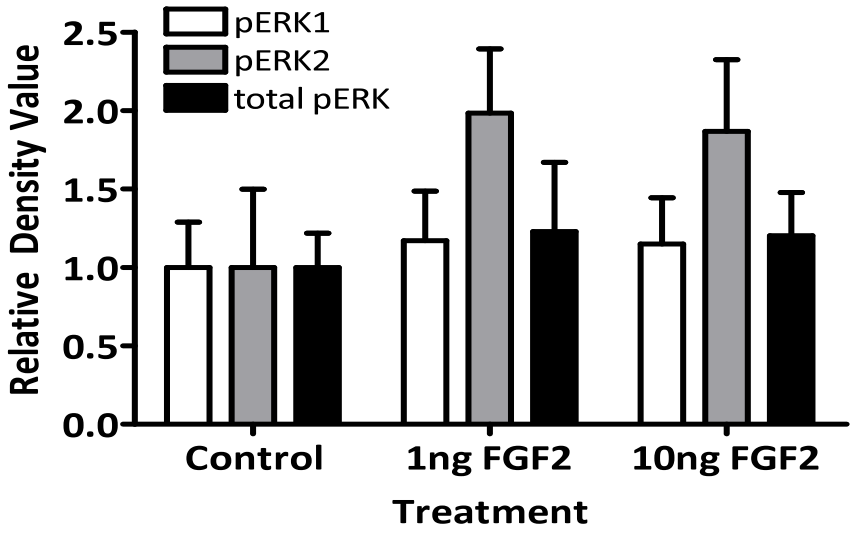


Figure 8: Effect of FGF2 on ERK phosphorylation.
(A) Representative Western blot for phosphorylated and total ERK. Lane 1 – Growth factor naïve OP; Lane 2 – OP treated with 1 ng/ml FGF2 for 10 min; Lane 3 – OP treated with 10 ng/ml FGF2 for 10 min.
(B) Relative density values represent band densities (pERK) relative to the GAPDH loading control and then normalized to a control (no PDGF-A treatment) value of 1. Three different cell preparations were analyzed three times and the mean RDV value was used. N= 3; 3 replicates each N. Error bars represent±SEM. (Vora, Pillai et al., 2011)

2.4.6 Receptor Tyrosine Kinase (RTK) mediated OP migration is regulated via ERK

It was previously shown that PDGF-A-induced migration of OP is regulated via the ERK signaling pathway (Frost, Zhou et al., 2009). In the current study, U0126 (an inhibitor of MEK1/2, the upstream activator of ERK1/2) (Favata et al., 1998; Newton et al., 2000), significantly inhibited 1 ng/ml PDGF-A-induced OP migration at a concentration of 10 μM, as used previously (Frost, Zhou et al., 2009). OP migration was reduced from 956.83 ± 15.60 μm after transient exposure to PDGF-A, to 389.83 ± 12.38 μm (60% decrease) in the presence of U0126 **(Fig. 10A)**. 10 μM U0126 is shown not to have any effect on OP survival or differentiation (Frost, Zhou et al., 2009).

FGF receptors are also members of the RTK receptor class. The intracellular signaling pathways activated by FGFs are essentially the same as those activated by PDGFs (Schlessinger, 2000). In this study, OP was incubated in 10 μM U0126 for 30 minutes to block ERK activation prior to the addition of 10 ng/ml FGF2. U0126 treatment significantly inhibited FGF2 induced OP migration as compared to untreated cells **(Fig. 10B)**. OPs migrated 968.25 ± 45.20 μm in the presence of FGF2 and 578.83 ± 14.41 μm (40% decrease) after pretreatment with U0126. This data indicates that FGF2-induced OP migration is regulated via the ERK signaling pathway.

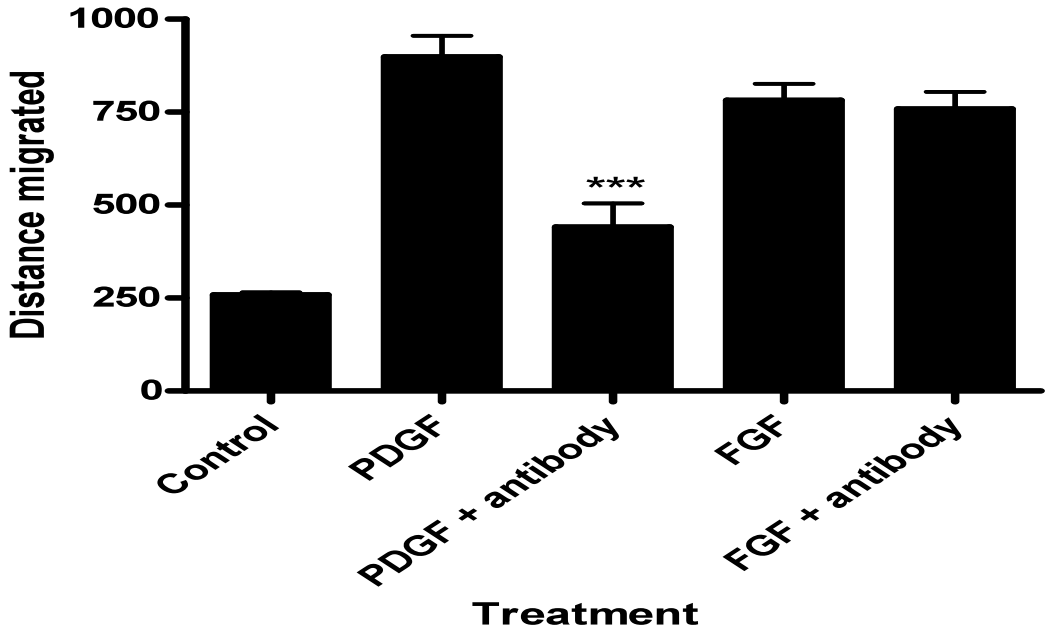
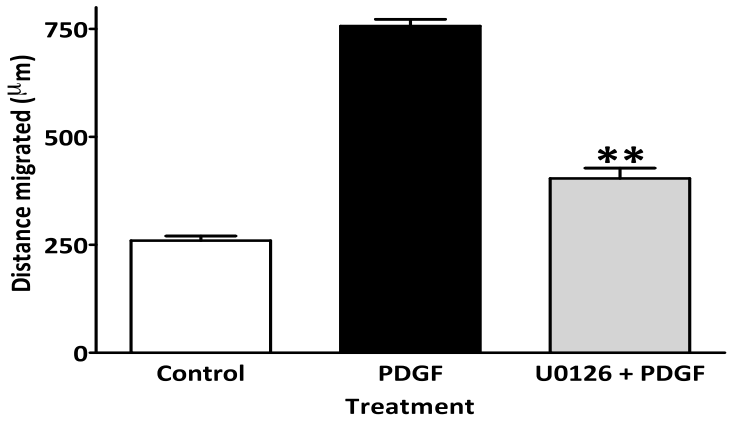


Figure 9: Effect of PDGFR-α neutralizing antibody (5µg/ml concentration) on OP migration following both, PDGF-A and FGF2 exposure. N= 3; 16 replicates each N. Error bars represent ± SEM. ***p < 0.001. (Vora, Pillai et al., 2011)

(A)



(B)

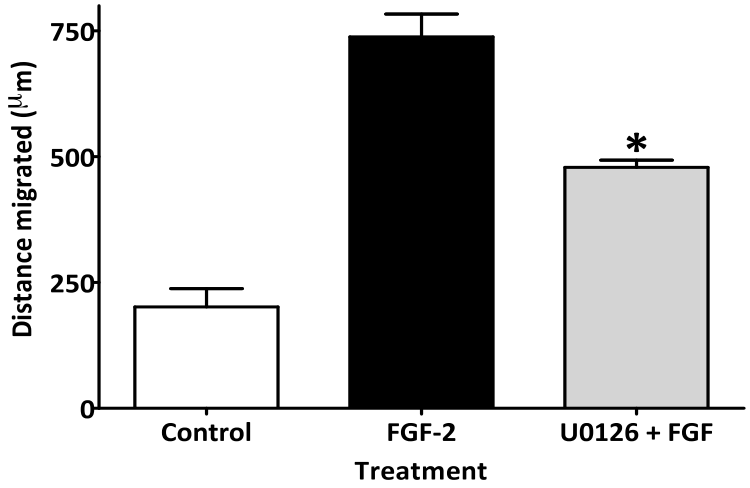


Figure 10: Effect of growth factors on OP migration following U0126 (MEK1/2 inhibitor) exposure.
(A) PDGF-A treated OP migration. (B) FGF2 treated OP migration. Migration measured 72 h after the addition of growth factor (distance measured in μm). N= 5 with 4–6 replicates. Error bars represent±SEM. **p < 0.005 and ***p < 0.001 vs. PDGF-A and FGF2, respectively. (Vora, Pillai et al., 2011)

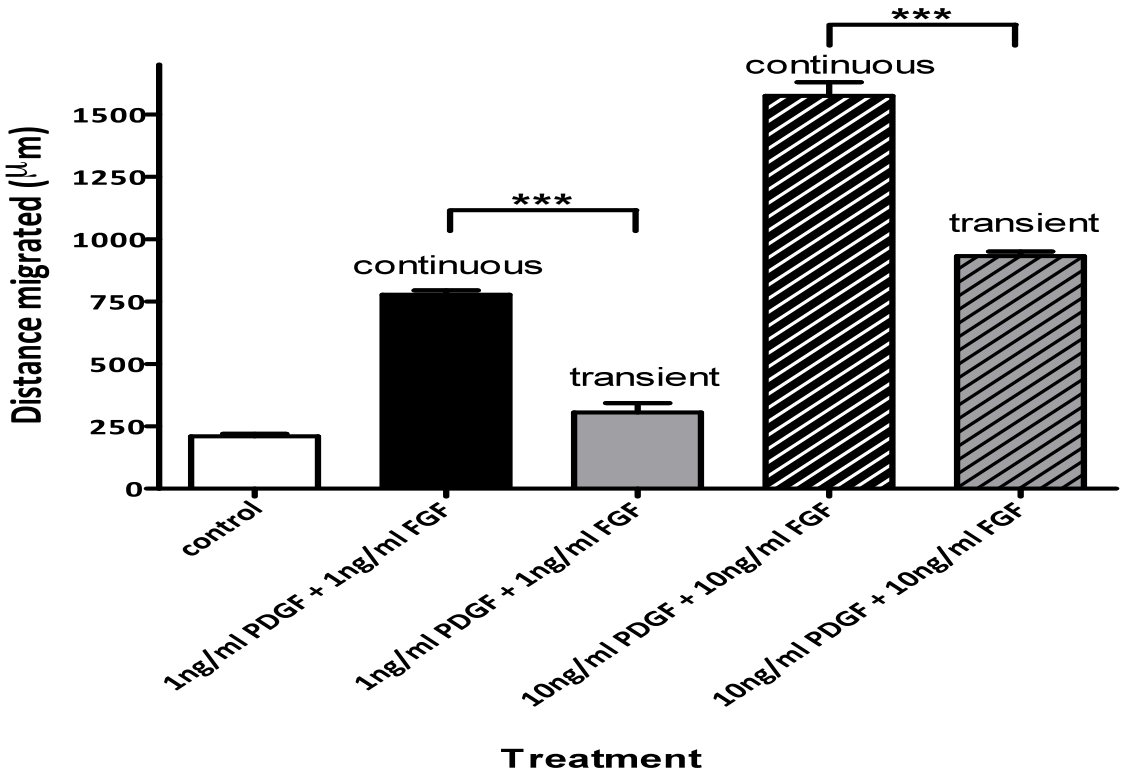


Figure 11: Effect of co-exposure to PDGF-A and FGF2 on OP migration.
Migration is measured 72 h after the addition of growth factor. N= 5 with 4–6 replicates. Error bars represent±SEM. ***p < 0.001 vs. continuous exposure. (Vora, Pillai et al., 2011)

2.4.7 Combinatorial effects of PDGF-A and FGF2 on OP migration

In response to transient (10 mins) exposure of a combination of PDGF-A and FGF2 at 1 ng/ml, there is no increase in migration as compared to control (untreated) levels (at 305.56 ± 37.84 and 210.05 ± 9.65 μm respectively). However, when the growth factors were exposed continuously (72 hours), OPs migrated 777.20 ± 18.07 μm compared to transient exposure (254% increase). When treated with a higher concentration of 10 ng/ml, the extent of migration was significantly increased with continuous exposure to combinatorial effects of PDGF-A and FGF2 as compared to transient exposure (10 mins), 1575 ± 54.84 μm and 932.29 ± 18.11 μm respectively (168% increase). **(Fig. 11)**

2.5 Discussion

Migration of OP is an essential process that precedes proliferation and differentiation of the cells to form myelinating OL. However, the mechanisms that regulate the temporal and spatial dispersal of OP remain to be fully clarified. PDGF-A and FGF2 are both well characterized as potent mitogens for OPs (Richardson, Pringle et al., 1988; Wolswijk and Noble, 1992; Ebner, Dunbar et al., 2000; Frost, Nielsen et al., 2003). Both growth factors have also been shown to be potent motogens for OPs *in vitro* (Armstrong *et al.*, 1990; Frost, Kiernan et al., 1996; Milner, Anderson et al., 1997; Frost, Zhou et al., 2009).

PDGF-A has been shown to be crucial for normal myelination of the CNS (Calver, Hall et al., 1998; Tsai, Frost et al., 2002) while FGF2 appears to be functionally redundant for oligogenesis (Miller *et al.*, 2000; Armstrong, Le et al., 2002; Murtie, Zhou et al., 2005a). The binding of PDGF-A or FGF2 to their respective receptors leads to receptor dimerization and phosphorylation, and recruitment of other intracellular proteins that associate with the tyrosine-phosphorylated receptors. It has already been established that PDGF-A and FGF2 are able to trigger a multitude of similar signaling pathways, including phospholipase C gamma (PLCγ) and Ras GTPase-activating protein (Mohammadi *et al.*, 1992; Claesson-Welsh, 1994b; Kanda *et al.*, 1997; Klint and Claesson-Welsh, 1999). However, to date, there are no published reports on the common intra-cellular pathways that regulate OP migration. The current study investigated both transient and continuous exposure to PDGF-A and FGF2 individually and in combination on OP migration. Differential effects of PDGF-A and FGF2 were observed in terms of both cell-migration and the signaling events that bring about OP migration.

PDGF-A is known to play a significant role in the regulation of OP migration (Armstrong, Harvath et al., 1991; Tsai, Frost et al., 2002; Frost, Zhou et al., 2009). It has been already shown that OP migration is regulated by the ERK signaling pathway and PDGF-A induced OP migration does not require continuous exposure to PDGF-A (Frost, Kiernan et al., 1996; Tsai, Frost et al., 2002; Frost, Zhou et al., 2009). In this study, it was found that at 1 ng/ml PDGF-A can only induce OP migration when the cells are continuously exposed to the mitogen, and these results are in line with earlier reports

(Frost, Kiernan et al., 1996; Tsai, Frost et al., 2002). The dose-dependency analysis suggests that saturation of PDGF-A receptors on the OP cell surface occurs, thus migration doesn't change with increased PDGF-A concentration. However, transient exposure to PDGF-A at low concentration (1 ng/ml), did not demonstrate any significant migration compared to control (untreated) cells. Transient exposure to PDGF-A at 10 ng/ml concentration showed comparable OP migration to that of continuously exposed cells (Frost, Zhou et al., 2009).

One possible explanation for this result is related to receptor activation threshold levels. According to this hypothesis transient exposure to higher concentrations of PDGF-A is sufficient for OP migration, and with lower concentrations, the minimum threshold levels of receptor phosphorylation are not achieved, hence the cells fail to migrate. In addition, according to this theory threshold levels of receptor activation are achieved with sustained exposure to low concentrations of PDGF-A, resulting in OP migration.

Internalization of the activated receptor/ligand complex has been shown to contribute to the duration, magnitude, and nature of a cell's response to the growth factor (Sorkin and Von Zastrow, 2002). OPs are known to require prolonged exposure to PDGF-A for longer than two hours in order for proliferation to occur (Frost, Zhou et al., 2009). Previous studies have shown that the PDGF-A receptor/ligand complex is internalized approximately 8 hours after ligand binding (Heldin and Westermark, 1999; Heldin and Ericsson, 2001). Thus it is entirely possible that OPs migration induced by PDGFRα activation is regulated by cell surface receptors, and proliferation is regulated by endocytosed receptors (Sorkin and Von Zastrow, 2002). Further studies are required to

clarify which mechanism is involved in the differential regulation of OP behaviour by PDGF-A binding.

It has been shown that threshold levels of ERK phosphorylation are required to activate different down-stream behaviours (Stork, 2002; Avrov and Kazlauskas, 2003). Specifically, a threshold level of ERK activation has been shown to be required for process extension in OPs (Stariha et al., 1997). Further, protein analysis has shown that ERK1 is the predominant ERK present in growth factor naïve OPs (Stariha, Kikuchi et al., 1997), which is concordant with our findings. Interestingly, in the current studies, the ERK-1 levels are not affected by growth factor treatment, whereas ERK-2 levels are significantly elevated at both high and low concentrations of PDGF-A, compared to untreated controls. Further, FGF2 treatment seems to induce ERK-1 or ERK-2 phosphorylation, albeit not at significant levels. These data indicate that ERK-2 dependent signaling regulates OP migration. It is noteworthy that ERK-1 null mice are viable, fertile and of normal size and ERK-2 can compensate for the loss of ERK-1, suggesting functional redundancy of ERK-1 (Pages and Pouyssegur, 2004). However, ERK-2-deficient mice die early in development (Hatano et al., 2003; Saba-El-Leil et al., 2003; Yao et al., 2003).

FGF2 is a key regulator of OL behaviour during brain development (Baron, Metz et al., 2000; Bansal *et al.*, 2003a; Bansal *et al.*, 2003b; Fortin, Rom et al., 2005). Our present findings demonstrated that transient exposure to FGF2 at both 1 ng/ml and 10 ng/ml is sufficient to activate ERK1/2 in OP. However, transient exposure failed to induce any

significant OP migration at either concentration. One possible explanation is that the transient activation of FGF receptor is not sufficient to induce the binding of the receptor to FGF Substrate 2 (FRS2). FRS2, is a key adaptor protein which is critical for recruitment of downstream signaling molecules (Eswarakumar et al., 2005). It is also thought to link the FGFRs to the multiple downstream pathways (Hadari et al., 2001). To the best of our knowledge, there is currently no published information on the timing of FRS2 activation post ligand binding and further studies are warranted. Further, our studies show that after FGF2 treatment, inhibition of MEK1/2 only partly inhibited OP migration, suggesting the involvement of an ERK-independent signaling pathway in FGF2 induced OP migration. FGF2 activates multiple signaling cascades through various different receptors (Powers et al., 2000). The best understood pathways are the RAS-MAPK (including ERK1/2, $p38^{MAPK}$ and JNK kinases); the PI_3K/AKT pathway, and the PLCγ pathway (Schlessinger, 2000). It is possible that the PI_3K/AKT and/or PLCγ pathways may also be involved in FGF2 induced OP migration. Further studies are required to fully clarify the exact pathways involved.

