CHAPTER ONE

1. INTRODUCTION

1.1 Central nervous system and their cell types

The nervous system is responsible for controlling various body functions such as perceptions, behavior, memories and initiation of voluntary movements. It is classified on the basis of its morphological and functional properties into central nervous system (CNS) and peripheral nervous system (PNS). The central nervous system consists of the brain and the spinal cord which integrates impulses received from the body and coordinates and influences the activity of all the body parts. The peripheral nervous system (PNS) is composed of the cranial nerves, spinal nerves and connections between the CNS and the targets organs of the body (Tortora & Derrickson, 2008).

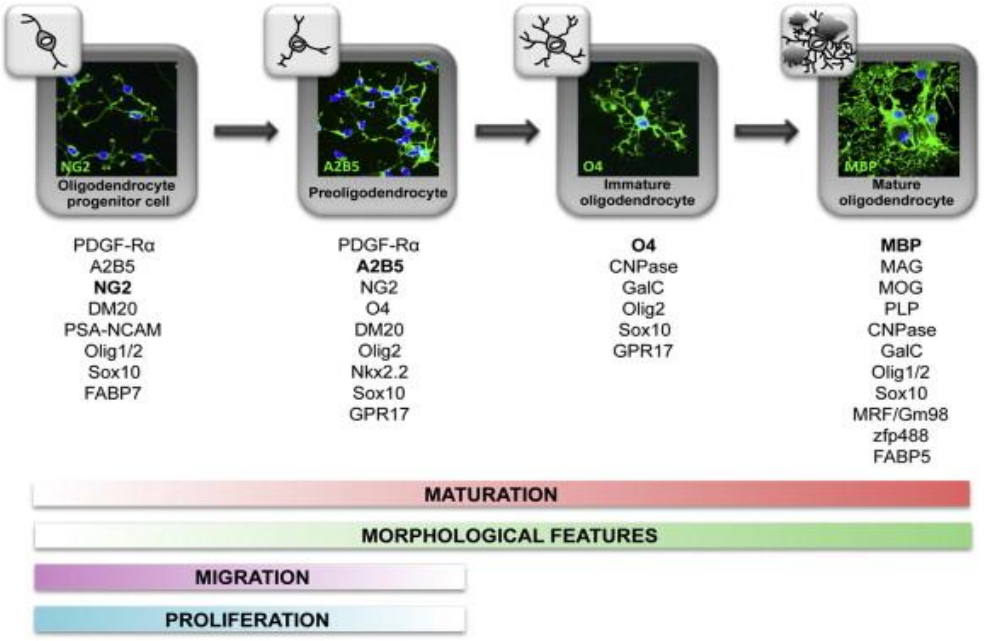
The smallest functional unit of the nervous system is neuron that elicits electrical impulses to be transmitted to other neurons or to other cell types by synaptic communications. Along with the neurons, various others supporting glial cells are present which provide homeostasis, nutrition, clearance of metabolites to the mammalian CNS etc. such as astrocytes, microglia and the myelin forming cells (oligodendrocytes in CNS and Schwann cells in the PNS). Astrocytes function to provide nutrition and maintain of homeostasis among cells in Central nervous system by secretion of growth factors and cytokines. Microglia is resident specialized macrophages which help in the clearance of the metabolites and provide immunity to the brain. Furthermore, oligodendrocytes are responsible for production of myelin for the proper conduction of nerve impulses across the system. The tissue of the central nervous system is divided into the white matter and gray matter. The white matter is majorly consisting of oligodendrocyte lineage cells and axons of the neurons while the gray matter is composed of unmyelinated nerve fibers and numerous neurons (Hickman et al., 2001; Kandel et al., 2000).

1.2 Oligodendrocytes

Oligodendrocytes (OLG) are glial cells with fewer processes which are responsible for the production and maintenance of myelin in the central nervous system. They are counted among the most vulnerable cells of the central nervous system due to their complex differentiation and unique metabolism or physiology.The primary role of

oligodendrocytes in the CNS is the synthesis and wrapping of layers of myelin around neuronal axons, which provides electrical insulation, effectively lowering the capacitance and increasing the resistance of the axonal membrane. Myelin is essential for rapid action potential conduction. Myelinated axons are wrapped by myelin in a series of sheaths, which are interrupted by unmyelinated segments rich in voltage gated Na+ channels, called the nodes of ranvier. Further, some oligodendrocytes can produce few myelin sheaths at a time while other are able to myelinate up to 50 internodes in other regions (Peters & Proskauer, 1969; Remahl & Hildebrand, 1990).

Before the myelination process, oligodendrocyte undergoes a developmental process with morphological distinct stages in its life span (Figure 1.1). The development stages begin with the migratory and mitotic bipolar cells oligodendrocytes progenitors cells (OPCs) to the more branched pre-oligodendrocytes. Further it differentiate into immature oligodendrocytes with 3-4 cellular processes and mature progressively with dense network of cellular processes into post-mitotic myelin-producing oligodendrocytes (Baumann & Pham-Dinh, 2001; Bradl & Lassmann, 2010).The consecutive and sequential expression of the developmental markers or surface antigens distinguish each stage of oligodendrocyte development according to its functional attributes such as migration capabilities, proliferation capacity and changes in its morphology(Figure 1.1).



Adapted from (Barateiro & Fernandes, 2014)

Figure 1.1Oligodendrocyte lineage: The stages include OPCs (Oligodendrocyte progenitor cells), pre-progenitor cells, immature oligodendrocytes and Mature oligodendrocytes cells. OPCs use guidance cues to migrate from the sub ventricular zone into all regions of the brain and spinal cord. Depending on the environmental signals, the migrating cells either differentiate into mature oligodendrocyte. The different stages of oligodendrocyte lineage are marked by the presence and absence of antigenic markers namely PDGFRα, O4, A2B5, MBP etc.

1.3 Oligodendrocyte origin and specification

Oligodendrocytes progenitors cells (OPCs) are bipolar cells and are marked by the presence of the surface antigen PDGFRα (Platelet derived growth factor) and proteoglycan NG2. The oligodendrocyte originates as pre-progenitors cells in the telencephalon of the brain which later migrate across the sub-pallial layer to the sub-ventricular zone (SVZ) to proliferate and develop into OL progenitors cells (OPCs)(Levison et al., 1993).The OPCs further migrate from the SVZ to populate the developing white matter tracts of the brain (Kakita & Goldman, 1999).During this migratory phase, OPCs remain proliferative and only exit the cell cycle when reaching their destination. The origin of oligodendrocytes has been much debatable topic among the developmental glial biologists (Richardson et al., 2006; Spassky et al., 1998). Studies and speculation have concluded two important sources of origin that includes the spinal cord and the forebrain. But, the distribution of the zonal population of OPCs is still not clear.

However, the most primitive source of emergence of OPCs has been the medial ganglionic eminence and the anterior entopeduncular area of the Forebrain (Bradl & Lassmann, 2010).Later on, multiple sources of origin were described according to the chronological order of OPCs emergence in the brain(Figure 1.2). The first wave of OPC generation starts in the developing spinal cord where oligodendrocytes are produced in two separate waves (Figure 1.2 a). Initially, OPCs arise from the pMN (motor neuron) domain of the ventral ventricular zone the spinal cord at E12.5(Q. Zhou et al., 2001). The embryonic development of the vertebrate nervous system comprises of number of transcription factors and growth factor which induce a gliogenic switch to generate cells with oligodendrocyte specification. Sonic hedgehog (Shh) (Tekki-Kessaris et al., 2001), one such signaling cue secreted from the notochord and floor plate induce cell type specific expression of Olig genes (Lu et al.,

2000; Q. Zhou et al., 2001) and enhance early OL production in brain and spinal cord (Orentas et al., 1999). Thus, pMN domain after producing motor neuron precursors, give rise to OPC after the neuron/gliogenic switch. Most of the oligodendrocytes in the spinal cord (about 85%) are derived from the pMN domain domain (Cai et al., 2005; Fogarty, 2005; R. B. Tripathi et al., 2011). Later, a second wave of OPC arises from the dorsal zone of the spinal cord independent of Shh signaling which contributes to 10-12% of the final spinal oligodendrocytes(Fernando de Castro et al., 2013).