One possible explanation for FGF2 induced migration is increased proliferation of cells distributed away from areas of high cell density. However, using a glutamate receptor antagonist, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA), we and others have already shown that this is not the case. AMPA inhibits Cdk2 without affecting OP migration (Gallo *et al.*, 1996; Frost, Zhou et al., 2009). In fact, there was marked reduction of OP cell density in the wells with AMPA. This confirms that the observed cell migration was not skewed by proliferation of the OP cells.

Previous studies have shown that FGF2 and PDGF-A interact to regulate the proliferation and migration of OP (McKinnon, Matsui et al., 1990; Wolswijk and Noble, 1992; Milner, Anderson et al., 1997; Baron, Metz et al., 2000; Lachapelle *et al.*, 2002; Frost, Nielsen et al., 2003). Therefore, our study assessed the effect of a combination of PDGF-A and FGF2 on OP migration. As with previous studies, OP migration was seen in response to PDGF-A and FGF2 in combination, at either 1 ng/ml or 10 ng/ml, is significantly enhanced when compared to either growth factor alone (McKinnon, Matsui et al., 1990; McKinnon *et al.*, 1993; Milner, Anderson et al., 1997; Frost, Nielsen et al., 2003). However, when OPs are transiently exposed to the growth factors added in combination, the extent of migration is not enhanced over OP migration seen in response to PDGF-A alone. Previous studies have shown that FGF2 induces PDGF-A receptor expression on OP (McKinnon, Matsui et al., 1990), which would result in enhanced response of the OP to PDGF-A. However, at lower concentrations of PDGF (1 ng/ml), the presence of FGF2 did not enhance the OP migration. This is likely because the transient FGF2 exposure was not sufficient to affect the gene expression. This hypothesis also fits with previous studies that have shown that prolonged activation of ERK1/2 signaling is required for gene expression (Pellegrino and Stork, 2006).

The differential regulation of cell differentiation by growth factors occurs in other cell types, for example during hematopoiesis (Tsiftsoglou et al., 2009). Differential roles for PDGF-A and FGF2 have been discussed previously (Gard and Pfeiffer, 1993). A combination of PDGF-A and FGF2 has previously been hypothesized to regulate

lineage progression of the cells from OP to mature OL (Gard and Pfeiffer, 1993). Our findings from the current study support this hypothesis. Our previous study, along with the data from this study, provides a unique and intriguing insight into the intricacies of the regulatory mechanisms underlying developmental myelination mediated by these RTK ligands.

Thus, it is established that PDGF-A and FGF2 regulate OP migration via an ERK1/2 signaling pathway. Further, PDGF-A-mediated migration occurs in response to transient exposure, in contrast to FGF2, which requires continuous exposure. Although the physiological relevance of these findings remains to be established, such regulation may provide a means for fine-tuning the dispersal of OP in the developing brain. Advanced understanding of the ways in which different growth factors promote OP migration may provide an opportunity for controlling myelination through the selective manipulation of specific signal transduction pathways. The enhanced knowledge of independent and combinatorial effects of these growth factors will enable appropriate use of therapeutic interventions in abnormal myelination of CNS.

Chapter 3: "The Role of Transcription Factor Methyl CpG Binding Protein 2 (MeCP2) in Regulation of Myelination"

3.1 Introduction

3.1.1 Transcriptional regulation of myelination

In order to understand the mechanisms underlying myelin formation by OLs, it is imperative to know how gene expression is controlled in these cells during both development and differentiation. The most common control of gene expression is through transcription factors (TFs). The TFs are proteins that bind to a typical domain on the DNA and influences transcription. The effect of a TF can be either repression or activation of transcription depending on the type of binding site, the distance to coding regions, or on the presence of other molecules. As opposed to TFs working in isolation, in OLs they function as a network of molecules bringing about induction, inhibition and/or permissive action leading to normal OL development. Several families of TFs have been recently implicated in OL development, in particular, homeodomain proteins, zinc-finger proteins and HMG-domain proteins. However, many TFs that prompt OL differentiation and maturation to myelinate axons are either unknown or poorly

understood. Methyl CpG binding protein 2 (MeCP2) was recently identified as one of the transcription factors expressed in OPs, the absence of which leads to abnormal expression patterns in myelin associated genes (Vora *et al.*, 2010).

3.1.2 Methyl-CpG Binding Domain Proteins

The Methyl-CpG binding domain (MBD) proteins were discovered by the research group of Dr. Adrian Bird, ironically in their quest to discover factors that bind to unmethylated DNA. These proteins, initially named MeCP1 and MeCP2, have the ability to bind specifically to DNA containing methylated CpGs islands (Meehan *et al.*, 1989; Lewis *et al.*, 1992). The CpG islands are genomic regions that contain a high frequency of CpG sites. The "p" in CpG refers to the phosphodiester bond between the cytosine and the guanine, which indicates that the C and the G are next to each other in sequence regardless of being single- or double- stranded. More specifically, both C and G are on the same strand of DNA/RNA covalently bonded by a phosphodiester bond. DNA methylation occurs exclusively at the CpG islands found in vertebrate DNA. In mammals, CpG islands are typically 300-3,000 base pairs in length. About 60-90% of all CpGs are methylated in mammals (Bird, 1986). DNA methylation is a major epigenetic modification that is implicated in transcriptional regulation.

In the following years, sequence homology searches for the conserved MBD encoding domain in MeCP2 lead to the discovery of four proteins, named MBD1, MBD2, MBD3

and MBD4 (Hendrich and Bird, 1998). In addition to having the common MBD, the proteins MBD1, MBD2, and MeCP2 contain a similar C-terminal transcriptional repression domain (TRD). MeCP1 was later found to be a large multi subunit complex consisting of a number of proteins, one of which (MBD2) binds directly to methylated DNA (Cross *et al.*, 1997; Ng *et al.*, 1999).

MBD1 is the largest member for the MBD family with a 70 kDa molecular mass (Cross, Meehan et al., 1997). It is known to play a role in histone methylation by forming a complex with histone H3 lysine 9 (H3K9) methylase, SETB1, and the chromatin assembly factor 1 (CAF-1). This complex become activated during the S phase of the cell cycle, interacting with the replication machinery and facilitating histone methylation (Sarraf and Stancheva, 2004).

The MBD2 protein has two isoforms, namely MBD2a (43.5 kDa) and MBD2b (29.1kDa) depending on initiation sites of translation (Hendrich and Bird, 1998). They are thought to regulate genetic silencing and have been characterized as DNA demethylases (Bhattacharya *et al.*, 1999).

MBD3, a 32 kDa protein and the most abundant member of the MBD family proteins, shares high homology to MBD2b (80% similar, 70% identical). It has been linked to transcriptional repression through its association with the nucleosome remodeling and histone deacetylase (NuRD) complex and MBD2 (Saito and Ishikawa, 2002). It also induces genomic DNA demethylation (Brown *et al.*, 2008) and its protein levels are

shown to increase in certain cancers such as gliomas (Schlegel *et al.*, 2002). MBD4 is a 62 kDa protein and is known to have homology with bacterial DNA-repair enzymes. A DNA glycosylase, MBD4 is shown to efficiently remove the thymine or uracil from mismatched CpG sites *in vitro*, helping to minimize mutations in methyl-CpG sites(Hendrich *et al.*, 1999).

The fifth member of the MBD family, MeCP2 is the 52 kDa protein which has both, a MBD and TRD domains. MeCP2 gained wider attention when the Dr. Huda Zoghbi's team at the Baylor College of Medicine found it to be mutated in the neurodevelopment disorder, Rett Syndrome (Amir *et al.*, 1999).

3.1.3 MeCP2 and Rett Syndrome

3.1.4 Clinical features of Rett Syndrome

Rett Syndrome (RTT) is a postnatal, progressive, neurodevelopmental disorder, which affects 1:10,000 to 1:15,000 births (Percy, 2002). First characterized by Dr. Andreas Rett in 1963, RTT is characterized by stereotypic movement disorder, microcephaly, communication dysfunction and cognitive impairment (Rett, 1966). It is an X-linked disorder which primarily affects females and is the major cause of intellectual disability in females (Carter and Segal, 2001). A classic Rett Syndrome female patient shows apparently normal perinatal and early postnatal development and the phenotypic symptoms develop only after 6 to 18 months of age. Until this time, the patient tends to

achieve normal motor, language and social milestones with the expected rate and age. As the phenotypic characteristic features of RTT start to appear, the patient begins to regress. The clinical progression of classic RTT can be divided four distinct stages (Chahrour and Zoghbi, 2007; NIH, 2011). After apparent normal development, entering Stage 1 (6-18 months) of the disease, patients cease to acquire new skills; they show deceleration of head growth and hypotonia (weakening of muscles). Also called the “developmental stagnation” stage, the characteristic phenotype microcephaly (Hagberg *et al.*, 1983) appears during this period. General growth arrest and weight loss are also observed. During Stage 2 or “rapid regression” stage (1-4 years), patients lose the ability to speak and the purposeful use of their hands. Autistic characteristics may start to appear along with social withdrawal and self-abusive behaviour. Many RTT patients also develop seizures followed by intellectual disability and loss of motor skills. It is at this stage, patients show the classic 'hand-wringing' or ‘hand washing’ activity, irregularities in breathing patterns, with episodes of apnea, and hyperventilation. In Stage 3 (2-10 years), which is the ‘pseudo-stationary stage’, the patients become more alert and interested both in people and their surroundings. There is an improvement in behaviour, with less irritability, crying, and autistic-like features. However, inability to speak, hand apraxia (loss of purposeful movements) and stereotypic hand activities persist. Some patients also develop severe scoliosis where the spine gets curved from side to side. During Stage 4 (5-15 years and older) or the ‘late motor deterioration’ stage, seizures become less frequent, but somatic and neurologic deterioration continues. Autonomic abnormalities, including reduced movement, rigidity, cold blue feet and cardiac abnormalities are commonly observed. Parkinsonian features start to

develop followed by loss of mobility (Ancel, Livinec et al., 2006). Patients might survive until their sixties or seventies with symptomatic support, but they exhibit severe physical disabilities (Bebbington *et al.*, 2008).

In males, MeCP2 mutation was initially thought to cause prenatal lethality, RTT being a dominant X-linked trait. It has been shown lately however, that MeCP2 mutations actually cause a variable phenotype in male patients (Ravn *et al.*, 2003). Phenotypes ranging from severe neonatal encephalopathy or Rett-like symptoms to mild mental retardation have been reported in males with MeCP2 mutations (Orrico *et al.*, 2000; Couvert *et al.*, 2001; Ravn, Nielsen et al., 2003; Masuyama *et al.*, 2005).

3.1.5 Cellular pathology of Rett Syndrome

Genetically engineered transgenic mouse model of Rett Syndrome have significantly advanced our current understanding of the cellular pathology of the disease. Along with phenotypical characteristic of RTT, these MeCP2-deficient mice have decreased brain weight and reduced size of neuronal soma (Chen *et al.*, 2001). Hence, most of the research into cellular mechanisms underlying RTT syndrome to date, has focused on neuronal cells. It is reported that MeCP2 regulated proteins are important for maturation and maintenance of neurons, including synapse formation and dendritic arborization, rather than cell-fate decisions (Kishi and Macklis, 2004). This was supported by the fact that the brain size of RTT patients is small because of smaller, immature neurons rather

than fewer cells (Nagai *et al.*, 2005; Miyake and Nagai, 2007). Moreover, reduced spine density, smaller somas and substantially decreased dendritic arborization was seen in many different parts of RTT brain (Armstrong *et al.*, 1998; Armstrong, 2002; Zhou *et al.*, 2006; Chapleau *et al.*, 2009). Recently, glial cells, including astrocytes and OLs were also shown to express MeCP2 protein, which may play an important role in pathogenesis of RTT (Ballas *et al.*, 2009; Maezawa *et al.*, 2009; Vora, Mina et al., 2010).

3.1.6 Role of MeCP2 in Rett Syndrome

In 1999, it was demonstrated that RTT is associated with a gene mutation of the X-linked transcription factor**,** methyl-CpG binding protein 2 (MeCP2)**,** located on the X chromosome (Xq28) (Amir, Van den Veyver et al., 1999). About 85% of RTT patients were revealed to have MeCP2 mutations (Bienvenu *et al.*, 2000). Most MeCP2 gene mutations appear to be spontaneous, novel mutations. These mutations could be either missense, nonsense, or frameshift mutations (Christodoulou *et al.*, 2003). It has been reported that there are over 300 unique pathogenic nucleotide changes (Christodoulou, Grimm et al., 2003), as well as deletions encompassing whole exons (Archer et al., 2006; Pan et al., 2006). The majority of these MeCP2 mutations are from C-terminal truncations of varying length (~40% of cases) as well as from missense mutations within the methyl-binding domain (~45% of cases). The common MBD mutations include

T158M (~10% of patients), R106W (~4%), and R133C (~4%) (Monros *et al.*, 2001; Vacca *et al.*, 2001; Laurvick *et al.*, 2006; Philippe *et al.*, 2006; Percy *et al.*, 2007).

This suggests the fact that even though males with MeCP2 mutation survive after birth, the severity of the disease is much more than in females. This is because only one copy of the MeCP2 gene is present in their genome. Moreover, one of the reasons of why this disorder is almost exclusively seen in females is because most *de novo* mutations in the MeCP2 gene arise from the male germline cells (Girard *et al.*, 2001; Trappe *et al.*, 2001). Thus, such predominant paternal origin of MeCP2 mutations likely prevents them from being transmitted to the male offspring.

3.1.7 Mouse models of Rett Syndrome

Currently, there exists no cure for the devastating RTT syndrome. “Gene targeting” procedures, which can introduce a predetermined mutation in a specific area of the mouse genome, is commonly used by researchers to study such monogenic disorders. A number of different transgenic mouse models were created with genetic aberrations characteristic of human RTT syndrome patients. These mice carrying endogenous MeCP2 mutation have been shown to recapitulate many RTT symptoms. The primary focus of these transgenic colonies is on the phenotype of hemizygous male or homozygous female mice. It is because of the unpredictability of the heterozygous female phenotype, deriving from the X-chromosome inactivation (XCI). As a result of

XCI, where one of the two chromosomes is randomly inactivated in every cell of the body, the severity of symptoms is extremely variable. The severity depends on the number of cells expressing the normal allele versus the mutated one. Hence, the hemizygous male or homozygous female mice are considered a more suitable model to investigate the role of MeCP2 gene mutations on the development of RTT-like symptoms.

The three most well characterized mouse model of RTT syndrome are: the $MeCP2^{y/308}$ (Shahbazian *et al.*, 2002a), expressing a truncated form of MeCP2, the $MeCP2^{Bird}$, lacking exons 3 and 4 (Guy *et al.*, 2001); and the $MeCP2^{Jae}$ (Chen, Akbarian et al., 2001), generated by targeted deletion of exon 3 **(TABLE 1)**.

Dr. Zoghbi (Baylor College of Medicine) generated a mouse model of RTT syndrome, by truncating the gene at amino acid 308, generating the $MeCP2^{y/308}$ mouse model (Shahbazian, Young et al., 2002a). These mice express a truncated MeCP2 protein with a full length MBD but a shorter TRD. This resulted in non-functional MeCP2 protein, which leads to the recapitulation of milder RTT-like symptoms in the mice. The mutant mice appear normal until six weeks of age and then start exhibiting tremors. The mice later develop ataxia, stereotypical forepaw motions, breathing abnormalities as well as seizures by about 4-6 months of age. Most $MeCP2^{y/308}$ male mice survive to at least one year of age (Shahbazian, Young et al., 2002a).

MOUSE TYPE	ZOGHBI (Shahbazian, Young et al., 2002a)	BIRD (Guy, Hendrich et al., 2001)	JAENISCH (Chen, Akbarian et al., 2001)
SYMPTOM START	6 week (tremors) Apparent in 4th month	3-8 weeks	5 weeks
DEATH	12 months	8 weeks	10 weeks
TYPICAL SYMPTOMS	Tremors Seizures Ataxia Breathing abnormalities	Uncoordinated gait Stiffness Reduced spontaneous movement Weight loss	Altered gait Weight loss Body tremors Hind limb clasping
mRNA/PROTEIN	mRNA present protein present (but non-functional)	mRNA present protein absent	mRNA absent protein absent

Table 1: Mouse models of RTT

A MeCP2^{null} mouse generated by the Dr. Adrian Bird's lab lacks both, exon 3 and exon 4 of the MeCP2 gene. Such an excision leads to a generation of an unstable mRNA transcript and ablation of MeCP2 protein expression (Guy, Hendrich et al., 2001). The males and females carrying the MeCP2 mutation appear normal for first three weeks of development. They then develop gross abnormalities including, stiffness, rapid weight loss, irregular breathing and abnormal gait. They also exhibit uneven wearing of teeth, anxiousness and hind limb clasping. Typical hemizygous males display internal testes, and thus are infertile and survive about eight weeks postnatally (Guy, Hendrich et al., 2001).

In the MeCP2^{Jae} mice, altered gait is usually identified at four weeks of age in mutant male mice. The knockout was created by targeted deletion of exon 3 using the Cre-LoxP recombinant system, which results in a slightly smaller MeCP2 transcript and no MeCP2 protein expressed. The mice exhibit body tremors, breathing difficulties, significant weight loss and piloerection. In later weeks of life, the mice also develop hind leg clasping as well as other motor deficits and die at 6-10 weeks of age (Chen, Akbarian et al., 2001).