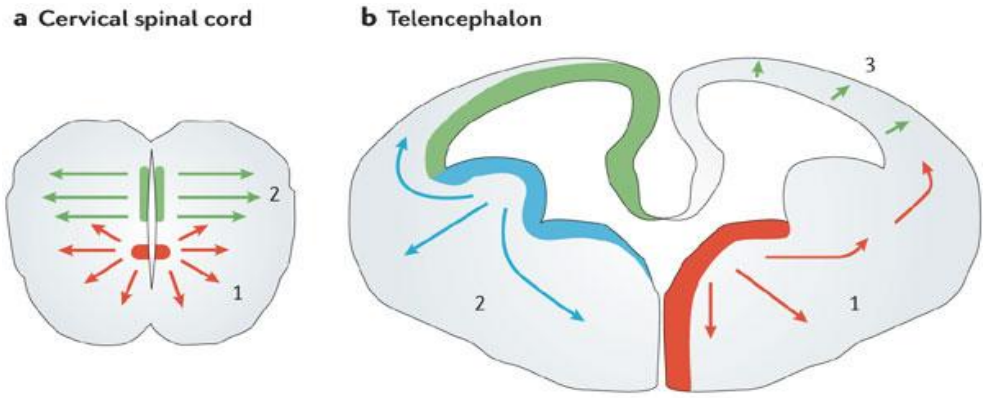


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Figure 1.2: Sources of origin and migration of oligodendrocyte progenitors in a) spinal cord and b) telencephalon or forebrain: a) In spinal cord, 85% of OPCs are produced from pMN (motor neuron domain) in the ventral ventricular zones (1), which starts at about embryonic day (E) 12.5. At E15, secondary wave of progenitors starts in respective dorsal regions (2). b) In the forebrain or telecephalon, the ventral-most the OPCs arise in the medial ganglionic eminence at about E12.5 (1.Red Area), from the lateral ganglionic eminence the migration starts a few days later (2.Blue area), and finally from of the cortex (3.Green area).

In the forebrain, the first OPCs originate in the medial ganglionic eminence and anterior entopeduncular area of the ventral forebrain (Figure 1.2 b). The embryonic origin of the oligodendrocyte in these regions has elucidated three home domains viz, Nkx2.1, Gsh2 and Emx1 wherein the Nkx2.1 population are the first to appear but later die down during its course. The other two Gsh2 and Emx1 domains colonize and myelinate the entire neocortex (Ventura & Goldman, 2006). These are met with second wave of OPCs originating from the lateral and/or caudal ganglionic eminences. Postnatal cortex acquaintance a third wave of OPCs arising from the sub

ventricular region which later spread across entire telecephalon (Ventura & Goldman, 2006). Although, oligodendrogliogenesis is distributed along multiple foci, yet we do not observe a chronological coordinated event in different vertebrates (F de Castro & Zalc, 2013)

OPCs, apart from their origin, have been studied for their migrating waves and pattern from different foci of the brain, optic nerve, and spinal cord (Kessaris et al., 2006; Menn et al., 2006; Richardson et al., 2006; Timsit et al., 1995). Along their route to destination, the cells migrate in response to growth factors/cytokines and adhesive proteins present in the extracellular matrix (ECM) (Keely et al., 1997; Lauffenburger & Horwitz, 1996). They express differential cell surface receptors and respond to a numerous neuronally derived signals including neurotransmitters, growth factors and cell adhesion ligands. Further, to understand the numerous signals and growth factor affecting the migration of OPCs, it is necessary to first comprehend the complex process of glial cell migration.

1.4 Glial Cell migration

Dynamic interaction between a cell and the substratum on which it is attached is a primary requisite for cell migration. Protrusion and retraction in an oscillatory mode complete a migration cycle of a cell. Protrusions are membrane extensions which are generated by actin polymerization and nascent adhesions at the leading edge whereas retractions are contractile events along with the formation of actomyosin bundles at the adhesions junctions(Lauffenburger & Horwitz, 1996; Newell-Litwa & Horwitz, 2011). Glial cells have the ability to migrate in one or two distinct modes in specific areas of the brain. The PNS encounters a collective mode of migration where in the Schwann glial cells trails behind in chains under the influence of positional cues (Figure 1.3b). Oligodendroglial cells in the CNS moves as single cells which can be in a saltatory or exploratory in nature (Figure 1.3a). Early OPCs disperse from the sub ventricular zone to the other areas follows a saltatory mode of migration which involves fast moving and stationary periods accompanied by dramatic changes in the cell shape. Later, these OPCs migrate to different regions of the white matter and gray matter following a exploratory mode accompanying integrated movements of the cell body and the leading edge without any changes in the cell shape(Hui-Hsin Tsai et al.,

2009). However, the integrated process of oligodendrocytes migration is the result of the combinatorial effort of transient localised adhesion and signalling.

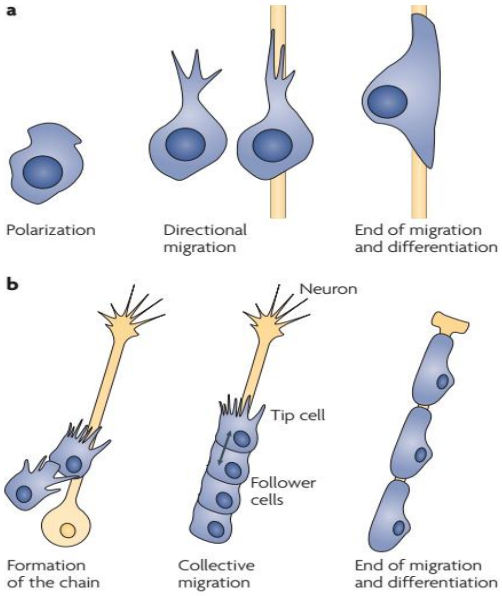

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Figure 1.3: Glial cell migration in CNS and PNS. The glial cells show two types of migratory modes. In the CNS, glial cells migrate as single cells, whereas in the PNS a collective migration is seen. a) Single cell migration: Before the beginning of migration, glial cells polarize and stimulate their cytoskeleton for instructive cues for the migration. On the route, glial cells are directed by diffusible cues from neuronal membrane and finally terminated from the migratory phase to differentiate by unknown signals at their final destination. b) Collective migration: Migration begins as soon as the migratory chain is assembled. Inside the migrating chain, the tip cell is distinct from its follower cells which forms filopodia at the protruding end and establishes cell-cell junctions with the followers (arrow).

Migrating OPCs, in the developing zebrafish, are characterized by continuous retraction and extension of the filopodium processes .Their migrating pattern and multiplication is also regulated by its association with other OPCs (Klämbt, 2009). Besides several dynamic changes in the cytoskeleton, a coordinated interplay of the ions and water fluxes plays across the cell membrane (Happel et al., 2013). Recognising the path along the extracellular matrix and triggering several molecular reactions, they reach the final destination from sub ventricular zone to the White matter and Gray matter where they differentiate proliferate and populate the area.

Amidst their journey, they are guided by several growth factors, cytokines and chemokines.

1.5 Factors regulating OPC migration

1.5.1 Intrinsic Factors

Several transcription regulators have been involved in the regulation of different developmental stages of oligodendrocytes which comprises of bHLH proteins of the Olig family, homeodomain proteins of Nkx family and Sox family proteins (Wegner, 2001; Wegner & Stolt, 2005). Sox 9 and Sox 10 are the members of HMG domain proteins of Sox E family which are involved in the neural crest development and the terminal differentiation of the oligodendrocytes (J. Kim et al., 2003; Maka et al., 2005; Paratore et al., 2001).In Sox10 deficient mice which later selectively lost Sox9 in OLPs during the process of specification showed altered pattern of precursor cell migration, decreased cell number and increased apoptosis rate. Also, PDGF receptor alpha expression were found to be absent in the mutant OPC which explained the dependence of PDGF receptor on its transcriptional control by Sox9 and Sox10.However, PDGFrα remained detectable in OPCs after Sox9 deletion indicating the Sox10 occurrence is sufficient to permit PDGFrα expression, but at reduced levels. Overall, this concluded that both the transcriptional regulator Sox9 and Sox10 are essential for OPC migration and survival through PDGF-A signaling. Interestingly, Sox9/Sox10 double deficient OPCs proliferated in vivo and showed mitogenic response to PDGF-A in mixed spinal cord cultures. PDGF-A thus retained its mitogenic activity on Sox9/Sox10 double deficient OPCs. The mitogenic effect of PDGF-A may therefore not be exclusively mediated by PDGFrα, but additionally by yet other unknown signaling pathways.

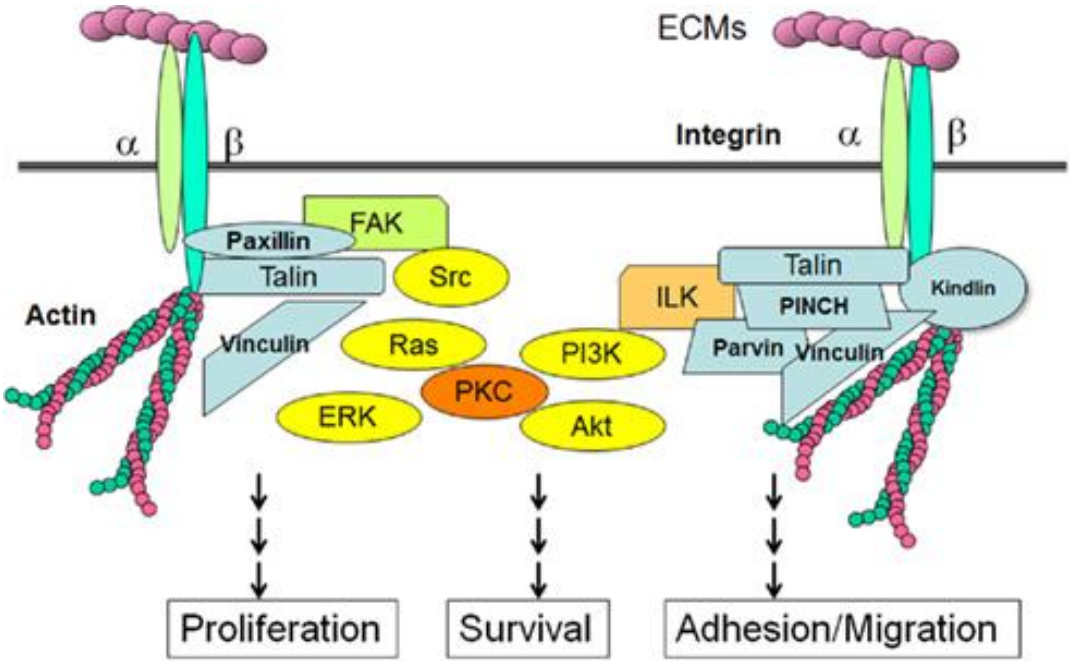
The Bone morphogenetic factors Bmp4, Bmp7 and TGF β1 one the members of TGFβ family proteins in the embryonic stages of development and corticogenesis (Choe et al., 2014).The meninges and pericytes surrounding the forebrain are a major source of secreted Bmps during cortical development (Choe et al., 2014; Furuta et al., 1997). Previous studies showed that the Bmp and TGF signaling pathways are also involved in the proliferation, differentiation, and specification of OPCs (Gomes et al., 2003; Gross et al., 1996; McKinnon et al., 1993; Sabo et al., 2011; See et al., 2004; Zhang et al., 2010). Bmp and TGF signaling pathways exhibit cross talk during

development(Keller et al., 2011; Spinella-Jaegle et al., 2001) and, deletion of Smad4, a co-regulator of the Smad1/ 5/8 and Smad2/3 signaling pathways, which signals in both pathways, has a significant effect on OPC distribution. The aberrant activation of these factors repels the ventral wave OPCs to the cortex (Choe et al., 2014).

1.5.2 Extrinsic Factors

Extracellular Matrix

Oligodendrocytes are regulated by a set of integrins receptors expressed throughout the developmental stages (O'Meara et al., 2011).Integrins has been known for mediating a link between the extracellular matrix (ECM) and actin cytoskeleton, thus coordinating signals between both compartments to govern various cellular processes(Figure 1.4). Reports and evidences have suggested the role of various ECM proteins in governing different physiological aspect of Oligodendrocyte biology.ECM components such as Fibronectin(FN),Vitronectin(VN) and Laminin-(LN2) have been responsible for driving Oligodendrocyte proliferation, survival and development(Figure 1.4)(Baron et al., 2005; Blaschuk & Frost, 2000; Buttery, 1999; E. E. Frost et al., 2009; Richard Milner et al., 1997; Richard Milner et al., 1996). Integrins receptors are heterodimeric structures composed of α and β subunits. Out of the different 18 subunits and 8β subunits, oligodendrocytes express αv subunits complexed with β subunits 1,3,5 and 8 (Richard Milner et al., 1997; R Milner & Ffrench-Constant, 1994).Further, Integrin αvβ1 have been shown to regulate OPC migration(Richard Milner et al., 1996), αvβ3 regulates OPC proliferation(Baron & Shattil, 2002), αvβ5 helps in regulating OPC differentiation (Blaschuk & Frost, 2000; Buttery, 1999) and α6β1 plays a role in the regulation of axo-OLG interactions (E. E. Frost et al., 1999). These functional attributes of Integrins was correlated with "internal switches" in the OPC cellular environment which govern the Integrin expression leading to changes in cellular behavior. Switching off or inhibiting αvβ5 integrin showed reduced OL differentiation (Blaschuk & Frost, 2000). Further, association of PDGFrα with αvβ3 integrin lead to the activation of PI3K and protein kinase (PKC) pathways which are found to be involved in OPC proliferation(Ebner et al., 2000; McKinnon et al., 2005).



Adapted from (Ozaki et al., 2011)

Figure 1.4: General Schematic Integrin signalling pathways

Chemokines and Growth factors

Several growth factors and chemokines are found to influence oligodendrocyte biology both in vitro and in animal models of remyelination. During the phase of migration from the SVZ region to the white matter of the brain, OPCs are guided by various growth factors such PDGF, FGF, VEGF etc. However, the early hypothesis that OPs migrate along a chemotactic gradient in the developing CNS, is now considered obsolete. Recent studies have provided evidence in support of an alternative explanation (Andrew A. Jarjour et al., 2003; N. Spassky et al., 2002; H. H. Tsai et al., 2003).Rather than moving away from the GM (Gray matter) in response to attractive cues present in a concentration gradient, OPCs are repelled by negative cues present in the GM. Several studies show that GM expression of Netrin-1 acts as a repellent to direct OPCs migration away from the GM in the spinal cord (K. Xu et al., 2014). Netrins comprise a conserved family of laminin-related secreted molecules with members originally implicated in axon guidance and neuronal migration. Netrin1 (NTN1) is the best-characterized member of the family and in CNS it signals mainly through transmembrane receptors belonging to the Deleted in Colorectal Cancer (DCC) and Unc-5 homolog (UNC5) families(Hakanen et al., 2011). The application

of netrin-1 to OP cells in vitro caused the retraction of OP processes, consistent with a repellent function. Furthermore, it is reported that the distribution of OP cells is disrupted in the spinal cords of mouse embryos lacking DCC or netrin-1(Andrew A Jarjour et al., 2003; Nathalie Spassky et al., 2002).Another study by Sugimoto et al. (2001) have provided evidence that both semaphorin-3A (Sema3A) and netrin-1 are chemorepellents for OP cells migrating from explants of newborn rat optic nerve. Semaphorins are secreted, transmembrane, and GPI-linked proteins, defined by cysteine-rich semaphorin protein domains, characterized for their importance in the development of the nervous system and in axonal guidance. Two members of the secreted class 3 semaphorins(Sema) family Sema3A and Sema3F, have an opposite effect on oligodendrocyte progenitor cell migration during development which is repulsive and attractive, respectively(Nathalie Spassky et al., 2002; Sugimoto et al., 2001). Both Sem3A and Sema3F bind to its co-receptors neuropilin (NRP1 for sema3A, NRP2 for sema3F) which use Plexin-A for signal transduction.

Also, it is clear that Oligodendrocyte progenitors stop migrating and proliferate in response to a localized concentration of the cytokine CXCL1, a homologue of interleukin-8, formerly known as Groα (Robinson et al., 1998; H.-H. Tsai et al., 2002). CXCL1, at a precise concentration of 0.5ng/ml, inhibits oligodendrocyte progenitor migration (H.-H. Tsai et al., 2002) and induces oligodendrocyte progenitor proliferation. The study was further supported by (Vora et al., 2012) which reports that CXCL1 inhibition of PDGF-A induced oligodendrocyte progenitor migration is regulated by changes in the intracellular calcium flux. In order to proliferate, cells must stop migrating, to allow the restructuring of the cytoskeleton necessary for spindle formation and cytokinesis. Calcium plays a critical role as secondary messenger in the process of myosin-actin complex activation, which is required for the cytoskeletal reorganization that accompanies migration (Vora et al., 2012).Furthermore, CXCR4, which utilizes CXCL12 for ligand activation, is shown to promote OPCs differentiation and remyelination in the injured adult CNS(Patel et al., 2010).