3.1.8 MeCP2 gene and its functions

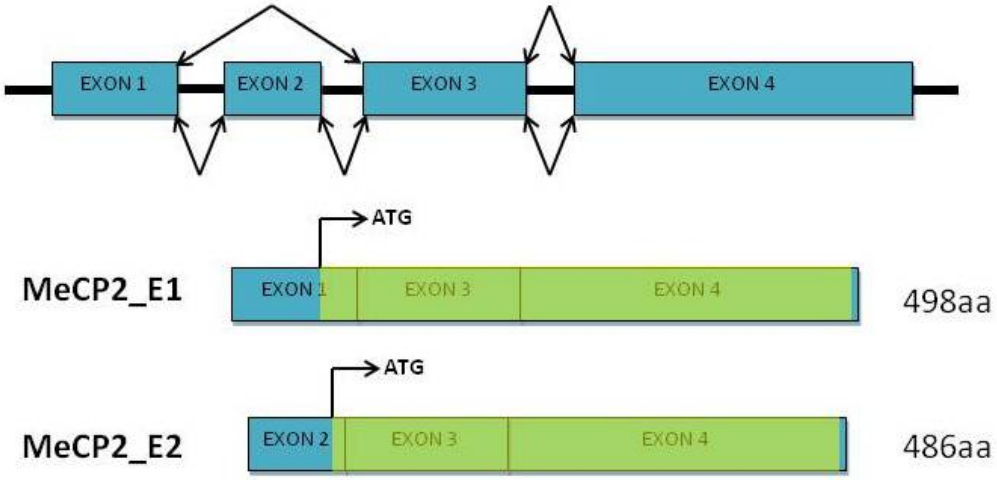


Figure 12: MeCP2 gene structure.
The human MeCP2 gene has two isoforms made of four alternatively spliced exons. The longer splice variant MeCP2_E1 consists of exon 1, 3 and 4, whereas MeCP2_E2 consists of exon 2, 3 and 4.

The human MeCP2 gene is positioned in chromosome Xq28 and is composed of four exons. MeCP2 also has four potential polyadenylation sites, which encompass a well-conserved 3'-untranslated region (UTR) (Quaderi *et al.*, 1994; Coy *et al.*, 1999; Reichwald *et al.*, 2000; Pelka *et al.*, 2005). As a result of alternative splicing, this gene results in two different isoforms of MeCP2, differing in only 21 amino acids at the N-terminus (Kriaucionis and Bird, 2004; Mnatzakanian *et al.*, 2004). The longer splice variant MeCP2e1 (also called MeCP2B) utilizes the translational site in exon 1, splices out exon 2 and generates a 498 kDa protein from exon 1, 3 and 4 **(Fig. 12)**. Another splice variant MeCP2e2 (also called MeCP2A) utilizes the ATG signal on exon 2, utilizes all the four exons to generate a 486 kDa protein. Even though differences in the structure and distribution are shown, no functional differences between these two MeCP2 isoform have been described. In fact, a recent paper indicates that the two MeCP2 splice variants can substitute for each other and fulfill the basic functions of MeCP2 in the mouse brain (Kerr *et al.*, 2011).

The MeCP2 protein contains a methyl-CpG binding domain (MBD), a transcription repression domain (TRD) and the N- and C-terminal domain with unknown functions. Shared by exons 3 and 4, the MBD domain binds preferentially to symmetrically methylated CpGs sequences with adjacent A/T-rich motifs (Klose et al., 2005). Such a binding to the methylated DNA using the MBD represents an epigenetic mechanism by which the hereditary epigenetic marks can used as functional effects to modify gene expression. The TRD, which is located on exon 4 regulates gene expression in various ways. One of the well-accepted hypotheses of MeCP2 function is the interaction of the

TRD with mSin3a and the subsequent recruitment of co-repressors and histone deacetylases 1 (HDAC1) (Nan et al., 1997; Martinowich et al., 2003). As shown in the case of MeCP2 target gene BDNF (brain derived neurotrophic factor), the non-phosphorylated MeCP2 binds specifically to the BDNF promoter III and represses BDNF expression (Chen et al., 2003). In response to neuronal activity dependent Ca^{2+} influx into the neurons, MeCP2 is phosphorylated and is released from the BDNF promoter, thereby allowing its transcription (Chen, Chang et al., 2003). Besides, the TRD of MeCP2 is also shown to bind with transcriptional co-repressors including nuclear receptor corepressor (N-CoR) and c-Ski (Kokura et al., 2001).

MeCP2 was discovered about 20 years ago as a protein that selectively binds to methylated DNA (Meehan, Lewis et al., 1989). Accordingly, MeCP2 was shown to act as a methylation-dependent transcriptional regulator *in vitro* and *in vivo* (Wade, 2004; Mann *et al.*, 2007). Additionally, MeCP2 can modulate chromatin architecture independently of mSin3a, without DNA methylation, ATP and histone deacetylases (HDAC) (Georgel *et al.*, 2003). MeCP2 is also implicated in chromatin loop formation, as in the case of Dlx5 and Dlx6, where MeCP2 gene upregulation increased aberrant chromatin looping (Horike *et al.*, 2005). MeCP2 is also shown to interact with nucleosomes (Chandler *et al.*, 1999), histone H3 methyltransferase (Fuks *et al.*, 2003), DNA (cytosine-5)-methyltransferase 1 (Dnmt1) (Kimura and Shiota, 2003) and transcription factor PU.1 (Suzuki *et al.*, 2003). Thus, MeCP2 is now thought to be a complex multifunctional nuclear protein with a significant role in regulating global chromatin architecture. In addition, MeCP2 is shown to bind RNA with a similar affinity

as it binds to methylated DNA (Jeffery and Nakielny, 2004). Studies have shown that MeCP2 can interact with the RNA splicing factor, Y box-binding protein 1 (YB-1) (Young *et al.*, 2005) as well as other splicing factors (Buschdorf and Stratling, 2004).

The ability of MeCP2 to preferentially bind with methylated DNA and bringing changes in transcriptional regulation suggested that RTT may be a result of inappropriate expression of a number of different genes. However, gene expression profiling experiments in neuronal and non-neuronal tissues from MeCP2 knockout mice has revealed differential expression of very few genes. This could be due to functional redundancy of genes regulated by MeCP2, in which case, related proteins may compensate for the loss of that specific protein.

3.1.9 Potential target genes for MeCP2.

A number of different groups have investigated the gene expression changes in neuronal and non-neuronal tissue from the brain, from mice lacking MeCP2 proteins. However, these studies failed to identify convincing target genes that could be held responsible for Rett syndrome pathogenesis (Tudor *et al.*, 2002; Jordan *et al.*, 2007). The table below **(TABLE 2)** summarizes the most significant studies of potential MeCP2 target genes and their function in the nervous system.

TARGETS	FUNCTIONS
Brain derived neurotrophic factor (BDNF)(Chen, Chang et al., 2003)	**Neuronal Survival, differentiation and dendritic outgrowth.**
Distal-less homeobox 5/6 (DLX5/6) (Horike, Cai et al., 2005)	**Promote differentiation in interneurons.**
Reln (Reelin) (Jordan, Li et al., 2007)	**Important for neuronal progenitor migration and role in PNS myelination.**
Early Growth Factor 2 (EGR2)(Swanberg *et al.*, 2009)	**Hindbrain development, important for PNS myelination.**
Myelin Basic Protein (MBP)(Vora, Mina et al., 2010)	**Structural component of myelin, important for fusion and stabilization of the myelin sheath.**
Myelin Associated Glycoprotein (MAG)(Vora, Mina et al., 2010)	**Structural component of myelin, important for maintenance of myelin and neuronal axon.**

Table 2: Potential MeCP2 target genes and their functions.

3.1.10 MeCP2 expression in brain

MeCP2 is a ubiquitously expressed protein (D'Esposito *et al.*, 1996). It is widely expressed in various human and rodent tissues. However, the level of MeCP2 protein is relatively high in brain, lung, and spleen compared to other tissues such as heart and kidney (Shahbazian *et al.*, 2002b). The expression level of MeCP2 is high during embryogenesis, but gradually declines during the postnatal period. There is a subsequent increase in expression levels later in adult life (Shahbazian, Antalffy et al., 2002b; Pelka, Watson et al., 2005). The MeCP2_e1 isoform is more abundant in brain, while the MeCP2_e2 isoform predominates in other tissues such as fibroblast and lymphoblast cells.

In brain, MeCP2 was thought to be expressed only in neurons (Mnatzakanian, Lohi et al., 2004). However, data from last few years suggests that MeCP2 is expressed in glial as well as neuronal cells (Ballas, Lioy et al., 2009; Vora, Mina et al., 2010). In addition, studies have shown that MeCP2 works as a multi-functional protein regulating transcriptional activation, chromatin remodelling (Georgel, Horowitz-Scherer et al., 2003; Horike, Cai et al., 2005) and regulated RNA splicing (Young, Hong et al., 2005).

3.1.11 Role of MeCP2 in other neurodevelopmental disorders

MeCP2 was found as a nuclear protein with an ability to bind methylated DNA (Lewis, Meehan et al., 1992; Meehan *et al.*, 1992). Upon establishment of links between mutations in MeCP2 and Rett in 1999 (Amir, Van den Veyver et al., 1999), research on MeCP2 has increased dramatically and is now linked with several other disorders (Gonzales and LaSalle, 2010). Autism and Rett Syndrome are both classified under the pervasive neurodevelopmental disorders named the “Autism Spectrum Disorders”. The two share significantly common phenotypic characteristics like repetitive or stereotypic behaviour, social and communication impairments, seizures and sleep abnormalities. MeCP2 mutations similar those found in RTT patients are identified in some autistic males and females (Lam *et al.*, 2000; Beyer *et al.*, 2002; Carney *et al.*, 2003). In addition, levels of MeCP2 protein in brain was found to be lower in 79% of autistic cortical samples (Nagarajan *et al.*, 2006). Moreover, recent studies show that polymorphic variants of MeCP2 gene are suspected to confer autism vulnerability (Loat *et al.*, 2008). Further research is warranted to understand the role of MeCP2 in Autistic patients.

Males with the classic RTT phenotype are very rare and are usually subject to somatic mosaicism or with XXY karyotype (Leonard *et al.*, 2001; Topcu *et al.*, 2002). However, males with normal karyotype who inherit the mutated MeCP2 gene from their mildly or unaffected mother, suffer severe neonatal encephalopathy. This disorder is characterized by static encephalopathy, severe developmental delays, hypotonia,

seizures and respiratory abnormalities, which usually lead to death in infancy (Schanen *et al.*, 1998; Villard *et al.*, 2000).

MeCP2 is also implicated in a number of other disorders, including Angelman's Syndrome (Watson et al., 2001), Attention deficit hyperactivity disorder (ADHD) (Nagarajan, Hogart et al., 2006), Prader-Willi Syndrome (Nagarajan, Hogart et al., 2006) and intellectual disability in males (Villard, 2007).

3.2 Rationale and Hypothesis

Considerable progress has been made in understanding the functions of MeCP2. However, it is still unclear how MeCP2 mutations contribute to the pathogenesis of RTT Syndrome. It is known that MeCP2 regulates proteins known to control maturation and maintenance of neurons, including dendritic arborization, and may play little or no role in cell fate decisions (Kishi and Macklis, 2004). These processes involve critical interaction of neurons with OL. This is supported by the characteristic reduction in brain size in Rett patients, which is a direct result of smaller, immature neurons, rather than fewer cells (Nagai, Miyake et al., 2005; Miyake and Nagai, 2007). Several animal models have been developed which exhibit phenotypic characteristics similar to those exhibited by Rett patients (Guy, Hendrich et al., 2001; Shahbazian, Antalffy et al., 2002b)., including impaired learning and memory, and changes in synaptic plasticity (Moretti et al., 2006). Most of the current research into the cellular mechanisms that underlie Rett Syndrome focuses on neuronal cells. However, neuronal maturation is dependent on signals

derived from the glial cells that make up 90% of the CNS cellular mass. To date, there has been virtually no research conducted on the role of glial cells in the pathophysiology of RTT.

Hence, we hypothesized that

1) The expression patterns of myelin associated genes are altered in the MeCP2^{null} mouse.
2) The MeCP2 gene and protein is expressed in different stages of OL maturation.

3.3. Methods

3.2.1 Cell Isolation and Culture

OL progenitor cells (OPs) were isolated from neonatal rat pups (P0-P1), by a previously described method (Armstrong, 1998). Please refer to Section 2.3.1 **[Chapter 2]** for detailed methodology.

3.2.2 MeCP2^{null} mouse

Various mouse models of Rett Syndrome are commercially available for breeding purposes **(Table 2)**. The MeCP2^{null} mice for the proposed experiments were purchased from The Jackson Laboratory (MeCP2^{tm1Hzo} breeding pair sold as a homozygote plus hemizygote Strain Name: B6.129S-MeCP2$^{tm1Hzo/J}$ Stock Number: 005439). Two pairs of

female MeCP2 homozygous mice (42 days) along with the age matched control were used. At this stage of development, myelination is almost complete in the mouse brain. The Zoghbi transgenic mouse (Shahbazian, Young et al., 2002a) was selected over the Bird mouse (Guy, Hendrich et al., 2001) for two reasons:

1) The nature of the symptoms displayed by the mutant mice: The Zoghbi mice demonstrate abnormal involuntary movement, tremors and impaired coordination, with surviving males 100% affected, and females 62% affected (Shahbazian, Antalffy et al., 2002b).

2) The Zoghbi homozygous females breed with hemizygous males to produce a full litter of knockout mice, negating the need for genotyping each mouse, and the possibility of loss of null status.

The mice were euthanized, and the whole brain was removed and placed into RNALater™ (RNA stabilization reagent) (Qiagen; Cat# 76104), sliced into 2 mm thick slices and stored in RNALater™ until preparation of whole RNA.

3.2.3 RNA Extraction

The RNeasy Lipid Tissue Mini Kit (Qiagen; Cat# 80004) was used for purification of total RNA from the different brain tissues. This convenient RNeasy Lipid Tissue protocol integrates QIAzol lysis with RNeasy RNA isolation for high yields of total RNA. Total RNA was isolated using the efficient combination of the stringency of phenol/guanidine-

based lysis with the speed and purity of silica-membrane purification (Qiagen RNA extractions protocols). Tissue samples were then homogenized in QIAzol Lysis Reagent. After addition of chloroform, the homogenate was separated into aqueous and organic phases by centrifugation. RNA partitioned to the upper, aqueous phase, while DNA partitioned to the interphase and proteins to the lower, organic phase or the interphase. The upper, aqueous phase was extracted, and ethanol was added to provide appropriate binding conditions. The sample was then applied to the RNeasy spin column, where the total RNA binds to the membrane, and phenol plus other contaminants were efficiently washed away. High-quality RNA was then eluted in RNase-free water.

3.2.4 Quantitative reverse transcription polymerase chain reaction

The QuantiTect™ SYBR® Green RT-PCR Kit (Qiagen Cat# 204243) was used to provide accurate real-time quantification of RNA targets. The kit was optimized for using the Applied Biosystems® 7500 RT-PCR machine. All reagents were kept on ice throughout the procedure, until placed in the cycler. The protocol detailed in the manufacturer's (Qiagen) kit was followed, and the expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH is an enzyme associated with cell metabolism and is used as a standard housekeeping gene for expression pattern comparisons (Sporkel et al., 2002; Wang et al., 2006). Several primers for the study were commercially available from SuperArray; others are obtained

from previously published studies **(Table 3)**. The quantification technique compared the results from both the Standard Curve Method and the comparative Ct method, using whole brain lysates from age-matched control wild type mice. Seven replicates of RNA extracted from different sample tissues were used to derive a statistically appropriate result.

Gene	Forward	Reverse	Reference
MeCP2	5'-aacggggtagaaagcctg-3'	5'-atgctccagactgccttg-3'	**Jackson Laboratories RT-PCR protocol**
BDNF	5'-catacttcggttgcatgaagg-3'	5'-cgaaccttctggtcctcatc-3'	**(Collin et al., 2007)**
PLP	5'-tcagtctattgccttccctagc-3'	5'-agcattccatgggagaacac-3'	**(Werner et al., 2007)**
NG2	5'-gaacgcatcagccaccgtaa-3'	5'-ggacgcttcttcctggtttc-3'	**(Ye et al., 1992)**
CNPase	5'-ccaaattctgtgactacggg-3'	5'Ggtttgcccttcccatagta-3'	**(Ye, Kanoh et al., 1992)**
GAPDH	PPM02946E		SuperArray
MBP	PPM04745E		SuperArray
PLP	PPM04717A		SuperArray
MOG	PPM33328A		SuperArray
MAG	PPM34494A		SuperArray

Table 3: Primers for RT-PCR analysis of gene expression

3.2.5 Protein Isolation and Western Blot analysis

OL progenitors (10^6) derived from new born pups were plated on poly-D-lysine-coated 6-well plates (Sigma-Aldrich; Cat#M8562) and cultured for up to 10 days DIV. Protein was isolated from the whole cell lysates prepared from purified OPs at different stages of maturation. Cells are washed in PBS, then lysed in 2X sample buffer (Laemmli, 1970) and boiled (at 95º-100º) for 5 minutes. Samples were stored at -80º until analyzed. Protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratories; Cat# 500-0001). Samples were diluted to give equal protein loads per sample, and aliquots of each lysate are resolved by SDS-PAGE (10-20% gels). The resolved proteins were transferred to PVDF membranes (GE Health Care; Cat# RPN2020F) using a Panther Semidry electroblotter (Owl Separation Systems; Cat# B1A). Blots were immediately rinsed in double distilled water for one minute then blocked with 5% skim milk or 5% BSA, in Tris-buffered saline containing 0.5% Tween-20 (TBS-T) for one hour at room temperature. The blot was incubated with an appropriate dilution of the primary antibody in TBS-T plus blocking reagent for 2 hours. Protein bands were visualized by incubating the blot in horse radish peroxidise (HRP) conjugated secondary antibody, anti-rabbit (Jackson Immuno Research; Cat# 211-032-171) or anti-goat (Santa Cruz; Cat# sc2020) in TBS-T with 5% blocking reagent for one hour. Detection of the HRP-conjugated secondary antibody is done using a luminol-

chemiluminescence reaction using the Chemilucent ECL Detection System (Sigma; Cat# S3511). The autoradiographic images were used to complete semi quantitative measurement of signal strengths by densitometry. Protein expression was normalized to the GAPDH (Santa Cruz; Cat# FL-335) protein. Protein expression changes were assessed using the primary antibodies as follows: MeCP2 (Upstate Cell Signaling; Cat# 07-013 and Santa Cruz; Cat# sc5758); PDGFR-α (R&D Laboratory; Cat# AF-307-NA) and MBP (1:100, Santa Cruz; Cat# 13914).

3.2.6 Immunohistochemistry

After isolation, OL progenitors were plated on poly-D-lysin (PDL) coated 12 mm glass coverslips (Fisher Scientific; Cat# 22-037-169) in Sato's modified medium, supplemented with 0.5% fetal bovine serum (Thermo Scientific; Cat# SH30070). Cells were fixed after 10 days DIV in 10% buffered formalin. The cells were washed with PBS-T (Phosphate buffered saline (PBS) (Sigma; Cat# P-5368) and 0.1% triton X-100 (Sigma; Cat# X-100)) three times for 5 minutes interval and blocked with 20% normal donkey serum (NDS) (Sigma; Cat# D9663), 20% normal goat serum (NGS) (Sigma, Cat# G9023) in PBS-T for 20 min at RT. The primary antibodies against MECP2 (Upstate Cell Signaling; Cat# 07-013) and PDGFRα (R&D Laboratory; Cat# AF-307-NA) or MBP (Santa Cruz; Cat# 13914) were diluted in PBS-T containing 20% NDS, and 20% NGS were applied to the sections and incubate overnight at 4°C. Following incubation with the primary antibodies the slides were then washed with PBS-T and incubated with

the mixture of secondary antibodies (Alexa 568 donkey anti goat, Invitrogen, Cat# A11057; Goat anti mouse IgG, (Jackson Immuno Research, Cat# 115-095-003) diluted in PBS-T containing 20% NDS and 20% NGS for 30 min at RT. The slides were then washed with PBS-T twice. Following washing, one drop of aqueous mounting medium (R&D Systems, Burlington, Ontario: Cat# CTS011) was added to the cells and mounted with a no.1 coverslip (Marienfeld, Cat# 0101242) and stored at 4°C. Omission of the primary and secondary antibodies during staining of the selected cell culture slides was conducted and used as omission controls to assess for autofluorescence.

3.2.7 Imaging

Primary imaging was conducted using an Inverted Olympus IX51 with c-mounted RETIGA 2000RV monochrome camera with EXFO X-Cite metal halide fluorescence system. Images are captured in Image Pro 6.0 and saved to DVD prior to colorization in Image Pro 6.0. Further, confocal laser microscopy was used to confirm the co-localization of immunoreactivity, to avoid misinterpretation of overlying cell structures in the tissue section. A series of Z-sections were obtained through the section, which enabled the visualization of the specific location of the cell markers within each cell. The confocal microscopy was used to identify the precise location of the antigenic markers in relation to the cell membrane. The advantage of confocal microscopy over regular fluorescence microscopy is the precision of location of immunoreactivity.

3.2.8 Statistical Analysis

The data was expressed as mean ± standard error of the mean. Differences between treatment groups were analyzed using Student's *t* test or a one way analysis of variance with Bonferroni's post-test where appropriate. Statistical analysis was performed with Prism 4.03 software (GraphPad Software, Inc). $p < 0.05$ was considered significant.

3.3 Results

3.3.1 MeCP2 expression in Glial Cells

The western blot results found a very clear presence of MeCP2 protein in OPs isolated from neonatal rat brain **(Fig. 13a and b)**. To further validate the finding, antibodies from two different companies, Santa Cruz and Upstate Biologicals were used, which confirmed MeCP2 expression in OLs. In addition to WB analysis, MeCP2 expression in OL was assessed using immunohistochemistry **(Fig. 13d–g)**.

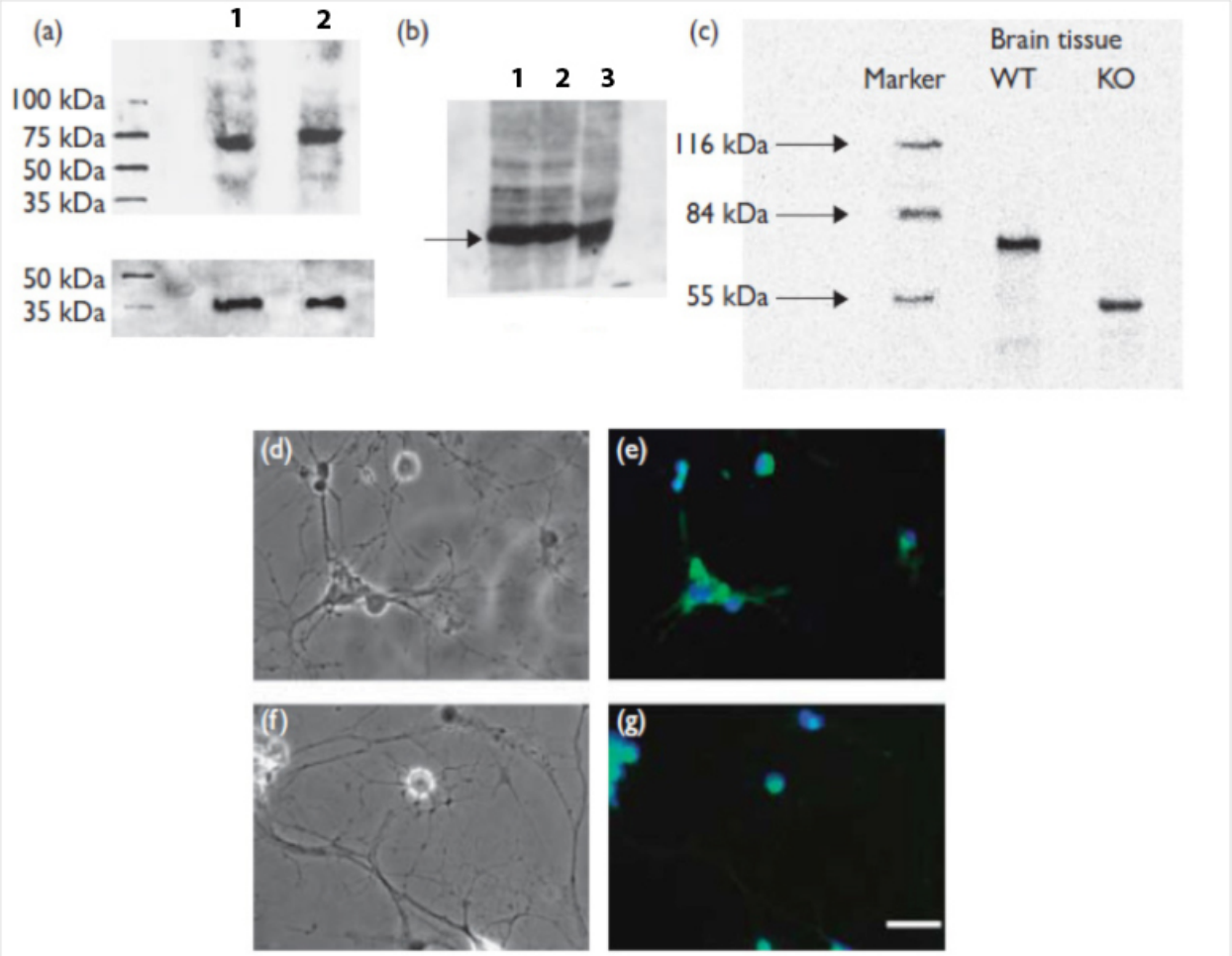
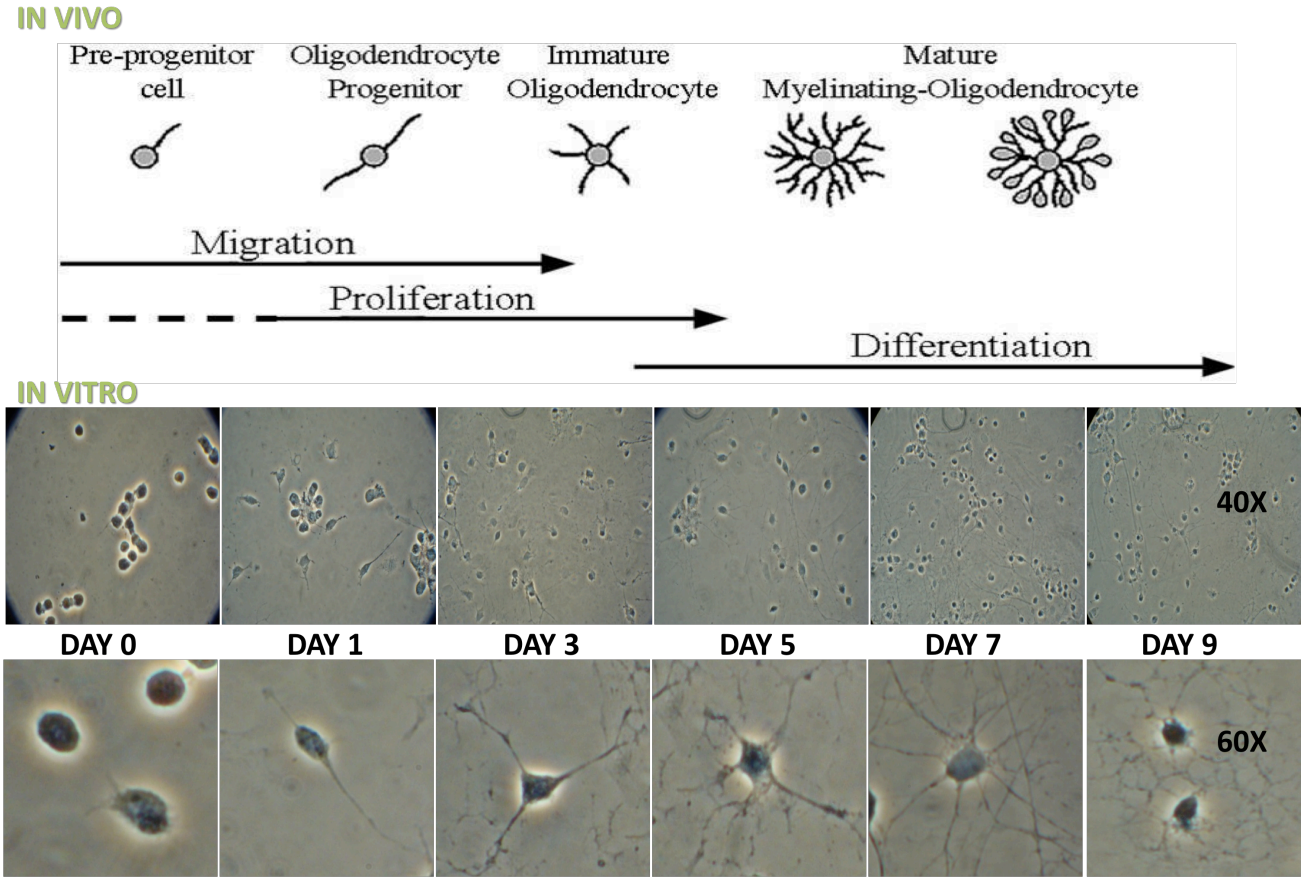


Figure 13: OL expression of methyl-CpG-binding protein 2 (MeCP2) analyzed by western blot and immunohistochemistry.
Panel (a) OP lysates (lane 2), and cortex tissue lysates (lane 1) (detected with Upstate Biologicals rabbit polyclonal anti-MeCP2 antibody).
Panel (b) cortex tissue lysates (lanes 1 and 2) and OP lysates (lane 3) (detected with Santa Cruz goat polyclonal anti-MeCP2 antibody).
Panel (c) western blot analysis of protein isolated from the cortex of $MeCP2^{null}$ knockout (KO) and age-matched control wild-type (WT) mice showing the truncated (52 kDa) MeCP2 protein in the KO mouse brain compared with the regular 74 kDa protein in the WT mouse brain.
Panels (d) and (f) phase contrast microscopy of OL grown for 10 days *in vitro*. Panel (e) cells stained with Santa Cruz anti-MeCP2 antibody (green) counterstained with 40,6-diamidino-2-phenylindole (DAPI) (blue). MeCP2 immunoreactivity is closely associated with the nucleus of all the cells in the culture. Panel (g) cells stained with Upstate Biologicals anti-MeCP2 antibody (green) counterstained with DAPI (blue). Bar=20 mm. (Vora, Mina et al., 2010)



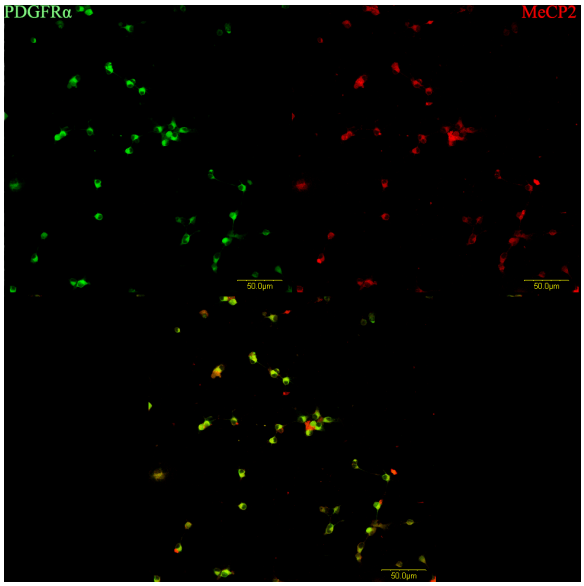
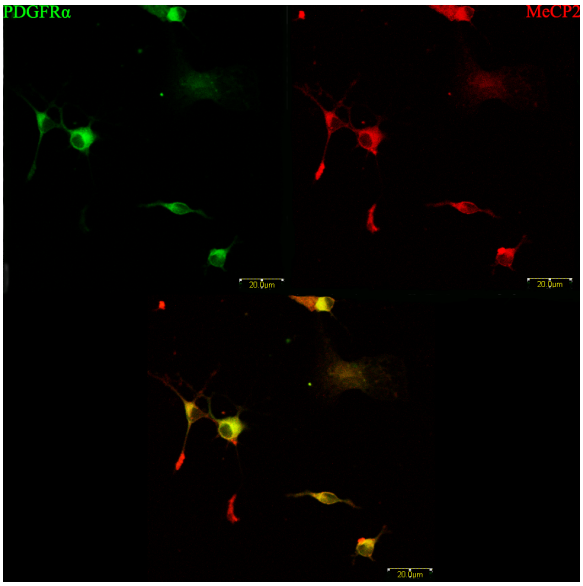
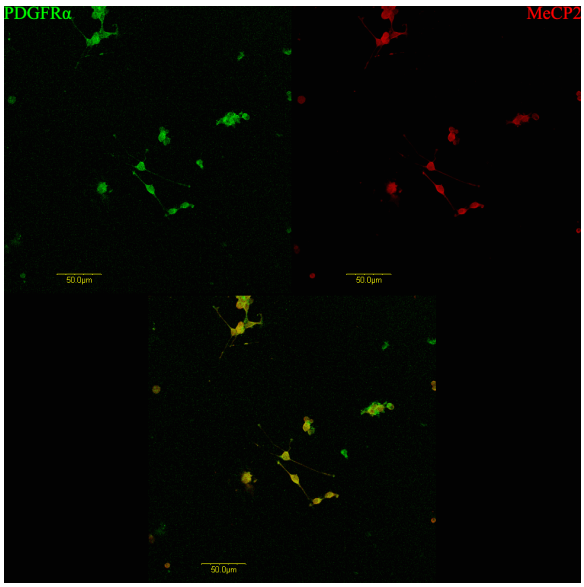
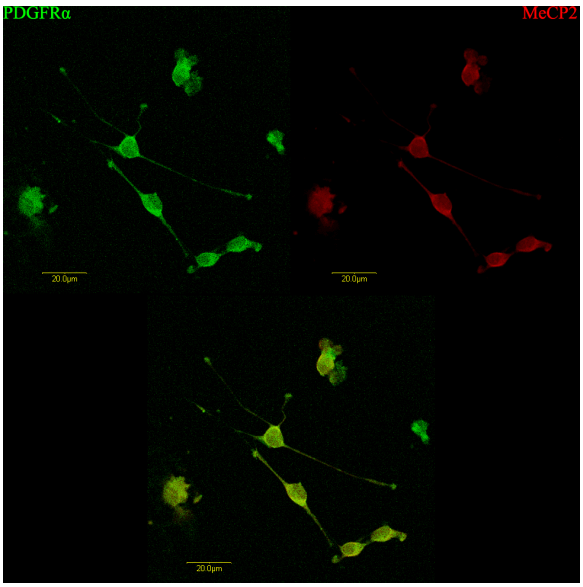
INVITRO OLIGODENDROCYTE LINEAGE RESEMBLES THAT SEEN INVIVO

Figure 14: *In Vitro* and *In Vivo* comparison of OL progenitor cell differentiation. The phase contrast image analysis shows increase in number of processes in the primary cell culture (Day 0 – Day 9) that are characteristic of the OL progenitor cell differentiation *in vivo* (*in vivo* picture provided by Dr. Emma Frost)

3.3.2 MeCP2 expression and OL progenitor differentiation

Cultured cells were isolated from different stages of OP differentiation (Day 0 – Day 9). This primary cell culture reproduced the discrete stages of OL differentiation *in vivo* and was confirmed using light microscopy **(Fig. 14)**. Furthermore, MeCP2 protein expression at different stages of OP maturation was done using double labelling with OP markers, PDGFRα and MBP. The OP cells express PDGFRα at early stages of differentiation, *in vivo* from the OL progenitor stage to the immature OL stage or *in vitro* from Day 0 to Day 5. The OPs in their later stages of differentiation express MBP, *in vivo* from immature OL stage to mature OL stage or *in vitro* from Day 6 to Day 9.