Among the growth factors, VEGF-A (Vascular endothelial growth factor) is the prime regulator of angiogenesis and vasculogenesis. This highly specific mitogen for vascular endothelial cells and considered to have prominent role in its migration and

proliferation. There are many isoforms of VEGF which includes VEGF A-D, which differs in their molecular masses and biological properties. All these isoforms binds to the two VEGF receptors two tyrosine-kinase receptors,VEGFR-1 (*flt-1*) and VEGFR-2 (*KDR/flk-1*)(Hayakawa et al., 2012)**.** Apart from the vascular endothelial cells, cerebral endothelial cells have also been reported to secrete the VEGF-A isoform in vitro and in vivo, which further regulates the CNS homeostasis.OPC has also been reported to express VEGF receptor2/ Flk-1/KDR. After binding to the VEGF-receptor2/KDR/Flk-1, VEGF-A promotes particularly the migration rates of OPCs by activating the FAK (focal adhesion Kinase) and thus the cytoskeletal actin reorganization(Hayakawa et al., 2011). Other VEGFs may also participate in the OPC function. VEGF-C was reported to promote OPC proliferation via VEGFR-3 but not VEGFR-2(Flk-1)(Le Bras et al., 2006). Although VEGF-C and VEGF-D are known as ligands for VEGFR-3, these VEGFs can also bind to Flk-1 under some conditions(Shibuya & Claesson-Welsh, 2006).

A detailed study on FGF8 (Fibroblast growth factor 8)suggests its role in the migration and proliferation of post natal OPCs in demyelinating mouse models both *in vivo* and *in vitro*. The migrating property of these OPCs in response to FGF8 was conducted in matrigel cultures where in the OPCs were placed at a certain distance of a FGF8-soaked heparin beads. Migration and proliferation of the OPCs was observed towards the heparin beads. Furthermore, in vivo demyelinating mouse model also showed the migration of the OPCs towards the grafted FGF-8 soaked heparin beads(Cruz-Martinez et al., 2014). Further, FGF2 have been shown to promote the proliferation of OPCs, and block the terminal differentiation of the OPCs. It also, specifically maintains the high level expression of the PDGF α receptors and not PDGF-β receptor. As PDGF α receptor give rise to the increased functional responsiveness to the growth factor PDGF and thus, FGF is also responsible for increasing the sensitivity of the OPCs towards PDGF (McKinnon et al., 1993).

Another chemotropic cue, anosmin-1 (A1) has been identified which contributes to the axonal outgrowth and collateralization. Anosmin-1 regulates the migration of different cell types through the Fibroblast growth factor receptor 1 (FGFR1)(Bribián et al., 2006; Clemente et al., 2011). A1 is an extracellular matrix (ECM) glycoprotein encoded by the human KAL1 gene(Franco et al., 1991; Legouis et al., 1991).The

KAL1 gene is responsible for the X-linked form of Kallmann syndrome (KS), associated with hypogonadotropic hypogonadism and anosmia(Kallmann, 1944). In A1overexpressed transgenic mice, proliferation of OPC was increased as giving rise to a higher number of OPCs with an enhanced migratory capacity due to the activation of ERK1/2 via FGFR1s (Murcia-Belmonte et al., 2016).

Lastly, PDGF-A ,a growth factor, serves as potent motogen and mitogen for the oligodendrocyte progenitor cells(Calver et al., 1998; E. E. Frost et al., 2009). PDGF-A is synthesized by astrocytes and neurons and interacts with the specific PDGF receptors on OPCs (Richardson et al., 1988). These PDGFRα receptors are the markers of Oligodendrocyte precursors which through PDGF-A ligand-ligand binding, autophosphorylate and dimerize to activate further several signaling cues and protein kinases (Hoch & Soriano, 2003). PDGFRα phosphorylation marks the activation of several other downstream signalling pathways which further are involved in multiple cellular functions. These signalling pathways include phosphoinositide-3 kinase (PI3K),mitogen activated kinases (MAPKs) and phospholipase C gamma (PLCγ) which are responsible for the growth, migration, proliferation and differentiation of OPCs(Ebner et al., 2000; Haines et al., 2008; J.-G. Hu et al., 2008; McKinnon et al., 2005). Also, PDGF-A has been shown mediate OPC proliferation and initiate primary process outgrowth and cell migration by Ca^{2+} dependent pathway(Simpson & Armstrong, 1999).Further, some PDGF-A activated signalling pathways have been reported to guide the process of migration which include MAPK-ERK, CDK5 etc. CDK5, cyclin dependent kinase 5 have been reported to regulate Oligodendrocyte migration by phosphorylation of WAVE2 through non receptor tyrosine kinase Fyn(Miyamoto et al., 2008).

In brief, a large number of locally and transiently expressed environmental cues have been reported to be involved in regulation of OPCs migration and as the modulating factors of differentiated OL which may include soluble signaling proteins and extracellular matrix protein(E. Frost et al., 1996; Richard Milner et al., 1997; H.-H. Tsai et al., 2002) .Furthermore, A little has been known regarding the molecular mechanisms regulating OPC migration. A little has been known regarding the different mechanisms taking place during OPC migration. Over the past decade, researchers have been debating on the action of several growth factors and

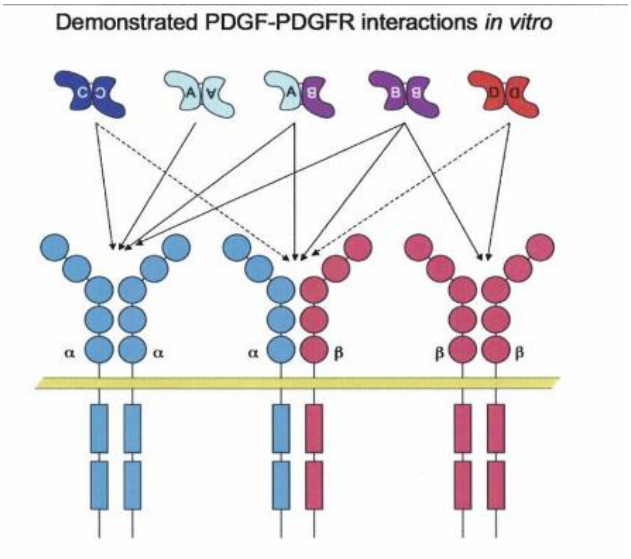
chemotactic repellents such PDGF, FGF, VEGF etc (Fernando de Castro & Bribián, 2005) influencing and activating signalling cascades reactions which further may lead to migration.

1.6 PDGF and its receptors

Platelet derived growth factor (PDGF) has been identified as the serum growth factor for fibroblasts, smooth muscle cells and glial cells(Kohler & Lipton, 1974; Ross et al., 1974; B Westermark & Wasteson, 1976). It is prime and potent neuron derived mitogen for OL progenitors in vitro which is expressed by astrocytes and neurons throughout the developing CNS(Noble et al., 1988; Richardson et al., 1988).Platelet-derived growth factor (PDGF) family members include PDGF-A, -B, -C and -D, which are assembled as disulfide-linked homo- or heterodimers (Funa & Sasahara, 2014).The PDGF dimers are assembled intracellularly and, in the case of PDGF-A and PDGF-B, without apparent bias towards a specific dimer configuration (Figure 1.5).A cell may therefore produce either PDGF-AA or PDGF-BB if either of these PDGF genes is solely transcribed. If both genes are transcribed simultaneously, a mixture of PDGF-AA, PDGF-BB and PDGF-AB will be produced (Betsholtz et al., 2001). The recently identified members -C and –D chains differ from the traditional A- and B-chains, in that PDGF-C and D-chains possess a long N-terminal CUB (complement protein C1r/C1s, VEGF, and Bmp1) domain, which has to be cleaved for binding to receptors (Bergsten et al., 2001; LaRochelle et al., 2001; Li et al., 2000). It is postulated that PDGF-CC and PDGF-DD are secreted as a latent, conditionally inactive , growth factor similar to the situation for Transforming growth factor β (TGFβ)(Miyazono & Heldin, 2008).