MeCP2 was expressed in all stages of OP differentiation. Interestingly, MeCP2 was present in both nucleus and cytoplasm of the OPs. Early stages of OP differentiation seem to have more cytoplasmic MeCP2, while the later stages of OP cell differentiation had more nuclear MeCP2 expression **(Fig. 15 and 16).**

	Scale bar: 50µm	Scale bar: 20µm
OL DAY 1		
OL DAY 3		

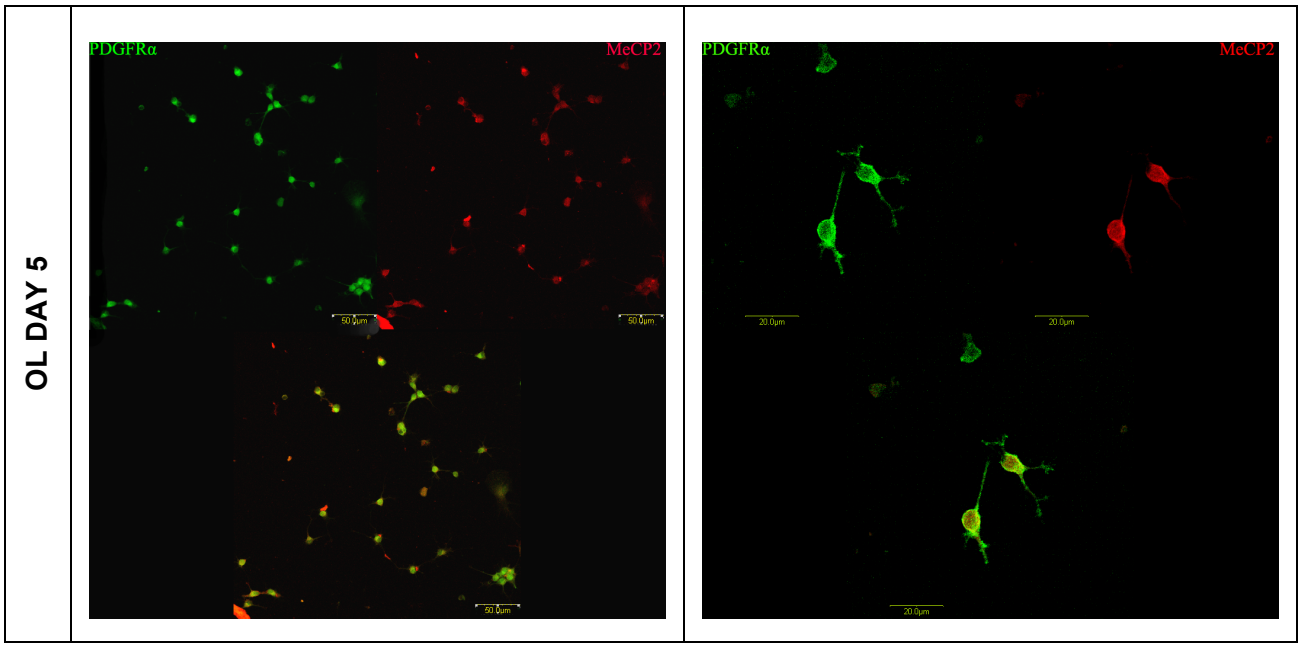


Figure 15: MeCP2 expression in early stages of OL differentiation.
OL in their early stages of differentiation (day 1, day 3, day 5) double immunolabeled with Tetramethyl Rhodamine Isothiocyanate (TRITC) (red, MeCP2 protein) and OL progenitor specific marker with fluorescein isothiocyanate (FITC) (green, PDGFRα protein). Merged color of green and red in the bottom shows co-localization. Bars represent 50μm and 20μm in column 1 and column 2, respectively.

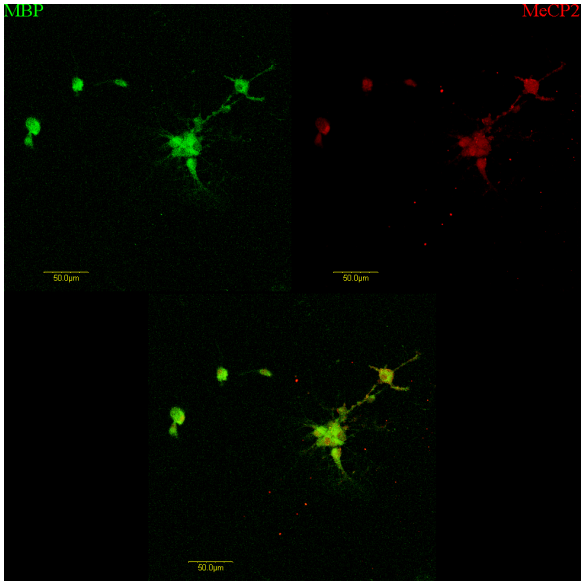
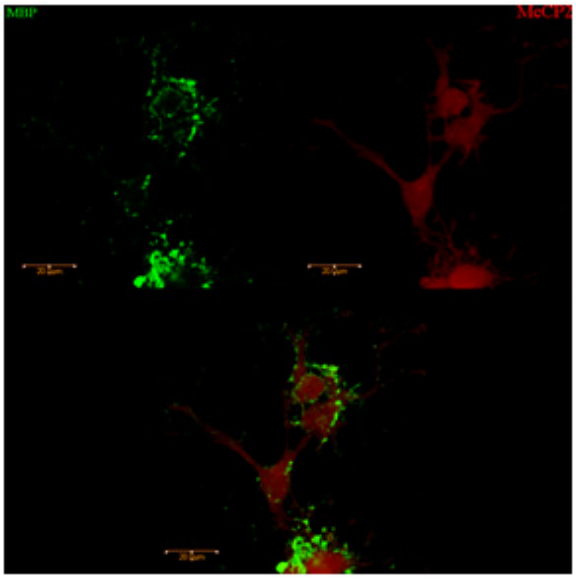
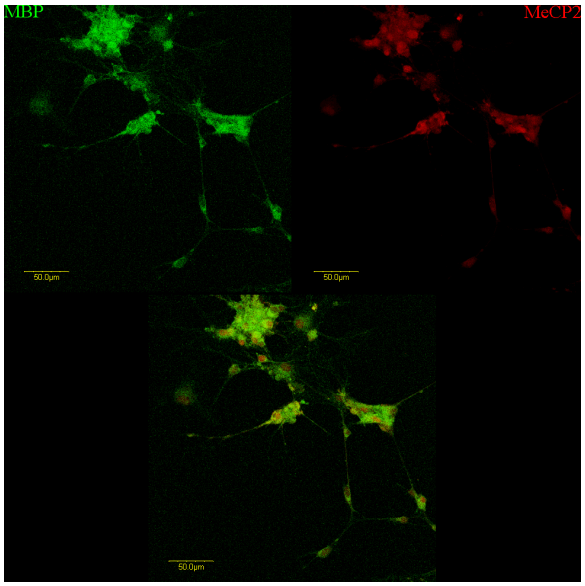
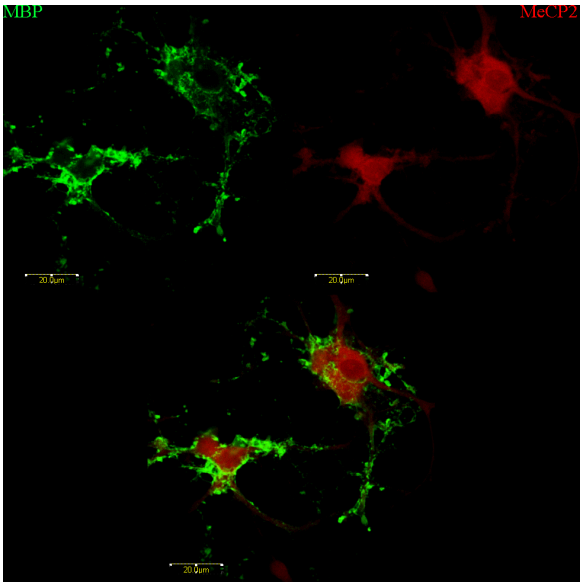
	Scale bar: 50µm	Scale bar: 20µm
OL DAY 7		
OL DAY 9		

Figure 16: MeCP2 expression in late stages of OL differentiation.
OL in their late stages of differentiation (day 7, day 9) double immunolabeled with TRITC (red, MeCP2 protein) and mature OL specific marker with FITC (green, MBP protein). Merged color of green and red in the bottom shows co-localization. Bars represent 50 µm and 20 µm in column 1 and column 2, respectively.

3.3.3 Differential myelin-related gene expression in MeCP2^{null} mice

Quantitative RT-PCR was used to assess gene expression levels in brain tissue derived from a MeCP2^{null} mouse. A significant increase in MBP mRNA levels was found in tissues from both forebrain (1.58-fold, $P<0.05$) and midbrain (2.90-fold, $P<0.005$) with no significant change in the cerebellar tissue **(Table 4)**. Similarly, significantly increased MAG mRNA levels (3.66-fold, $P<0.001$) were seen in the midbrain of the MeCP2^{null} mouse. However, in contrast to MBP, there was no change in MAG gene expression levels in the forebrain or cerebellum. The qRT-PCR analysis also showed no significant differences in the mRNA expression levels of PLP in the forebrain and midbrain of the MeCP2^{null} mouse as compared with age-matched wild-type controls. However, in contrast to MBP and MAG, we found significantly lower levels of PLP mRNA in the cerebellum.

Two other OL-specific proteins, CNPase and NG2, were also assessed for expression changes at the RNA level. The expression of CNPase, which remains constant throughout the OL lineage, remains unchanged in the MeCP2^{null} mouse. Expression levels of NG2, which is expressed by progenitors but not mature OLs, also remain unchanged in all assessed areas of the brain.

GENE	FOREBRAIN	MIDBRAIN	CEREBELLUM
MBP	1.58 ± 0.51*	2.90 ± 0.68**	0.73 ± 0.39
MAG	1.13 ± 0.79	3.66 ± 1.05***	1.33 ± 1.25
PLP	1.63 ± 0.59*	0.81± 0.41	0.45 ± 0.59
CNPase	1.27 ± 0.23	0.97 ± 0.37	1.37 ± 0.12
NG2	1.37 ± 0.56	0.92 ± 0.27	0.96 ± 0.59

Table 4: Myelin associated gene expression in MeCP2^{null} mice

Furthermore, during OL differentiation, alternative splicing of MBP, MAG and PLP occurs. If OL cell differentiation is affected because of MeCP2 gene mutation, it may affect the ratio of isoform expression of these myelin associated genes. However, PCR analysis of gene expression did not show any apparent difference to suggest dysregulation of OL cell differentiation **(Fig. 17)**

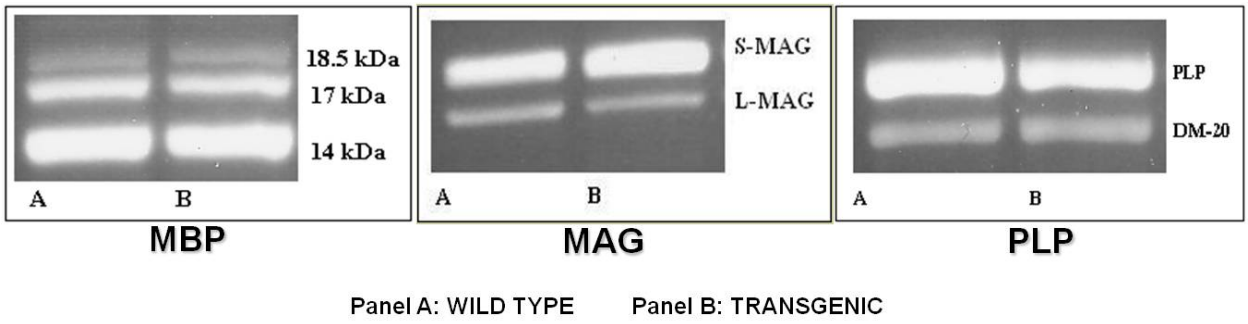


Figure 17: Expression of different isoforms of myelin associated genes.
RT-PCR results of expression of genes of MBP, MAG and PLP in the midbrain region of $MECP^{null}$ mouse (Panel B) and the wild type mouse (Panel A). No apparent difference was found in the ratio of gene isoform expression.

3.4 Discussion

Considerable progress has been made since the discovery of the RTT-causing gene, providing further understanding into the pathogenesis of the disease. Despite this advancement, the precise biological function of MeCP2 in the normal mammalian nervous system remains unclear. We know MeCP2 is a multi-functional protein, is expressed in many different cell types, works together with different molecular cues and is influenced by a number of genetic, epigenetic and environmental factors, all of which work in concert to insure proper development of the human brain.

Until recently, MeCP2 expression was considered limited to neuronal cells. In fact, it was reported that MeCP2 is expressed at very high levels in neurons and is undetectable in glia (Shahbazian, Young et al., 2002a; Jung *et al.*, 2003; Kishi and Macklis, 2004). Lately, magnetic resonance imaging and spectroscopy studies in RTT mouse show not only neuronal, but oligodendrocytic cell metabolism is also affected (Saywell *et al.*, 2006; Viola *et al.*, 2007). However, our studies conclusively show that OLs express MeCP2.

Furthermore, MeCP2 protein is continually expressed at high levels in primary rat OL lineage cells. The process of differentiation appears to involve changes in the nuclear and cytoplasmic levels of MeCP2 protein. The cytoplasmic MeCP2 appears to decrease, and nuclear MeCP2 appears to increase gradually in the differentiating OLs. It is interesting to note that MeCP2 localization in the cytoplasm and nucleus of the

neuronal cells was reported earlier (Miyake and Nagai, 2007). In accordance with the previous study in neurons, this data indicates that MeCP2 is expressed in cell bodies on the first day of the OP culture while after 7 DIV, MeCP2 starts to translocate into the nucleus in association with OL maturation. This nuclear translocation of oligodendrocytic MeCP2 is possibly induced during differentiation and/or maturation. The molecular mechanisms of this hypothesized translocation of MeCP2 remain unclear. Further studies are warranted to identify the exact role of MeCP2 in OLs.

In addition, this study also revealed that myelin associated gene expression is altered in the MeCP2^{null} mouse. These findings are consistent with the hypothesis that dysfunctional myelin may contribute to the pathophysiology of disorders associated with MeCP2 mutations, including autism, ADHD, schizophrenia, and Rett syndrome.

The regulation of myelin gene expression is critical for normal myelin formation. Proteomic analysis of myelin has shown that PLP, MBP, CNPase, MAG, and myelin OL glycoprotein are the five major proteins of the myelin sheath at 17, 8, 4, 1, and 1%, respectively (Jahn, Tenzer et al., 2009). Studies using transgenic mice have shown clear roles for these proteins in the normal functioning of myelin (Jahn, Tenzer et al., 2009). Based on our hypothesis that myelin is abnormal in the MeCP2^{null} brain, the mRNA expression levels of these myelin associate genes were investigated. Using a transgenic mouse, expressing a truncated form of MeCP2, the qRT-PCR results show significant changes in expression levels of MBP, PLP, and MAG compared with wild-type age-matched control tissue. PLP has been linked to several different functions,

including ion exchange, cell migration, apoptosis, and myelination (Baumann and Pham-Dinh, 2001). PLP gene mutations result in significant loss in myelin compaction and interestingly, overexpression of PLP causes a neurological disease that is more severe than the complete absence of PLP (Griffiths, Klugmann et al., 1998; Tanaka, Ma et al., 2009). MBP, the second most predominant protein in the myelin sheath, is primarily an adhesion molecule. Deletion of MBP results in almost complete lack of myelin in the CNS, with severe neurological defects and a significantly shortened lifespan (Wolf and Billings-Gagliardi, 1984). MAG is known to promote neurite outgrowth, with MAG^{null} mice showing structural abnormalities in the periaxonal areas of the myelin sheath (Johnson, Abramow-Newerly et al., 1989; Li, Tropak et al., 1994; McKerracher, David et al., 1994; Montag, Giese et al., 1994).

Interestingly, our data shows that levels of NG2 and CNPase are unaffected. NG2 is associated with OL progenitors, and expression is downregulated as the progenitors differentiate into mature OL, and CNPase expression is maintained through the OL lineage. Thus, our data suggest that total numbers of OL are unchanged in the $MeCP2^{null}$ mouse. The $MeCP2^{null}$ mouse has several characterized motor deficits that are not a result of abnormalities in muscle development (Shahbazian, Young et al., 2002a). These deficits have been explained as a result of impaired neuronal signaling, since there was no impairment of muscle strength in the null mice. (Shahbazian, Young et al., 2002a)

Consequently, a change in the expression levels of any of these proteins significantly impacts the quality of the myelin sheath, which in turn significantly affects nerve conductance. Changes in the expression patterns of MBP, PLP, and MAG mRNA indicate that MeCP2 plays a direct or indirect role in the regulation of myelin gene expression. Myelination occurs in a temporal pattern in the developing mammalian brain. The corpus callosum is one of the first areas of the brain to be myelinated, followed by the forebrain, with the cerebellum being myelinated last (Baumann and Pham-Dinh, 2001). Consequently, our finding that different areas of the brain show differential levels of expression fits with the temporal pattern of myelination during development. Further studies are required to fully characterize the effects of MeCP2 deletion on the myelin of the CNS.

Chapter 4: “Role of CXCL1 in regulation of OL Progenitor cell (OP) behaviour via calcium signaling”

4.1 Introduction

4.1.1 CXCL1 and the chemokine family

The family of small (6-10kDa), secreted cytokines known as chemokines are well known to play a significant role in cell trafficking systems in mammalian biology. They comprise a large protein family, which is divided into sub-families on the basis of structural motifs (C, CC, CXC, CX3C). These chemokines exert their biological effects via a large family of 7-transmembrane G-protein-coupled receptors. Based on the specificity of the chemokine, a logical receptor nomenclature system was established in which each receptor was designated by chemokine subfamily name (C, CC, CXC, CX3C) followed by the letter "R" (for "receptor") and a number based on the chronological order in which it was identified. However, subsequent discovery of more than 50 human chemokines and 18 human chemokine receptors to date (Nomenclature, 2003), has resulted in a considerable challenge with respect to identifying the specific chemokines and receptors required for complex and essential functions. Moreover, the fact that several

chemokines can activate multiple receptors, and many chemokine receptors are responsive to numerous chemokines, has added to the complexity of understanding the downstream signaling pathways activated by this system.

The role of chemokines was first studied and established as inflammatory cytokines in controlling leukocyte migration (Yoshimura *et al.*, 1987). However, later studies revealed that, in addition to the immune system, chemokine signaling may also have an important role in the CNS (Zou *et al.*, 1998; Bajetto *et al.*, 1999; Rostene *et al.*, 2007). The chemokine CCL2, a member of CC subfamily has been shown to mediate acute excitotoxic injury in neonatal rat brain (Galasso *et al.*, 2000) and is also shown to induce astrocytic migration in neonatal and adult mice *in vitro* (Ambrosini and Aloisi, 2004). The CXC subfamily of chemokines, which possibly evolved early in vertebrate biology, have been shown to be involved in CNS tissue patterning and development (Huising *et al.*, 2003). CXCL12 in the developing brain has been shown to regulate interneuron migration (Stumm *et al.*, 2003) and to stimulate proliferation of astrocytes (Lazarini *et al.*, 2003). Another such chemokine, CXCL1 (a homologue of interleukin-8, formerly known as Groα) and its receptor CXCR2 have been implicated in proliferation and migration of OL precursors during CNS development (Robinson, Tani et al., 1998b; Tsai, Frost et al., 2002). So far, CXCR2 is the only chemokine receptor that has been detected in primary OL precursors (Nguyen and Stangel, 2001; Filipovic *et al.*, 2003). CXCL1 was shown to enhance the proliferative response of immature rat spinal cord OLs in the presence of PDGF-A (Robinson, Tani et al., 1998b). Furthermore, in the dysmyelinating jimpy mouse mutant where there is extensive OL death, elevated levels

of CXCL1 mRNA and protein lead to an increase in the number of OP cells, suggesting the supportive role of endogenous CXCL1 in OP proliferation (Wu *et al.*, 2000). Later studies of CXCL1 on OP behaviour showed that CXCL1 signaling through the CXCR2 receptor inhibits PDGF-A induced migration of OP cells *in vitro* (Tsai, Frost et al., 2002).