Apart from the ligands, there exist two types of PDGF receptors (PDGFR-α,-β), the PDGFRA binding A-,B-,C- chains, while the PDGFRB binds B- and D-chains(Heldin et al., 1998; Shim et al., 2010). Binding of dimerized PDGF-ligand caused receptor dimerization which induces autophosphoryltion of intracellular kinases, activating the downstream signalling molecules that bind to the phosphorylated tyrosine residues of the intracellular domain of PDGF receptors in order to propagate signals (Heldin et al., 1998).Through these phosphotyrosine residues, the receptor dimmers contact a number of SH2 domain-containing molecules that connect to different intracellular signalling pathways which include cytosolic

proteins phospholipase C(PLC-ϒ),Phosphatidylinostol 3kinase p85 subunit(PI3K), Ras GTPase-activating protein(Ras-GAP), SHP phosphatase and SRC family kinase (Betsholtz et al., 2001).



Adapted from (Andrae et al., 2008)

Figure 1.5: Interactions among PDGF–PDGFRs. The PDGF dimer chains cooperate with each of the receptor subunits respectively. The active Ligand-receptor configuration is therefore elucidated by the ligand dimer configuration.

PDGF-A is responsible for migration, proliferation, survival and differentiation of OP cells (Regina Armstrong et al., 1991; Baron & Shattil, 2002; E. E. Frost et al., 2003; E. E. Frost et al., 2009). OPCs are reported to express only the alpha PDGF receptor subunit(Baumann & Pham-Dinh, 2001). PDGFR**α** is a Receptor Tyrosine Kinases (RTK); such receptors include PDGF, EGF, or FGF receptor. In vivo, PDGF-A is synthesized by neurons and astrocytes and interacts with these receptors PDGFRα present on OPCs(Noble et al., 1988; Richardson et al., 1988). Phosphorylation of the PDGFR-α results in activation of several different intracellular signalling pathways, including Phosphoinositide 3-Kinase (PI_3K)(Ebner et al., 2000), mitogen activated protein kinase (MAPK)(Bhat & Zhang, 1996) and phospholipase C**ϒ** (PLCϒ)(Heldin et al., 1998).

PDGF activated PI3K signalling also play an important role in OPC migration. Mckinnon and their group used a transgene rescue approach to assess the migration of OPCs from PI3K activation defective mice with respect to wild type PDGFR expressing mice. Transfection with wild type PDGFR expression vectors rescued the

migration defect on spinal cord explants, whereas transgenes incapable of activating PI3K could not. Moreover, they assessed the migration of OPCs away from the spinal cord explants to show that PDGFRα defective cells can be rescued with a PDGFRα transgene (McKinnon et al., 2005).In addition, the study also explains the role of PI3K and PLCϒ coupled pathways in PDGF-A induced OPC proliferation. The analysis of the proliferation was carried out on both primary rat OPCs and with an OPC cell line. It suggested that PDGFRα activated proliferation required PI3K coupling at low ligand concentration and PLCϒ-coupling at high concentration. However, Frost et al. contradicted the role PI_3Kinase in the regulation of PDGF induced OP migration and suggested the involvement of ERK 1/2 signalling pathway in OPC migration. PDGF-A activated ERK1/2 signalling was sufficient to sustain OP migration for up to 72 hours without affecting proliferation. Further, the studies has also suggested a positive feedback loop regulated by pERK activated cytoplasmic Phospholipase A_2 ($cPLA_2$) signalling pathway which is responsible for the sustained migration of OPCs (Vora et al.,2011).Recent studies also provide support for the role of MAPK in the regulation of OP distribution in the developing cortex. Extracellular regulated kinase (ERK) is one of three major groups of MAPKs.ERK has various functions in cells, and is involved in the proliferation, differentiation and survival of neurons during development(Cobb, 1999). Moreover, PDGF-A has also been known to induce oligodendrocyte differentiation mediated by the extracellular signal-regulated kinases 1 and 2 (ERK1/2)(Guardiola-Diaz et al., 2012; J.-G. Hu et al., 2008)
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1.7 PDGF-A activated signaling pathways in OPC migration

Among the PDGF family of growth factors, PDGF-A promotes OPCs migration through the non receptor tyrosine kinase Fyn which phosphorylates Cdk5. Inhibition of Fyn and Cdk5 function impairs the PDGF-A induced OPC migration. Cdk5 regulates PDGF-dependent OPC migration through the direct phosphorylation of WASP (Wiskott–Aldrich syndrome protein)-family verprolin homologous protein 2 (WAVE2) at Ser-137. Mutation of WAVE2 at Ser-137 results in reduced PDGF-A migration(Miyamoto et al., 2008). Further, Study by Frost and colleagues (2009) found that PDGF-A regulate the OPC migration through the ERK signalling pathway. PDGF-mediated migration occurs in response to transient exposure, in contrast to FGF2, which requires continuous exposure. A transient exposure (30 mins) of PDGF-

A is enough to drive the OPC migration up to 72 hrs via ERK pathway. There are positive feedback loops within the ERK cascade that may play a role in the maintained signalling seen after transient activation of the receptor. A positive feedback loop, utilizes cPLA2 to maintain ERK phosphorylation, help in OPC migration up to 72 hrs (E. E. Frost et al., 2009). Another mechanism by which PDGF-A stimulate the OPCs migration is by increasing intracellular calcium level. PDGF-A induce the calcium influx via modulation of VOCC (Voltage-operated Ca^{++} channel). PDGF receptor tyrosine kinase activity phosphorylates the VOCC(Paez et al., 2009; Paez et al., 2007). The PDGF effect on OPC Ca^{++} entry was abolished in the presence of AG-1296, a selective PDGF receptor tyrosine kinase inhibitor, confirming that the TK activity of PDGFR is essential for VOCC modulation.

1.8 Oligodendrocytes migration and cytoskeleton

OPCs proliferate and migrate from their origins, eventually becoming uniformly distributed throughout the gray and white matter of the brain and spinal cord(Kirby et al., 2006). Migratory OPCs are typically bipolar in vitro and in vivo, and cultured OPCs posses many properties similar to OPCs in situ(Armstrong et al 1990;Schmidt et al.,1997).Time lapse imaging studies during normal development of the OPCs expressing fluorescent proteins in Zebrafish gives an insightful key points to remember(Kirby et al., 2006). First, OPCs constantly remodel numerous filopodium (bundles of actin) like processes as they migrate and for many hours before they wrap axons. Second, OPCs often retract processes and change their course of migration after coming in contact with the neighbouring OPCs(Kirby et al., 2006). Both these observations suggest that OPCs inspects their environment by its filopodial activity and change their course of migration according to the density and distribution of the nearby OPCS and oligodendrocytes.

Oligodendrocytes leading edge consists of numerous F-Actin assembly and remodelling proteins, such as Arp2/3 complex, N-WASP, WAVE1, myosin, and the small Rho GTPase, Rac1, Cdc42 and RhoA (Bacon et al., 2007; Fox et al., 2006; Song et al., 2001). Further, Cytoskeletal entities assists a variety of cellular functions, including migration(Challacombe et al., 1996; Novak & Titus, 1997).The mechanisms and cytoskeletal entities involved differs with cell type and the nature of the migrational stimulus. In neuronal growth cone- mediated cell movement, actin and

tubulin interact to generate directed force and structural support(Challacombe et al., 1996; Lauffenburger & Horwitz, 1996).Focal adhesion kinase(FAK) and paxillin are adhesion associated proteins that transmit extracellular stimulus into changes in the cell motility. Paxillin acts as platform for tyrosine kinases such as FAK and also binds to protein that reorganize actin cytoskeleton during VEGF-A induced OPCs migration(Hayakawa et al., 2011). FAK and paxillin are also considered to be the cytoskeletal targets of ERK signalling pathway known to be involved in cell migration(Z.-X. Liu et al., 2002).Paxillin binds with ERK and unfolds a series of morphological changes (Ishibe et al., 2004; Teranishi et al., 2009).Further , the ERK activation lead to its localization to the cell periphery where there is a direct binding of the ERK to the actin filaments. The phosphorylated ERK(pERK) remains associated with the focal adhesions in its active form(A. Tripathi et al., 2017).Recent studies also suggest the role of WASP family, N-WASP and WAVE1, in cytoskeletal rearrangements during OPC migration. N-WASP and WAVE1, remodelling proteins, interacts with Arp2/3 to generate branched F-Actin network at the cell leading edge(Miyamoto et al., 2008).