4.1.2 Role of calcium in OP migration

Various growth factors and neurotransmitters that regulated OP cell development and pathology have been shown to evoke changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) (Soliven, 2001; Fields and Stevens-Graham, 2002; Belachew and Gallo, 2004). Activation of a range of cell-surface receptors and/or ion channels evoke Ca^{2+} changes in OP cells, either by triggering extracellular Ca^{2+} influx through plasmalemma or by release of Ca^{2+} from intracellular stores, the mitochondria and the endoplasmic reticulum. The subsequent recovery of $[Ca^{2+}]_i$ is brought about by sequestration of Ca^{2+} back into intracellular stores and by efflux of Ca^{2+} ions through ATP-dependent Ca^{2+}-pumps and Na^{+}-Ca^{2+} exchangers (Verkhratsky and Kettenmann, 1996; Deitmer *et al.*, 1998; Alberdi *et al.*, 2005)

Furthermore, Ca^{2+} changes are evoked in OP cells by several different signaling proteins. Many of the signaling pathways involved in regulating OP migration have been reported to be Ca^{2+} sensitive (Fay, 1995; Kohama *et al.*, 1996). However, the exact role of Ca^{2+} in OP migration remains largely unknown.

4.2 Rationale and Hypothesis

On reaching their destination, OPs undergo proliferation, before maturing into the myelinating OL. Early studies of the regulation of OP migration provided evidence for a putative chemotactic gradient of growth factor (PDGF-A being the most likely candidate) in the developing CNS (Armstrong, Harvath et al., 1990; Frost, Kiernan et al., 1996). However, studies of PDGF-A distribution in the developing brain suggest that no such chemotactic gradient exists (Yeh *et al.*, 1991; Hutchins and Jefferson, 1992; Reddy and Pleasure, 1992). More recent studies have shown that OP migration does not require continuous exposure to PDGF-A (Frost, Zhou et al., 2009; Vora, Pillai et al., 2011), negating the necessity of a chemotactic gradient of PDGF-A to drive it. In addition, the identification of chemorepellent factors in the germinal matrix of the developing brain provides further evidence against a motogenic signaling gradient in the developing CNS (Sugimoto *et al.*, 2001; Spassky, de Castro et al., 2002; Tsai and Miller, 2002; Jarjour *et al.*, 2003; Tsai, Tessier-Lavigne et al., 2003).

The precise positioning of OPs during development is critical to ensure the correct myelination of white matter tracts. In the absence of a motogenic gradient, cell migration may be precisely regulated by the presence of localized morphogens, which alter the cells' response to its local environment based on ligand concentration, and/or receptor occupancy (Freeman and Gurdon, 2002). Previous studies have shown that OPs stop migrating to proliferate in response to a localized concentration of the cytokine CXCL1 (Robinson *et al.*, 1998a; Tsai, Frost et al., 2002; Tsai and Miller, 2002). It was

shown earlier that CXCL1, at a precise concentration of 0.5ng/ml, inhibits OP migration (Tsai, Frost et al., 2002) and induces OP proliferation (Robinson, Tani et al., 1998a), regardless of PDGF-A concentration. The exact mechanism of this has yet to be identified. CXCR2, the receptor of CXCL1 is coupled to a pertussis toxin sensitive G-protein (Richardson *et al.*, 1998), and activates several signaling cascades including Rho kinase, and phosphatidyl inositol (L'Heureux *et al.*, 1995; Richardson, Pridgen et al., 1998; Schraufstatter *et al.*, 2001). Such coupling and activation of the heterotrimeric G-protein, leads to activation of phospholipase C (PLC), which further generates the second messengers diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). The binding of IP3 to its receptors on endoplasmic reticulum leads to release of Ca^{2+} from the intracellular stores (Hirsch *et al.*, 2000). IP3 and DAG act in combination with elevated iCa^{2+} to finally activate protein kinase C (PKC) (Smith *et al.*, 1986; Arai and Charo, 1996).

It is already known that changes in the $[Ca^{2+}]_i$ play a crucial role in the regulation of cell cycle progression (Balk, 1971) as well as OP migration (Simpson and Armstrong, 1999). In the current study, we hypothesizes that CXCL1 inhibition of PDGF-A induced OP migration is regulated by changes in intracellular Ca^{2+} flux.

4.3 Methods

4.3.1 Cell isolation and culture

OL progenitor cells (OPs) were isolated from neonatal rat pups (P0-P1), by a previously described method (Armstrong, 1998).

4.3.2 Agarose Drop Migration Assay

Migration was assessed using the agarose drop assay (Frost, Milner et al., 2000). Please refer to Section 2.3.2 **[Chapter 2]** for detailed methodology.

4.3.3 Calcium measurement by Fura-2AM dye

Fura-2 is a fluorescent dye which binds to free iCa^{2+}, and is commonly used to assess changes in $[Ca^{2+}]_i$ (Tucker *et al.*, 1989). Fura-2-acetoxymethyl ester (Fura-2AM) is a strongly hydrophobic dye that very easily diffuses through the lipid bilayer of the plasma membrane of the cell (Gunter *et al.*, 1988). Once inside the cell, the AM derivatives are rapidly hydrolyzed by nonspecific cytoplasmic esterases to become hydrophilic free acids, which are non-permeable and thus are trapped in the cell (Gunter, Restrepo et al., 1988). Fura-2 is excited at 340 nm and 380 nm. When bound to Ca^{2+} Fura-2 is excited at 340 nm and unbound Fura-2 is excited at 380 nm, it emits at 510 nm whether

bound or unbound. The ratio of emission at those wavelengths is directly correlated to the concentration of intracellular calcium, using the formula shown in **Table 5**. The use of a ratio automatically negates uncontrollable variables, such as dye concentration and cell thickness. Purified OPs were incubated with 2 μM Fura-2AM (Invitrogen, Burlington, ON) in Ca^{2+} free Locke's buffer (154 mM NaCl; 3.6 mM NaHCO3; 5.6 mM KCl; 1.0 mM MgCl2; 5.0 mM glucose; 5 mM Hepes; pH 7.0) with 0.1% BSA, for 1 hour. After washing, the cells were suspended in Locke's + BSA, and then placed into a Jasco CAF-110 intracellular ion analyzer for Ca^{2+} flux analysis.

$$\left[Ca^{2+}\right]_i = K_d\left(\frac{R - R_{min}}{R_{max} - R}\right)\left(\frac{\lambda_{380\ nm}\ \text{at minimum}}{\lambda_{380\ nm}\ \text{at maximum}}\right)$$

Table 5: Calculation of intracellular calcium concentration
R is the ratio of 510nm emission intensity, exciting at 340nm, to 510nm emission intensity, exciting at 380nm. Rmin is the ratio at zero free Ca^{2+} (i.e. prior to the addition of $CaCl^2$ to the Locke's buffer); Rmax is the ratio at saturating Ca2+ (i.e. after the addition of digitonin to perforate the cell membranes allowing extracellular Ca^{2+} to flood into the cell). 380nm at minimum is the fluorescence intensity, exciting at saturating free Ca^{2+}; 380nm at maximum is the fluorescence intensity, exciting at zero free Ca^{2+}. Kd is an empirical value determined from Fura-2 containing Ca^{2+} standards run on the analyzer prior to the use of the Fura-2 for each experiment (Grynkiewicz et al., 1985).

4.3.4 Materials

All materials were obtained from Sigma (St. Louis, MO, USA) unless otherwise stated. Platelet derived growth factor-A (PDGF-A) and fibroblast growth factor 2 (FGF2) was

purchased from R&D Systems (Minneapolis, MN, USA). The pharmacological inhibitors 1,2-Bis(2-amino-5-methylphenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis (acetoxymethyl) ester (MAPTAM) and U0126 were obtained from Sigma-Aldrich (Cat# 16609 & Cat# U120, respectively). MAPTAM was dissolved in DMSO at 50 mM stock concentration.

Sato defined medium was prepared as previously described (Frost, Zhou et al., 2009).

4.3.5 Statistical Analysis

Statistics were performed using GraphPad Prism version 4.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. One-way ANOVA with Dunnett's post test was used to test differences between migration curves. Student t-test was used to test the difference between means of cell count experiments.

4.4 Results

4.4.1 Calcium is required for PDGF-A and FGF2 induced OP migration.

Previous studies have shown that chelation of extracellular Ca^{2+} does not affect PDGF-A induced OP migration, whereas chelation of intracellular Ca^{2+} does inhibit PDGF-A induced OP migration (Simpson and Armstrong, 1999). The calcium chelating reagent MAPTAM was used to confirm the requirement for Ca^{2+} signaling in OP migration in the

agarose drop assay (Pende *et al.*, 1997). MAPTAM, is a cell permeant analog of EGTA which prevents any rise in $[Ca^{2+}]_i$. OPs were preincubated with 45 μM MAPTAM for 30 minutes prior to the addition of growth factor. Migration was measured 72 hours after addition of growth factor. In the presence of MAPTAM, both PDGF-A and FGF2 induced migration was inhibited significantly **(Fig. 18)**. PDGF-A induced OP migrated 973.25 ± 42.61μm (compared to 262.15 ± 31.79 μm in control wells), and in the presence of MAPTAM migration was significantly reduced by 45% to 536.62 ± 38.32 μm (p<0.001). FGF2 induced OPs also migrate 893.25 ± 32.14μm, and in the presence of MAPTAM, OP migration was inhibited by 44% to 502.44 ± 36.324μm (p<0.001).

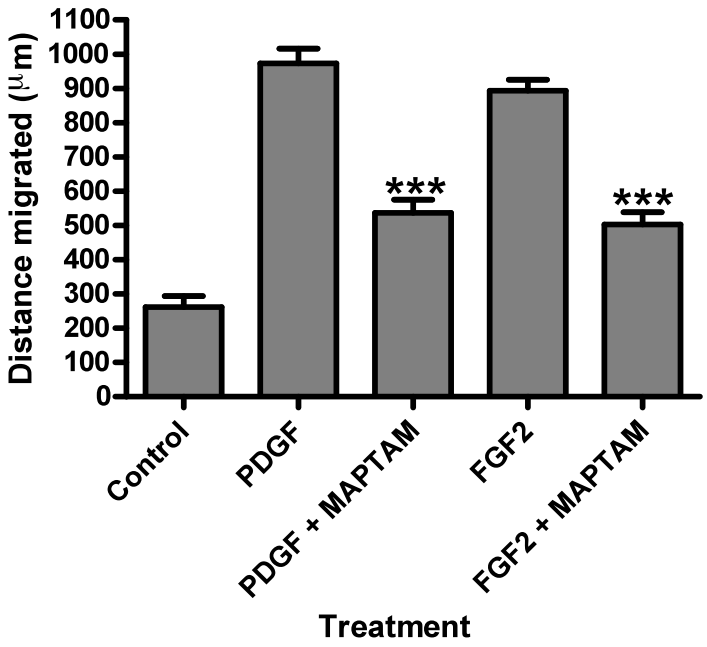


Figure 18: MAPTAM inhibits growth factor induced migration of OP.
OPs pre-treated for 20 minutes with 45μm MAPTAM showed significantly reduced migration in response to PDGF-A and FGF2 compared to growth factor treatment alone. (n=6, with 4-6 replicates) ***p<0.001

4.4.2 Active inhibition of PDGF-A induced OP migration by the chemokine CXCL1 is specific to CXCL1 concentration.

Previous studies have shown that the chemokine CXCL1 acts as a stop signal for OPs migrating in response to PDGF-A, using a microchemotaxis chamber migration assay (Tsai, Frost et al., 2002). For this study, the agarose drop assay was used, which allows manipulation of the media during the experiment, as well as to study migration over a longer period of time (Frost, Milner et al., 2000). To further understand the inhibition of PDGF-A induced OP migration by CXCL1 the effect of two concentrations of CXCL1 (0.5 and 5 ng/ml) on a dose response curve to PDGF-A (0.5-10 ng/ml) was assessed. Our results concurred with our previous findings (Tsai, Frost et al., 2002). In the presence of 0.5 ng/ml CXCL1, PDGF-A induced OP migration is inhibited regardless of growth factor concentration. In the presence of 5ng/ml CXCL1, there is no inhibition of PDGF-A induced OP migration regardless of growth factor concentration **(Fig. 19A)**.

To further analyze the effect of CXCL1 on OP migration, 10 ng/ml PDGF-A was used, and CXCL1 added after 48 hours. Even after OP migration was initiated by PDGF-A, CXCL1 blocked further migration indicating that it is an active inhibitor of migration **(Fig. 19B)**. After 24 hours of PDGF-A exposure, OPs migrate 401.21 ± 55.30 μm compared to 263.43 ± 38.35 μm in control (untreated) wells. After 48 hours of PDGF-A exposure, OPs migrate 688.98 ± 62.25 μm compared to 344.07 ± 64.23 μm in control (untreated) wells. CXCL1 (0.5 ng/ml) was added to the wells at T48, and 24 hours later (T72) OP

migration in the presence of PDGF-A was stopped at 671.75 ± 89.16 μm, compared to 989.00 ± 61.65 μm in the presence of PDGF-A alone.

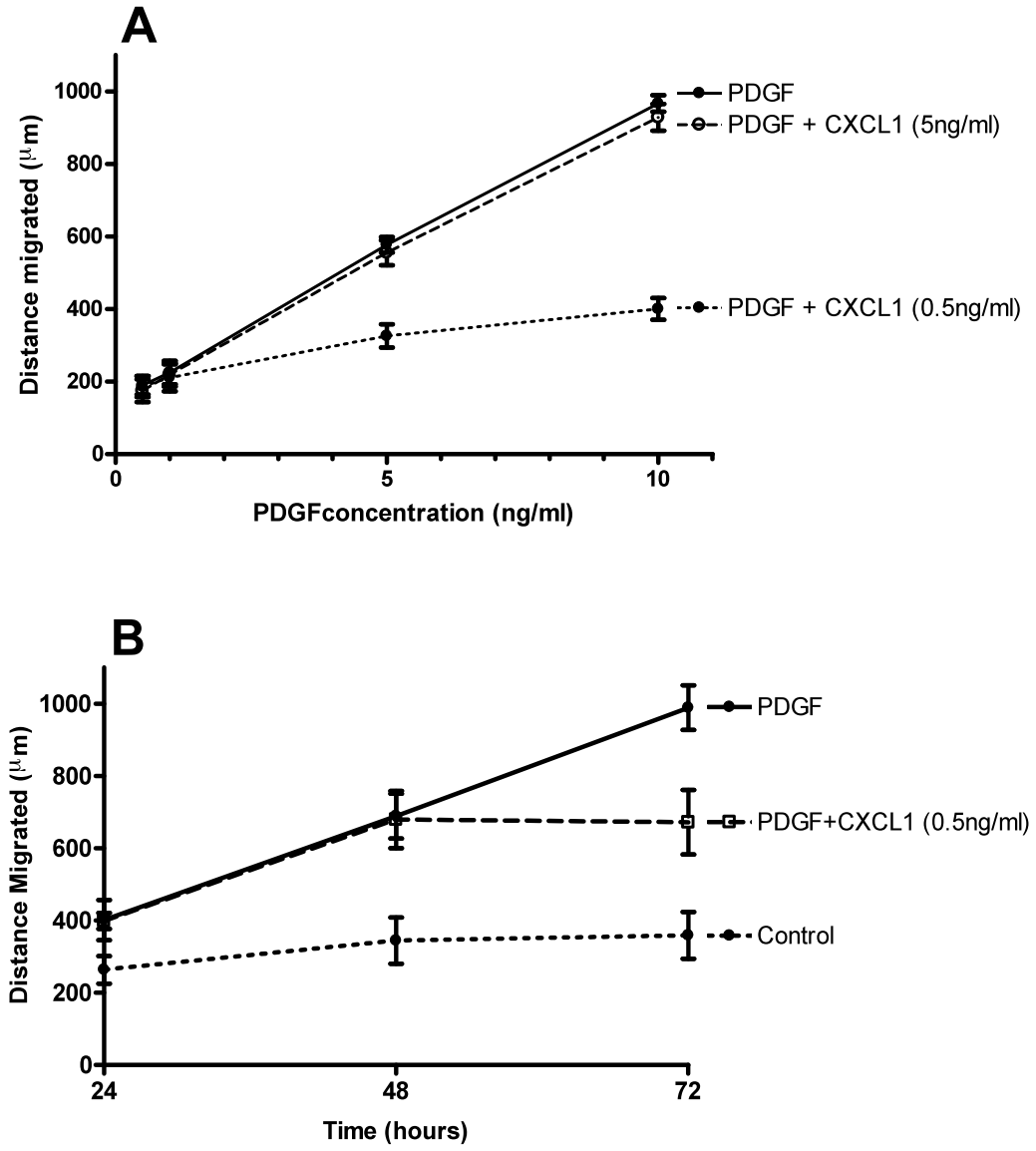


Figure 19: Inhibition of PDGF-A induced OP migration by CXCR2 activation.
Panel A - CXCL1 inhibition of PDGF-A induced OP migration is concentration dependent. At 0.5ng/ml, CXCL1 inhibits OP migration induced by all concentrations of PDGF-A (closed circles, dashed line), whereas at 5ng/ml, CXCL1 has no effect on PDGF-A induced OP migration (open circles, dotted line), at any concentration. (n=3 with 4-6 replicates).
Panel B - OP migration induced by PDGF-A (solid circle) is inhibited by CXCL1 added 24 hours after the initiation of migration (closed square). Inhibition of OP proliferation by the Glutamate agonist AMPA does not affect OP migration in response to PDGF (open circle dashed line. Inhibition of the cell cycle by AMPA does not prevent CXCL1 inhibition of migration (open square dashed line). (n=5, with 4-5 replicates per treatment).