Also, members of the Rho family of small GTP- binding proteins, including Rho, Rac and Cdc42 have been shown to participate in cell growth, differentiation and motility. To be precise, Rho regulates stress fiber formation and cell contraction , whereas Rac and Cdc42 regulate the formation of lamellipodia and filopodia ,respectively, and promote protrusive activities (Hall, 2005).Rho GTPase have also been reported to control other cellular activities, such as the JNK (c-Jun N-terminal kinase)and p38 MAPK(Mitogen-activated protein kinase) cascades, an NADPH oxidase enzyme complex, the transcription factor NF-κB (nuclear factor κB) and SRF(serum-response factor). Studies have demonstrated that activation of RhoA and ROCK is required for the reduction in OPC process length triggered by Netrin-1 and for the chemorepellent response make OPCs to netrin-1.However, application of netrin-1 to oligodendrocytes decreases RhoA activity (Rajasekharan et al., 2010). Pharmacological Inhibition of Rho signalling pathway promotes OPC process outgrowth and differentiation by potentially reducing Fyn kinase activity (Lingor et al., 2007; Siebert & Osterhout, 2011).Another study by Liu et al.,2012 demonstrates that treating OPCs with Slit2 , an extracellular matrix protein, deactivates Fyn kinase and increases the level of activated Rho-GTP. The study suggests that Fyn formed complexes with Robo1, but

the association later decreased with Slit stimulation. Liu et al. 2012 concludes that Robo1 interacts with Fyn to repel the migration of OPCs through RhoA activation(X. Liu et al., 2012). Lysophosphatidic acid(LPA) and Sphingosine-1-phosphate (S1P) are phospholipids with growth factor like signalling properties which activate several cellular activities such as proliferation, increased Ca2+ levels, MAPK activation and actin-cytoskeleton rearrangement (Contos et al., 2000; Panetti et al., 2001). Previous findings indicate the involvement of these phospholipids in the directional guidance and modulation of OPC migration via the Rho kinase signalling pathway(Dawson et al., 2003; Novgorodov et al., 2007).

Further, MLC (myosin light chain) phosphorylation also plays an essential role in the formation and maintenance of stress fibers and focal adhesions during cell migration. Several kinases can phosphorylates MLC, among which, myosin light chain kinase (MLCK) (Totsukawa et al., 2004) , and Rho-associated kinase (ROCK), a major downstream effectors of RhoGTPase (Amano et al., 1997) are well established. Previous studies suggest that actin associated motor protein non muscle myosinII (NMII) decreases as oligodendrocyte differentiate and that inhibition of NMII increases the branching and myelination which classify NMII as the negative regulator of oligodendrocyte differentiation(Wang et al., 2012; Wang et al., 2008).Myosin studies in OPCs indicates its role in force generation(at the front) ,in detachment(at the rear) and in process spreading during a migratory response. The migratory OPCs had myosin overlapping with F-actin bundles at the leading edged of the cells(Simpson & Armstrong, 1999). Thus, progress in understanding signal transduction will aid in connecting gaps between extracellular signals, actin polymerization and cell motility.

1.9 Demyelinating diseases/Neurodevelopment disorders perspective - OPC migration defects

Myelin is a lipid rich membrane, highly specialized in its structure, forming an insulating sheath around the nerve axons. The need for myelination occurs to support the effective transduction of action potentials via ‘saltatory’ impulse propagation and also providing an additional trophic and metabolic support to the axons of vertebrate nervous system (Fields, 2009) Myelin forming cells includes the oligodendrocytes and the Schwann cells in the central nervous system (CNS) and peripheral system (PNS)

respectively. Most of the studies on oligodendrocytes till date have focused on the proliferation and differential aspect which is governed by different molecular mechanisms. Migration of OPC is one of limiting factor in demyelinating diseases such as Multiple sclerosis and Leukodystrophies.

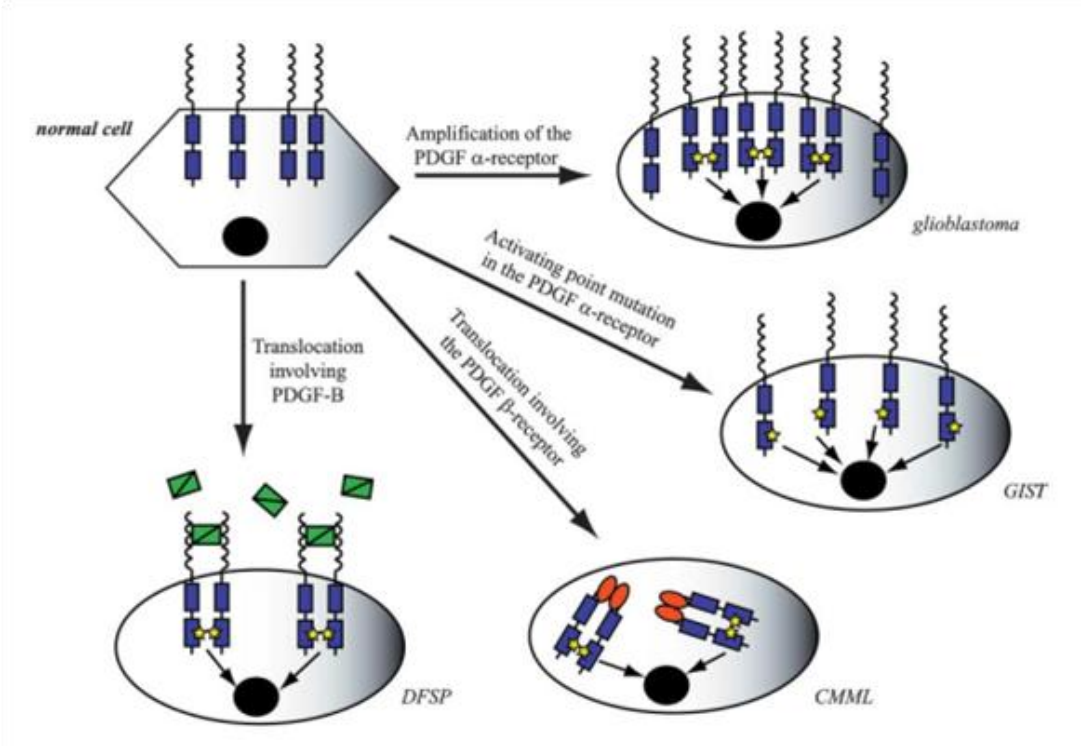
The demyelinating disease Multiple Sclerosis (MS) is probably the most well known disease of the adult white matter. It is a disabling inflammatory disease in which macrophage phagocytosis destroys the myelin sheath in an autoimmune fashion and it is characterized by the development of multiple, diffuse foci of demyelination (Peru et al., 2008).The inability to repair the areas where the myelin has been eroded, results in chronic inflammation, glial cell activation, further myelin degeneration and eventual loss of axons (Adams et al., 1989; M Melanson et al., 2009; Prineas & Raine, 1976; Ransohoff, 1999). The cellular and molecular mechanisms causing tissue degeneration in MS brain is still unclear, recent studies suggests that demyelination and oligodendrocyte death is mediated by activated parenchyma CNS cells and infiltrating immune cells that includes mostly T cells and monocytes (Ransohoff, 1999).The inflammatory response caused by the immune cells lead to the activation of microglia which degenerates and disrupts the crosstalk between the oligodendrocyte and axons and eventual OL death. This results in demyelination and further phagocytosis of myelin, axon and Oligodendrocytes which recruitment of T cells and B cells triggering more autoimmune response. The whole cycle of events eventually leads to the secondary axonal damage impairing the normal propagation of electrical impulses essential for neuronal functioning (Peterson & Fujinami, 2007). MS patients experience weakness, insensitivity to pain, ataxia and gait instability (Roxburgh et al., 2005). The final stages of the disease comprises of deterioration of bladder function, fatigue, cognitive deficits, impaired attention and memory loss (Maria Melanson et al., 2010; M Melanson et al., 2009).

Leukodystrophies, however, corresponds to genetic determined disorders either due to diminished myelin production, abnormal myelin development or complete loss /destruction of myelin (Powers, 2004). The characteristic symptoms involves neurological dysfunction, loss of motor skills, cognitive decline(Suzuki, 2003), seizures, ataxia and sometimes death in infancy (Hagberg, 1971).

Several groups have investigated the potential of transplants to provide new myelinating cells for demyelinated areas of the CNS, however, to date there are few successful transplant studies. Some success has been achieved using myelinating cells derived from the PNS, and more recently, stem cells (Halfpenny et al., 2002; Pearse et al., 2004). However, transplanted cells are unable to migrate the distances required to effect repair of scattered CNS lesions(Franklin & Blakemore, 1997; Targett et al., 1996).Understanding the regulatory mechanisms of oligodendrocyte progenitor migration is thus crucial to being able to dissect out the subsequent processes that culminate in myelination.

1.10 PDGF and Cancer

Apart from the role of PDGF activated intracellular signaling in OPC migration and neurodevelopment disorders, PDGF signaling has been known to play a prominent role in Cancer and its metastasis (Farooqi & Siddik, 2015).The discovery of the transforming retroviral v-sis oncogene from the PDGF-B chain gene has lead the PDGF signaling pathway an interesting target for cancer treatment and cancer therapy. Several clinically antagonists to PDGF receptors such as Glivec(STI571/ Gleevec),evaluates the importance of PDGF receptor signaling in malignancies (Buchdunger et al., 1996; Capdeville et al., 2002). Genetic alterations caused by constitutive activation of PDGF receptors and autocrine growth simulation, has been identified in different types of cancer cells (Figure 1.6). Amplification of the PDGF receptor gene occurs in a subset of high grade gliomas (Fleming et al., 1992) and ligand independent deletion mutant of the PDGF receptor fusion has also been observed(Clarke & Dirks, 2003)(Figure 1.6).Rabaptin-5 or the transcription factor Tel has been identified to dimerization, fusion and thereby constitutive activation of PDGF receptors in patients with chronic myelomonocytic leukemia(CMML) (Golub et al., 1994; Magnusson et al., 2001). Small deletion and activation of point mutations in PDGFRα gene has been found in patients with GIST (Heinrich et al., 2003). Further, translocations involving the fusion of PDGF-B chain with the collagen1 A1 gene, has been observed in dermatofirbrosarcoma protuberans (DFSP) which leads to constitutive production fusion proteins processed into PDGF-BB(O'Brien et al., 1998; Shimizu et al., 1999; Simon et al., 1997)(Figure 1.6).



Adapted from (Pietras et al., 2003)

Figure 1.6: Genetic alteration that cause the dysregulation of PDGF receptors signaling in different tumors. Different types of malignancies in each category in given in Italics. Active PDGF receptor kinase, yellow star; PDGF ligand green; dimerization domain not derived from the PDGF receptor, red.

Moreover, the Cancer genome atlas and other organizations have accumulated evidences in about 80 studies which supports the occurrence of several types of cancer based on the above defects including mutations , deletions and copy number aberrations in any of the PDGF-A/B/C/D and PDGFR A/B genes in about 30 percent of the patients. The incidence of these defects is specific to certain cancer types which includes melanoma (~10-30%), bladder (~ 15-20%), glioblastoma(~15-20%), prostate (~20%),colorectal(~10-15%), ovarian (~10-20 %) and a lower percentage(<10%) in breast, acute myeloid leukemia , liver and renal cancer.

1.11 GLIOMA

Gliomas of central nervous system are among the most common and aggressive forms of tumor recognized only by its pathological characteristics rather than the genetic causes. Gliomas are considered to be more rapid and invasive by nature and are usually fatal. The origin and events leading to formation of glioma remains unclear

but is been reported to be caused by genetic alterations (Grobben et al., 2002; Maher et al., 2001; M Nakada et al., 2007). These includes increased activities in the receptor tyrosine kinases and mutations in genes coding downstream signalling molecules (Lind et al., 2006; Nazarenko et al., 2012) which leads to increased proliferation, tumor invasiveness, genomic instabilities (Maher et al., 2001) and disruptions in the cell cycle arrest pathways (Y.-H. Zhou et al., 2005; Y.-H. Zhou et al., 2010).Glioma is the most malignant brain tumor that has the ability to migrate and invade the CNS. The genetic alterations due to the aberrant expression of the various growth factors such as Hepatocyte growth factor receptor (HGFR) ,C-met, Epidermal growth factor receptor (EGFR) and Platelet derived growth factor receptor (PDGFR) has been continuously reported to trigger glioma progression(Able Jr et al., 2011). One of the common genetic alteration or defect observed in glioma is the presence of PDGF autocrine loop characterized by the co-expression of the PDGF and its receptor(Barth, 1998; Lokker et al., 2002a).

1.12 C6 glioma as model system for Glioblastoma

Rat C6 glioma cell line exhibits similar morphology to glioblastoma multiforme (GBM) after it is introduced in the neonatal rat brains. It was formerly generated in random-bred Wistar-Furth rats by exposure to N, N”-nitroso-methylurea (Grobben et al., 2002). The C6 glioma model continues to be used for numerous studies related to tumor metastasis which includes tumor growth, invasion, growth factor regulation and production, BBB disruption and migration. Further, single cell clonal study demonstrates that C6 glioma cells have cancer stem cell like characteristics including self-renewal, the capacity of tumor formation in vivo and the potential for multilineage differentiation in vitro(Barth, 1998). Apart from this, C6 gliomas also show some characteristic of glial precursors and express some oligodendrocyte and astrocyte genes including PLP, MAG and GFAP (Salvati et al., 2004; Ye et al., 1992; Zhu et al., 1994).

However, the purpose of using Rat C6 glioma cell line as a experimental model system is due to presence of well-characterized PDGF autocrine loop (Grobben et al., 2002; Strawn et al., 1994). Blocking the PDGF receptors in glioma cell lines causes the inhibition of survival or mitogenic pathway widening the chances of its use as a therapeutic strategies in treating glioblastoma (Lokker et al., 2002a). Several studies

on overexpression/ hypersensitivity of PDGF and its ligands has proved PDGFRA the second most frequently amplified RTK gene in the tumors after EGFRs (McLendon et al., 2008). Furthermore, significant reduction in the migration and proliferation rate has been seen on inhibition of the Raf/MEK/ERK and PI3K/AKT/MTOR pathways in C6 glioma cells (Bloxham, 2013). Both the two pathways are activated by similar sets of growth factors receptors such as PDGFR and EGFR (Nazarenko et al., 2012; Thompson & Lyons, 2005).Inhibition of the ERK pathway has been reported to cause reduced migration and proliferation and increased GFAP expression in the C6 cells (Lind et al., 2006).MSAP (MRLC-interacting protein (MIR)-interacting saposin-like protein) has been reported to enhance the migration of rat C6 glioma through increase in phosphorylation of Myosin regulatory light chain (MRLC) (Bornhauser & Lindholm, 2005).The study stated that apart from the MLCK (Myosin light chain kinse) and Rho kinase (ROCK) conventional pathway to migration, MSAP phosphorylation can be activated through other protein kinases ERK, death associated protein kinase (DAPK) (Jin et al., 2001), zipper-interacting protein kinase (ZIPK) (Murata-Hori et al., 1999) and PAK family members(PAK2) (Zeng et al., 2000).

1.13 Rationale of the present study

Most of the studies on oligodendrocytes till date have focused on the proliferation and differential aspect which is governed by different molecular mechanisms. Migration of OPC is one of limiting factor in demyelinating diseases such as multiple sclerosis and Leukodystrophies. Understanding the regulatory mechanisms of oligodendrocytes progenitor migration is crucial to being able to dissect out the subsequent processes that culminate in myelination (Figure 1.7). A little has been known about the molecular mechanism which may help the migration of OPCs from the SVZ to white matter regions of the Brain

This study unravels the pathways and molecular mechanism behind the OPC migration.