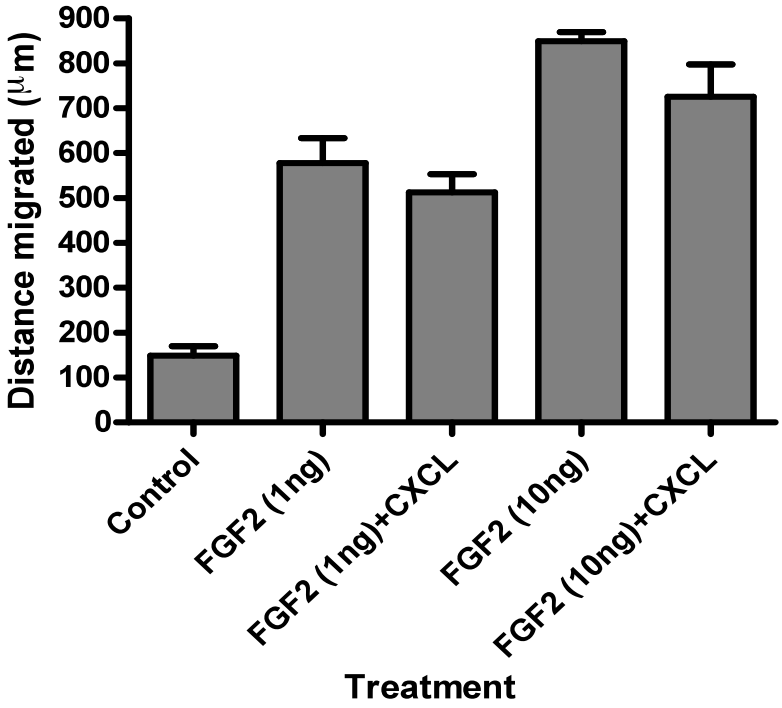


Figure 20: FGF2 induced OP migration is not inhibited by CXCL1.
OPs migrate significant distances in response to 1ng/ml and 10ng/ml FGF2. However, the chemokine CXCL1 does not inhibit FGF2 induced migration, in contrast to PDGF-A induced migration. OP Migration was measured 72 hours after the addition of growth factor. N=3 with 4-6 replicates.

4.4.3 Inhibition of OP migration by the chemokine CXCL1 is specific to PDGF-A induced migration.

As discussed in **Chapter 2**, PDGF-A and FGF2 play an important role in inducing OP migration. PDGF-A acts through the PDGFRα, which is a tyrosine kinase receptor (RTK). Another growth factor known to induce OP migration is FGF2. The members of the FGF receptor family (FGFR) are also RTKs, with similar intracellular signaling cascades activated by ligand binding. OPs express three of the four FGFR family members (Bansal, Kumar et al., 1996; Bansal, Lakhina et al., 2003a). In order to assess the potential for CXCL1 as the primary stop signal for OP migration, the effects of CXCL1 on FGF induced OP migration were analysed. In contrast to PDGF-A, in the presence of FGF2 OP Migration was not affected by the addition of CXCL1. In the presence of 1 ng/ml FGF2, OP were induced to migrate 578.10 ± 95.85 μm compared to 512.89 ± 70.13 μm in the presence of CXCL1 (0.5 ng/ml) p=0.395. In the presence of 10 ng/ml FGF2, OP were induced to migration 849.21 ± 40.80 μm compared to 725.38 ± 144.23 μm in the presence of 0.5 ng/ml CXCL1 p=0.150 **(Fig. 20)**.

4.4.4 PDGF-A induces significant, dose-dependent, calcium flux in OPs.

In order to analyse Ca^{2+} flux in OP, freshly isolated OPs were preincubated with Fura-2 for 2 hours, and then changes in $[Ca^{2+}]_i$ in the presence and absence of PDGF-A was assessed. It was found that in the presence of both 1 ng/ml and 10 ng/ml, PDGF-A evoked a significant increase in $[Ca^{2+}]_i$ of 230.35 nM compared to baseline of 183.2 nM; and 338.668 nM compared to a baseline of 208.6 nM **(Fig. 21)**. This represented 82.83% of the peak ATP value and 98.82% of the peak ATP value, respectively.

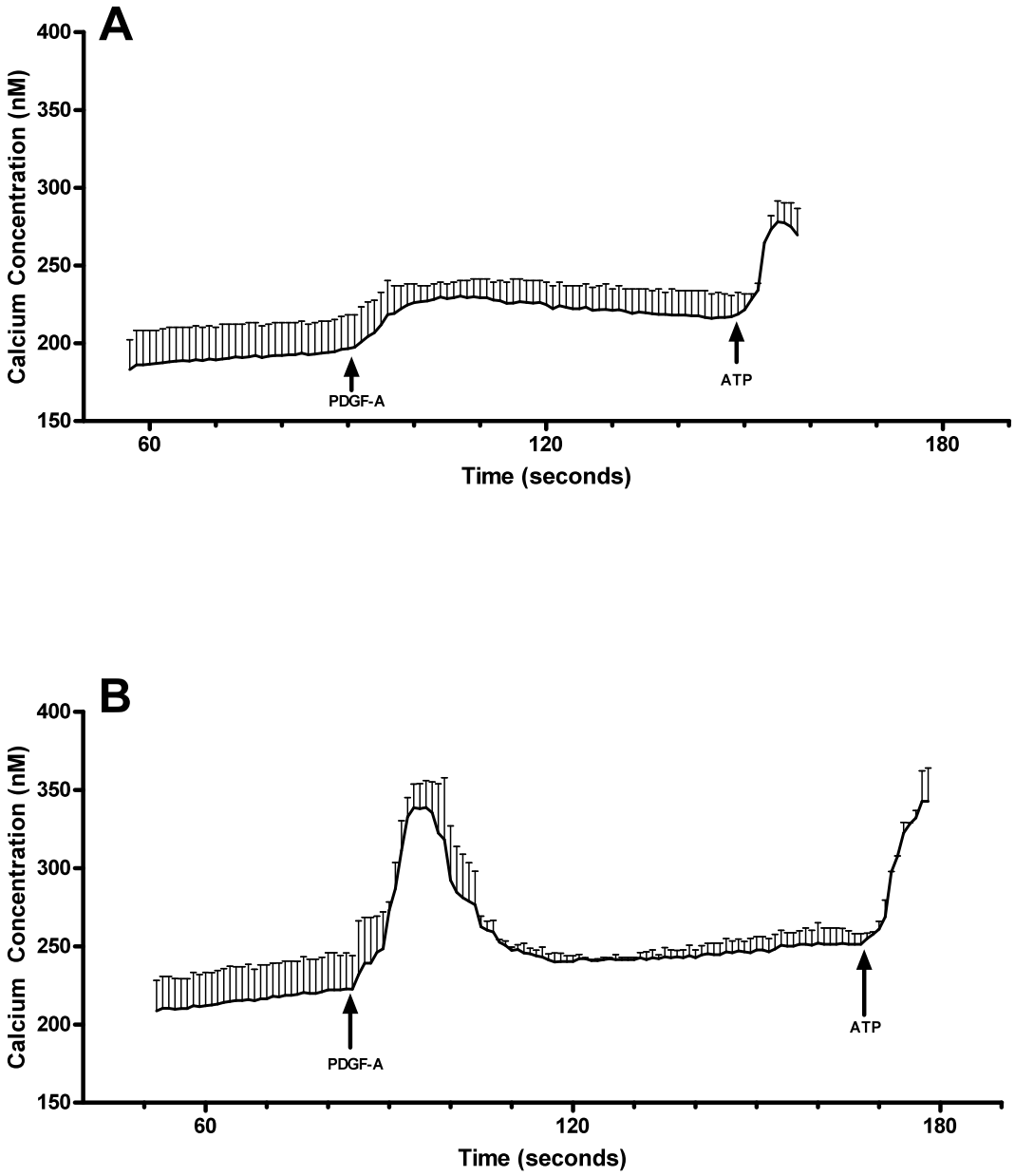
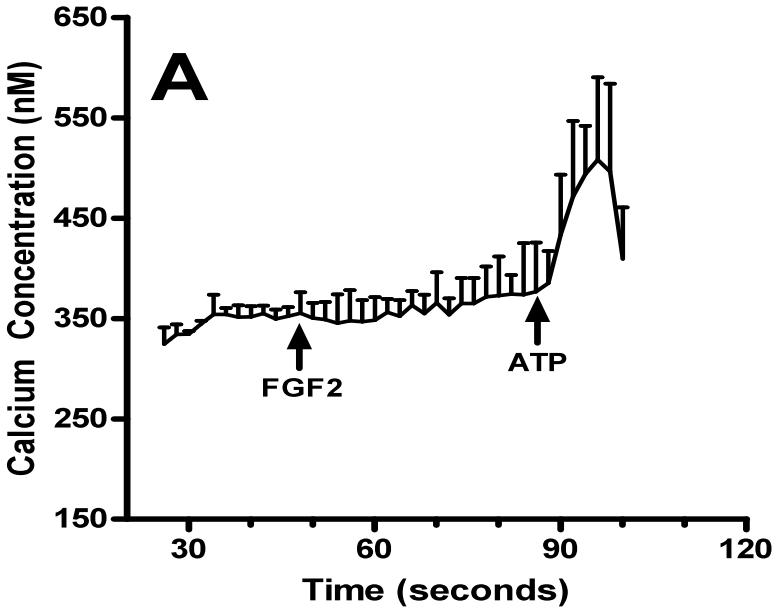


Figure 21: Calcium flux in response to PDGF-A
Calcium flux in primary OPs is induced by PDGF-A in a dose dependent manner. Panel A – PDGF-A is added to the cells at 1 ng/ml. A significant increase in intracellular calcium is seen. Panel B – PDGF-A is added to the cells at 10 ng/ml concentrations. A significant increase in intracellular calcium is seen. ATP is added to the cells to show that they are responding as expected. Arrows indicate the addition of PDGF-A and ATP. The values are given for Calcium concentration in nM (n=3).


A
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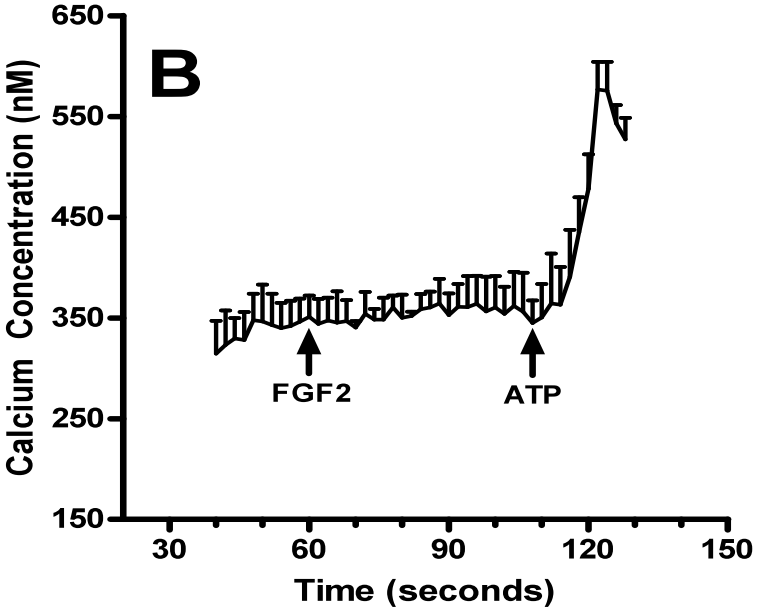
550
450
350
250
150
Calcium Concentration (nM)
FGF2
ATP
30
60
90
120
Time (seconds)


B
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Calcium Concentration (nM)
FGF2
ATP
30
60
90
120
150
Time (seconds)

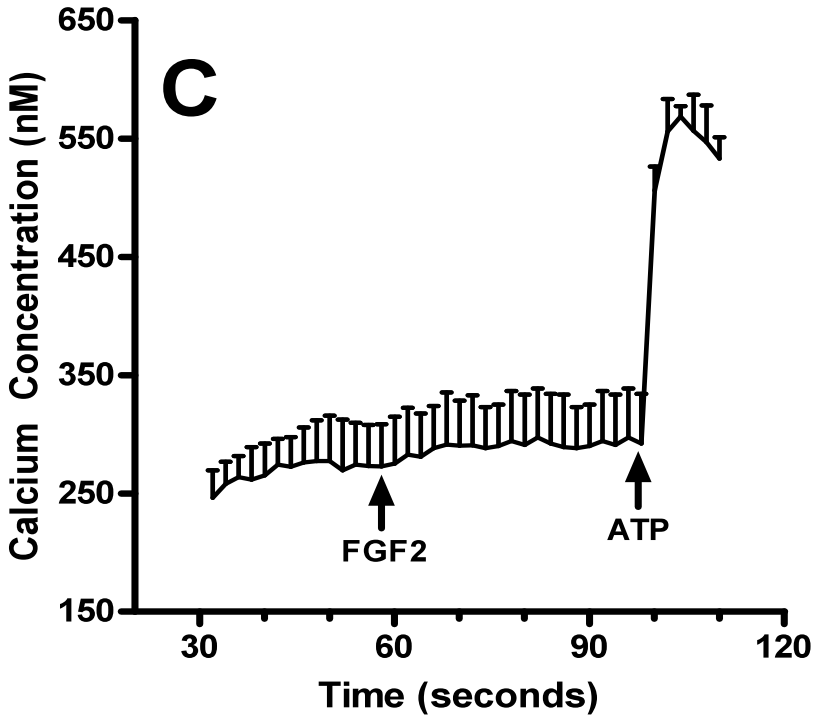

Figure 22: Effect of FGF2 on OPC calcium flux *in vitro*
Calcium flux in primary OPs is unaffected by FGF2. ATP is added to the cells to show that they are responding as expected.
Panel A – FGF2 at 0.1ng/ml,
Panel B – FGF2 added at 1ng.ml and
Panel C – FGF2 added at 10ng/ml. Arrows indicate the addition of PDGF-A and ATP. The values are given for Calcium concentration in nM (n=3).

4.4.5 FGF2 does not induce calcium flux in OP.

FGF receptor studies have indicated that $[Ca^{2+}]_i$ is altered in response to FGF ligand binding (Tsuda *et al.*, 1985; Eswarakumar, Lax et al., 2005). However, several studies have shown that the effect of receptor activation differs between cell types, with different intracellular signaling pathways being activated as a result of ligand binding (Satoh *et al.*, 1993; Stork and Schmitt, 2002; Murakami *et al.*, 2008). Therefore, for completeness, Ca^{2+} flux in response to FGF2 in OP was assessed. Even at a concentration of 10 ng/ml, FGF2 did not induce Ca^{2+} flux in the OP. The changes in $[Ca^{2+}]_i$ in response to 0.1 ng/ml FGF2 was assessed, in case a lower concentration threshold is required for Ca^{2+} flux. However, there were no apparent changes in $[Ca^{2+}]_i$ in response to any concentration tested (**Fig. 22)**.

4.4.6 CXCL1 does not affect intracellular calcium levels in OP.

CXCl1, acting through the CXCR2 receptor, is shown to induce Ca^{2+} flux in other cell types (Puma *et al.*, 2001; Shibata *et al.*, 2002). Moreover, other chemokines have shown to invoke rapid mobilization of Ca^{2+} from intracellular store in neurons (Giovannelli *et al.*, 1998; Meucci *et al.*, 1998). However, in OPs, contrary to the expected results, it was found that at 0.5 ng/ml, CXCL1 had no effect on $[Ca^{2+}]_i$ **(Fig. 23A)**. Even at concentrations as high as 50 ng/ml no changes were seen in $[Ca^{2+}]_i$ **(Fig. 23B)**.

4.4.7 CXCL1 does not affect PDGF-A induced $[Ca^{2+}]_i$ in OP.

In order to test the hypothesis that CXCL1 inhibits OP migration via a Ca^{2+} dependent pathway, CXCL1 was added prior to the addition of PDGF-A, and assessed changes in $[Ca^{2+}]_i$. CXCL1 was found to have no effect on PDGF-A induced OP Ca^{2+} flux **(Fig. 24)**. CXCL1 was added to the OP at 0.5 ng/ml and 30 seconds later 1 ng/ml PDGF-A was added to the cells. PDGF-A elicited a significant increase in $[Ca^{2+}]_i$ to 340.50 nM after pre-treatment with CXCL1, which represents 87.6% of the ATP response (388.9 nM), compared to 338.7 nM in the presence of 10 ng/ml PDGF-A alone **(Fig. 24)**.

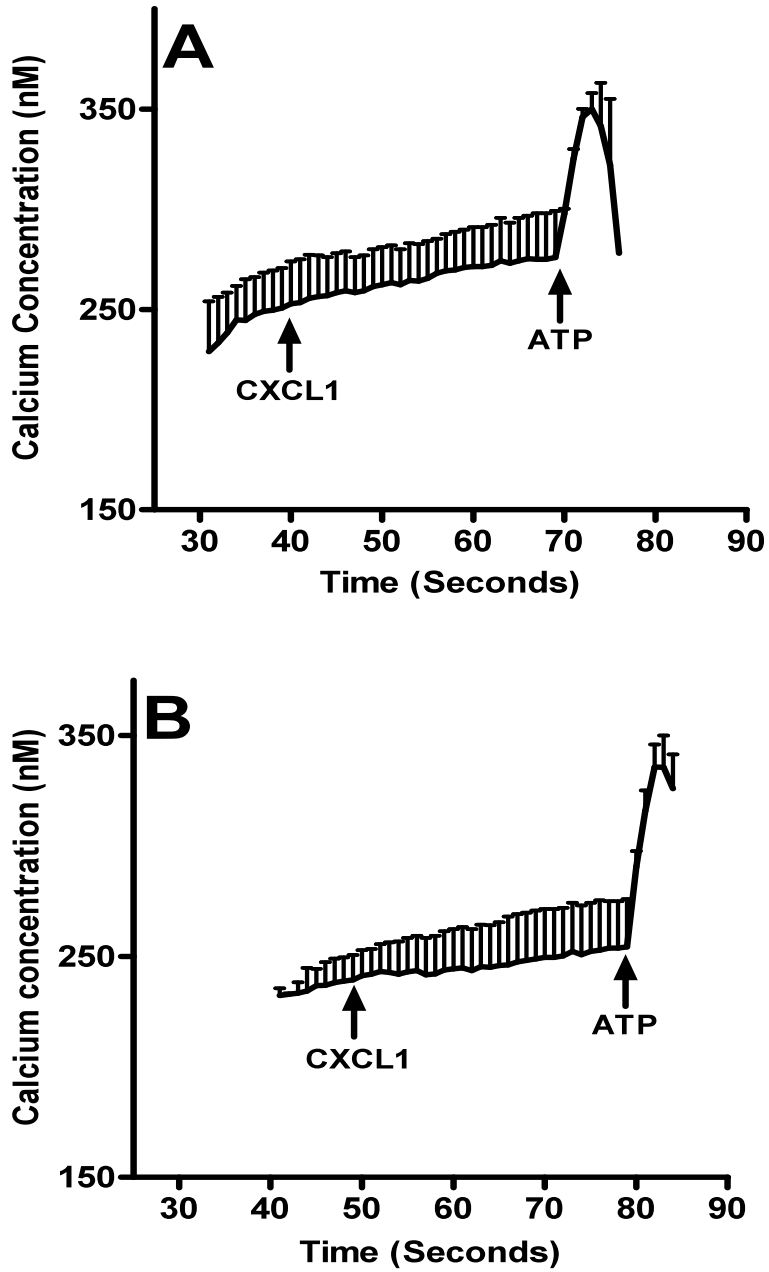

Figure 23: Calcium signaling in response to CXCL1
Calcium flux in primary OPs is unaffected by CXCL1. Panel A - CXCL1 is added to the cells at 0.5 ng/ml concentration. Panel B - CXCL1 is added to the cells at 50 ng/ml concentration. ATP is added to the cells to show that they are responding as expected. Purple arrows indicate the addition of CXCL1; green arrows indicate the addition of ATP. The values given for Calcium concentration are nM (n=3).