Apart from oligodendrocyte migration, aberrant expression of PDGF and its receptor is also responsible for cancer progression by promoting the migration and invasion. Malignant glioma exhibits a high rate of cell motility and migration that contributes the invasiveness. Glioma invasion into other brain tissue makes the disease difficult to treat by surgery and chemotherapy. Rat C6 glioma cell line is morphological similar

to glioblastoma multiforme (GBM). Rat C6 cell line have PDGF autocrine loop which is responsible for growth, motility and survival of tumor. Thus the understanding of the molecular mechanism of PDGF which regulate glioma cell migration is very important in order to develop potential target for treatment that can limit the invasion (Figure 1.8). The present study is focused on the role of PDGF-A activated intracellular signaling pathways during the process of migration in Oligodendrocytes precursors cells and Rat C6 glioma. C6 glioma cell line as experimental model system is well- characterized by the presence of a PDGF autocrine loop. Blocking the PDGF receptors in glioma cell lines causes the inhibition of survival mitogenic pathway widening the chances of its use as a therapeutic strategy in treating glioblastoma (Lokker et al., 2002a).

Thus the understanding of the molecular mechanism of PDGF which regulate glioma cell migration is very important in order to develop potential target for treatment that can limit the glioma progression.

Hence we **hypothesize** that:

PDGF-A induced activation of ERK1/2 lead to activation of downstream molecules involved in cytoskeleton reorganization and thus migration of oligodendrocytes progenitor cells and glioma. Thus, the objectives of the present study were defined as follows:

1.14 Objectives:

- ***To determine the role of ERK1/2 in the regulation of OPCs migration and cytoskeletal reorganization.***
- ***To determine the crosstalk of ERK1/2-ROCK signaling in OPCs.***
- ***To determine the role of ERK and ROCK signaling in PDGFR inhibition mediated intracellular signaling in C6 glioma growth and migration.***

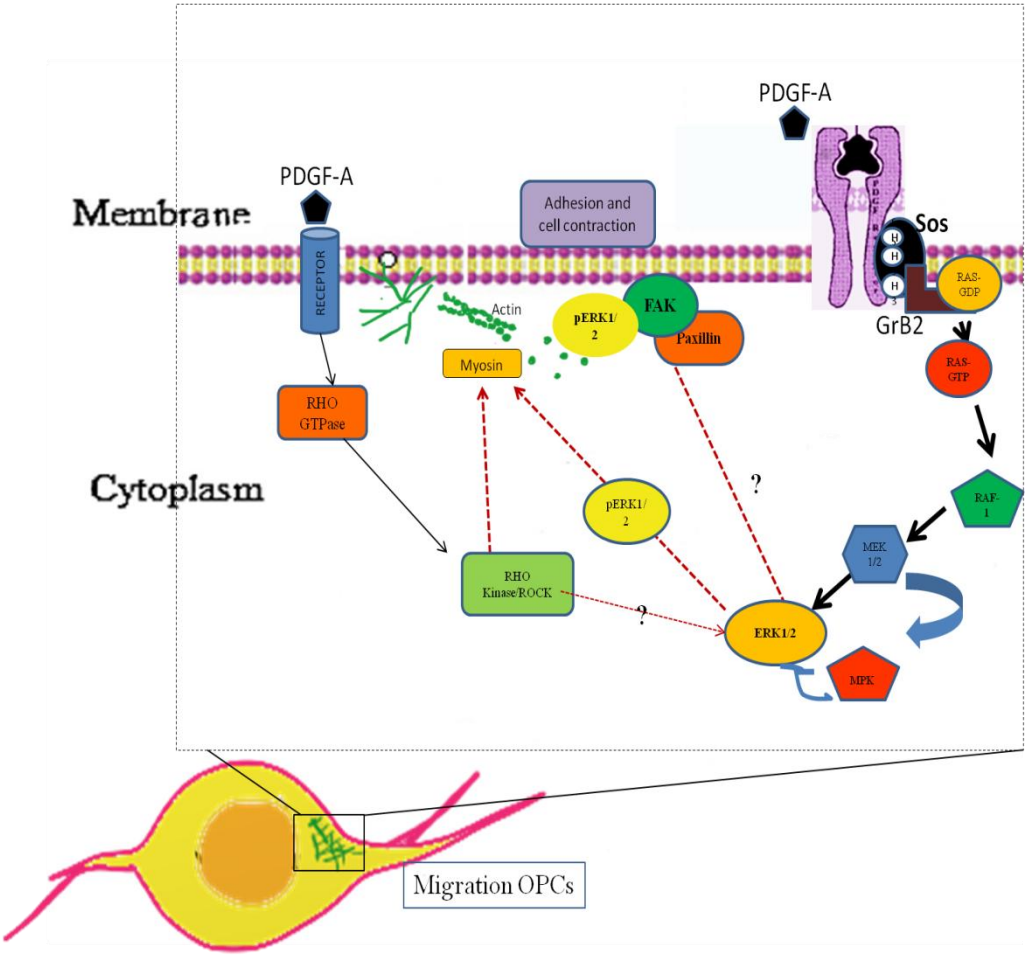


Figure 1.7 PDGF-A activated signalling pathways in OPC migration: Given straight lines shows the known signalling pathway whereas the dashed lines indicates the hypothetical signalling pathways

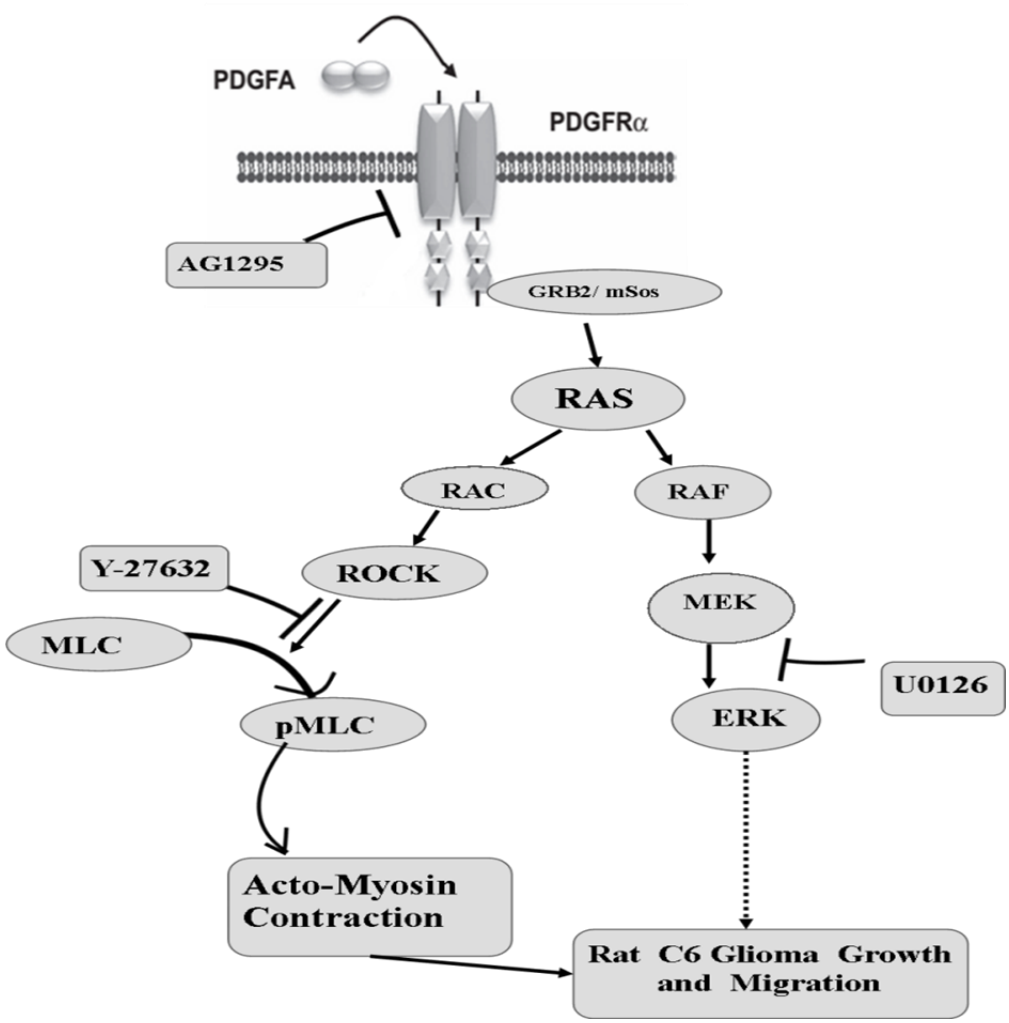


Figure 1.8 PDGF-A activated signaling pathways in C6 glioma migration: Given straight lines shows the known signalling pathway whereas the dotted lines indicates the hypothetical signalling pathways