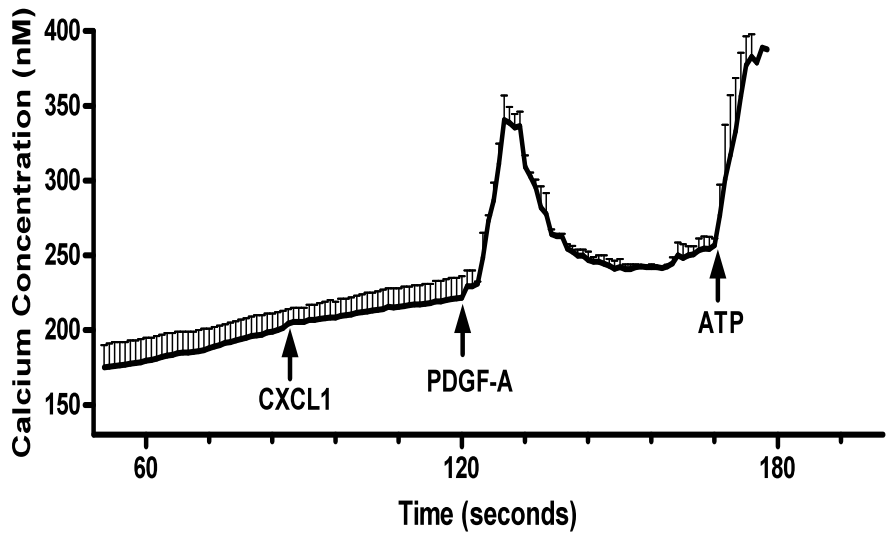

Figure 24: Calcium flux in response to PDGF-A after CXCL1 treatment
PDGF-A induced calcium flux in primary OPs is unaffected by the addition of CXCL1 at 0.5ng/ml prior to the addition of the PDGF-A. ATP is added to the cells to show that they are responding as expected. Arrows indicate the addition of CXCL1, PDGF-A, and ATP. The values given for Calcium concentration are in nM (n=3).

4.4.8 Inhibition of cell cycle activation does not affect CXCL1 induced inhibition of OP migration.

To check whether CXCL1 induced inhibition of OP migration is via activation of the cell cycle, we used 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid (AMPA). AMPA is shown to inhibit cyclin E-cyclin-dependant kinase 2 (cdk2) formation, leading to cell cycle arrest (Ghiani and Gallo, 2001a), without affecting OP migration (Gallo, Zhou et al., 1996; Frost, Zhou et al., 2009). The results show that AMPA had no effect on the CXCL1 inhibition of PDGF-A induced OP migration **(Fig. 25)**. Results were normalized and shown as a percentage of the migration elicited in response to 10 ng/ml PDGF-A. PDGF-A alone produced a 100 ± 6.23 % migration. PDGF-A in combination with AMPA resulted in a slight reduction in migration to 94.57 ± 6.66 %. In the presence of CXCL1, PDGF-A treatment resulted in 44.7 ± 6.99 % migration. In the presence of AMPA and CXCL1, PDGF-A induced 40.91 ± 7.08 % migration **(Fig 25)**.

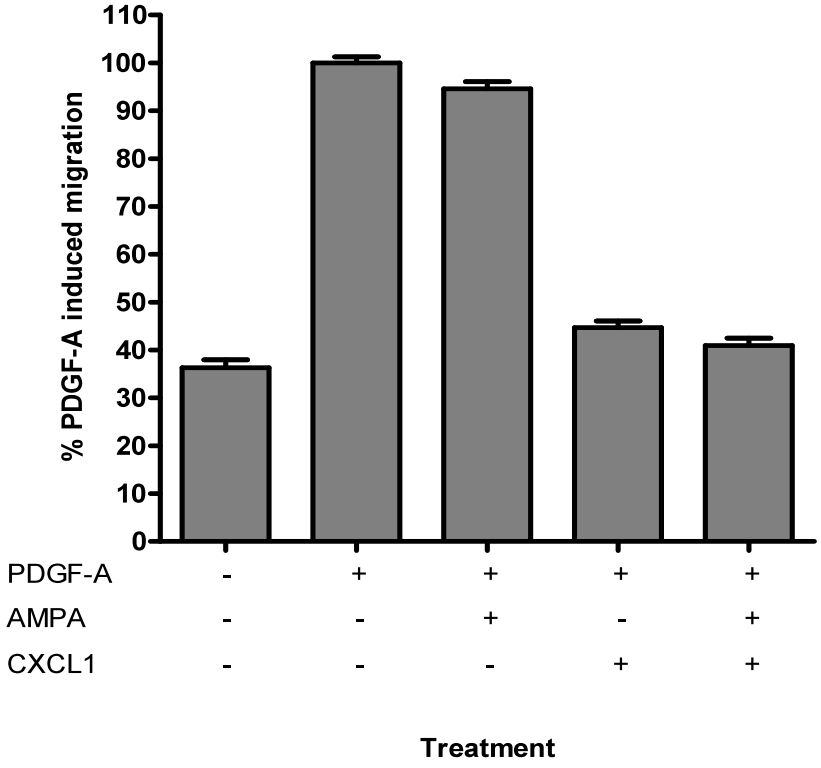

Figure 25: Cell cycle inhibition does not block CXCL1 inhibition of OP migration. OPs were treated with AMPA and or CXCL1 and PDGF-A to assess the effect of inhibiting the cell cycle on CXCL1 inhibition of PDGF-A induced OP migration.

4.5 Discussion

Normal myelination is critical for the successful functioning of the nervous system. Aberrant myelination is characteristic of several disorders of the brain, including cerebral palsy (Juliet, Frost et al., 2009), Schizophrenia (Carter, 2006; Peirce *et al.*, 2006), and leukodystrophies (Schiffmann and van der Knaap, 2004; Costello *et al.*, 2009). Further, the demyelinating disease, Multiple Sclerosis (MS) is also characterized by defects in brain myelin. OPs migrate extensively during development of the CNS (Marshall and Goldman, 2002; Rakic and Zecevic, 2003; Jakovcevski and Zecevic, 2005). The immature OPs migrate away from the germinal matrix in response to local cues like netrin (Spassky, de Castro et al., 2002; Tsai *et al.*, 2006), to populate the putative white matter tracts of the developing brain. Similarly, in the spinal cord, the OL pre-progenitors arise in the ventral ventricular zone (VVZ) during embryogenesis in part, as a result of the local sonic hedgehog (Shh) signaling (Ono *et al.*, 1995; Orentas and Miller, 1996a; Davies and Miller, 2001). They migrate away from the VVZ in response to local chemorepellent cues (Orentas and Miller, 1996b; Jarjour, Manitt et al., 2003; Tsai, Macklin et al., 2006) to populate the white matter tracts of the developing spinal cord. Several proteins have been identified as regulating the dispersal of OPs throughout the brain, including the growth factors PDGF-A and FGF2 (Armstrong, Harvath et al., 1990; Milner, Anderson et al., 1997; Frost, Zhou et al., 2009; Vora, Pillai et al., 2011). The exact molecular mechanisms underlying the regulation of OP migration has yet to be fully elucidated.

A morphogen gradient of the chemokine CXCL1 was previously shown to regulate the migration as well as spatial distribution of OP in the developing CNS (Tsai, Frost et al., 2002). The current study further analyzes the mechanism by which CXCL1 treatment affects OP migration. It shows that not only is CXCL1 inhibition of OP migration specific to PDGF-A induced migration, but it is unrelated to PDGF-A induced intracellular Ca^{2+} flux and OP cell cycle activation.

Numerous studies have shown that PDGF-A and FGF2 both regulate OP migration (Armstrong, Harvath et al., 1990; Milner, Anderson et al., 1997) and this process is shown to involve the ERK signaling pathway (Frost, Zhou et al., 2009; Vora, Pillai et al., 2011). Furthermore, it is known that CXCL1 interacts with PDGFRα to regulate the dispersal and proliferation of OP (Robinson, Tani et al., 1998a; Tsai, Frost et al., 2002). The current study assesses the effect of CXCL1 on FGF2 induced OP migration and shows that CXCL1 inhibition of OP migration is growth factor specific. Even at low concentrations of FGF2 (1 ng/ml), CXCL1 did not affect the extent of migration of OP in the agarose drop assay. Previous studies have shown a role for Ca^{2+} in the regulation of OP migration (Simpson and Armstrong, 1999; Agresti *et al.*, 2005a; Agresti *et al.*, 2005b) and hence further investigation to study the effects of PDGF-A and FGF2 on OP Ca^{2+} were performed.

Cell migration is a complicated cyclical process involving cytoskeletal reorganization, focal adhesion construction and deconstruction, cell membrane trafficking and a complex series of signaling cascades (Horwitz and Webb, 2003; Webb *et al.*, 2005).

Calcium plays a critical role in the process as it is involved in activation of the myosin actin complex required for cytoskeletal reorganization (Adelstein and Hathaway, 1979). Calcium activates myosin light chain kinase, which results in phosphorylation of myosin. The phosphorylated myosin then interacts with actin in order to bring cytoskeletal changes in the cell (Adelstein and Hathaway, 1979). Earlier study by the Simpson and Armstrong group showed that OP migration is a Ca^{2+} mediated response to PDGFRα activation (Simpson and Armstrong, 1999). In addition, it was demonstrated that FGF2 induced OP migration is also $[Ca^{2+}]_i$ dependent, corroborating with a previous study (Tucker, Chang et al., 1989). However, yet another group showed that FGF induced migration is not Ca^{2+} dependent (Clyman *et al.*, 1994). In order to assess the specificity of the Ca^{2+} signaling the Ca^{2+} flux associated with PDGFRα and FGFR activation was analysed. My data clearly shows that PDGF-A induces an immediate increase in $[Ca^{2+}]_i$ in a dose dependent manner. However, in contrast to previous studies (Tucker, Chang et al., 1989; Simpson and Armstrong, 1999), there was no increase in $[Ca^{2+}]_i$ in response to FGF2 treatment. One explanation for this is that FGFR activation induces localized $[Ca^{2+}]_i$ changes that are not strong enough to be detected by a whole cell suspension assay. Further studies are warranted in order to understand the relationship between FGF2 signaling and [Ca2+]i flux..

The factors that evoke changes in the intracellular calcium levels at different stages of oligodendrocyte development are also known to regulate their development and pathology. The key hypothesis of this study was that one such factor, CXCL1, which was previously shown to inhibit OP migration (Tsai, Frost et al., 2002), uses intracellular

calcium stores to bring about its effect. Interestingly, we didn't see any changes in the calcium levels even at high concentrations of CXCL1 treatment. Furthermore, we hypothesized that perhaps calcium stores are evoked upon inhibition of growth factor induced OP migration by CXCL1. However, the current study clearly shows that inhibition of OP migration is a calcium-independent event. Moreover, blocking the intracellular calcium ions with the calcium chelating agent, MAPTAM failed to inhibit CXCL1 action on OP migration.

It was also hypothesized that CXCL1 induction of the cell cycle inhibited OP migration. To test the hypothesis, a known inhibitor of OP proliferation, the glutamate receptor agonist AMPA was used, which blocks the cell cycle in G1 by inhibiting cdk2 activation (Gallo, Zhou et al., 1996; Ghiani and Gallo, 2001b). In the presence of CXCL1, the expected outcome of inhibition of cell cycle activation would be no change in OP migration. However, the results show that in the presence of AMPA, CXCL1 still blocks OP migration. It indicates that inhibition of the cell cycle does not appear to prevent the inhibitory effects of CXCL1. Thus, we show for the first time that CXCL1 actively blocks OP migration via a cell cycle independent pathway.

The mechanism by which CXCL1 inhibits OL precursor migration remains an enigma. The inhibitory effect of CXCL1 on OPs is directly regulated via CXCR2 (Tsai, Frost et al., 2002). The current study shows that this essential inhibition of OP migration is not arbitrary, but may be specific to PDGF-A activity in the developing brain. There are several alternative explanations for the molecular mechanisms underlying the effect of

CXCL1 on OPs. For example, CXCL1-mediated inhibition of OP migration may be a result of increased cell-substratum interactions. In monocytes, CXCL1 regulates the activation of integrins as a result of the shear forces of flowing blood (Huo et al., 2001). Integrins play a critical role in the regulation of OP behaviour, and it may be a decisive switch between β subunits that result in an inhibition of migration and a switch to proliferation. Understanding the molecular regulation of tissue patterning during development will further our understanding of tissue remodelling seen during disease-related processes, including neurodegeneration, neoplasia and tissue repair.

Chapter 5: General Discussion

More than one and half-century ago, Rudolf Virchow first described a non-neuronal structure in the nervous system by the name “Nervenkitt” (or the nerve glue) (Virchow, 1846). To a large extent, this terminology entrenched the view of glial cells as merely supporting cells of the nervous system. Since then, many glial scientists have gradually build-up a case to shatter this conventional view of glial function. Rio Hortega, using silver carbonate impregnation, found OLs (Rio Hortega, 1928). OLs became well-known because of their ability to interact with neurons in the CNS and form myelin. Over the past twenty years, knowledge regarding the formation of myelin during development has increased considerably. However, the current state of information concerning the intrinsic and extrinsic factors which cause OL migration and differentiation is still incomplete.

A series of intricate steps, which may or may not occur sequentially, leads to normal myelin formation in the CNS. First is the OL specification and differentiation step, which involves a network of transcription factors that regulate the development of the OL progenitors. The current studies add one more transcription factor, MeCP2 to this list. Similar to several other OL lineage transcription factors such as Nkx2.2, Olig1/2, Sox10 and YY1, MeCP2 is expressed in both cell types - OPs and OLs. Dysregulation of myelin associated genes indicate that the role of MeCP2 is not restricted to OL specification or differentiation per se. MeCP2 could also be coordinating subsequent

maturation/myelination by the OLs. It seems to regulate a complex series of regulatory events surrounding myelin gene expression, the consequences of which have yet to be fully identified. To date there have been no published studies on the ultrastructure of myelin in the Rett brain. Any changes in the expression of myelin-related proteins are expected to be reflected in defects in the ultrastructure of myelin. As a part of future study, we need to electron microscopy is warranted to specifically look at the thickness and compaction of the myelin sheaths around the axon. Disruptions, such as ballooning and infolding, and intermittent densities between the terminal loops of the paranode and the axolemma will also be examined. All of these structural defects will result in impaired myelin function, leading to decreased nerve impulse conduction. The next step to normal myelin formation is the migration of OL precursors to the site of myelination. Another part of the current study tries to explore the role of growth factors in the OP migration process. The precise regulation of migration depends on cross-talk between the intracellular signaling pathways activated by different receptors of these growth factors. There seem to exist multiple signaling pathways activated by the same extracellur kinase receptor in order to induce OL precursor migration. These alternative signaling pathways which could include the PI3K/AKT, PLC/PKC, JAK/STAT or SRC need to be further characterized. Additionally, once the target cell is found, various extracellular cues contribute to stop the OL precursor migration and allow the cell to differentiate into mature OL. The current study reflects the importance of such cues like CXCL1, which inhibits migration independently of calcium signaling. This inhibition of OP migration by CXCL2 was also found to be specific to CXCL1 concentration. Further investigation is required to elucidate the mechanism by which CXCL1 inhibits OP

migration and perhaps look at signaling pathways like cAMP and Rac as secondary messengers. The knowledge of the mechanisms of OP migration may assist in strategies to facilitate the local redistribution of endogenous and/or transplanted OPs for effective remyelination (Milner, 1997).

Thus, myelination is not simply a developmental process; it is an elegant event which, along with all its complexity, continues for decades in humans and continues to remain as an important contributor to a number of neurologic disorders. Loss of myelin or demyelination in CNS is usually the consequence of a direct insult targeted at the OLs. CNS demyelination can occur because of genetic abnormality (i.e. Leukodystrophy) or inflammatory damage (i.e. Multiple Sclerosis). Regardless of the cause, the consequences that follow are usually irreversible and devastating. Remyelination is the only way to restore neuronal conduction by formation of a new myelin sheath on the demyelinating axons and resolving the functional deficits (Smith *et al.*, 1979). However, as seen in MS patients, remyelination occurs initially, but becomes incomplete/inadequate later and eventually fails in the majority of lesions. At present, there are no therapies in the clinic that promote remyelination. The two popular strategies that are been tested in animal models of demyelination are 'endogenous cell transplantation' and 'exogenous promotion of repair' by inducing the OP cells. Both of these approaches invariably need an 'environment' which is conductive to remyelination. Numerous studies have shown that to create the 'right' environment there is need for a multitude of interacting factors, both 'extrinsic' (Baron *et al.*, 2005; Chesik *et al.*, 2007) and 'intrinsic' (Gokhan *et al.*, 2005; Shen *et al.*, 2008) , that guide

the OPs through the various stages of remyelination. Efficient remyelination might depend as much on the precise timing of action as on the presence or absence of these factors. The current study attempts to uncover additional molecular factors needed to clarify the basic principles of the remyelination process. Identification of these new therapeutic factors that can directly or indirectly promote myelin repair, protect neural cells from injury, and block the disease progression can have a profound implication for the treatment of various demyelinating disorders.

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