CHAPTER 1

INTRODUCTION

1.1 Central Nervous System glial cells

The nervous system coordinates the actions of complex organisms through electrochemical signal transmission. In the higher animals, it is divided into two main parts: CNS (Central nervous system)- made up of the brain and spinal cord which integrates information received from peripheral nerves and coordinates bodily responses and PNS (Peripheral nervous system)- made of peripheral nerves which link the CNS to the body's receptors and effectors (Brady et al., 2011).

Central nervous system is a complex and extremely co-ordinated network of neurons and glial cells (Hansson and Rönnbäck 2003). Neurons are responsible for collecting information from the surroundings, generating action potentials, processing and transmitting it to other neurons by synaptic communication by transmitting electrical or chemical signals (Pereda 2014) whereas glial cells provide metabolic (Deitmer 2001), structural, synaptic (Bacci et al., 1999) and trophic (Ohgoh et al., 2000) support to the surrounding neurons (Tortora and Derrickson, 2008).

The name neuroglia or glia is of Greek origin where glia means "glue". The name was derived from the idea of early scientists that they were the "glue" that held the nervous tissue together. Since a long time after their discovery, they were thought to be passive bystanders and merely neuronal supporters. But as a result of several recent studies it is now understood that brain activity is not just a result of neuronal activity but is rather extensively regulated by glial cell controlling several aspects of nervous system development, plasticity and disease (Barres, 2008; Greener, 2015; Todd et al., 2006). Glia, generally, are smaller than neurons, and they form a larger part of the nervous tissue- about 90% in human brain. These estimates, however, seem to be incorrect and recent reports suggest that the human brain has approximately equal numbers of neuronal and non-neuronal cells (Azevedo et al., 2009; Verkhratsky and Butt, 2013; Herculano-Houzel 2014). Of the six types of glia, astrocytes, oligodendrocytes, microglia, and ependymal cells, are found in the CNS whereas Schwann cells and satellite cells are present in the PNS.

1.1.1 Astrocytes

Astrocytes are largely a homogeneous cell population with a star-shaped morphology which extend numerous processes surrounding neighbouring neurons and blood vessels, and contain intermediate filaments (glial fibrils). Astrocytes were classically defined by their morphology and expression of glial fibrils, however with recent molecular and genetic tools; it seems that astrocytes represent a diverse population of cells with a number of functions. Moreover, neural progenitor or stem cell features have been displayed by a sub-population of GFAP expressing cells suggesting that astrocytes also possess certain neuronal properties. This widens the definition of an astrocyte while also making it more confusing.

Astrocytes are involved in a wide range of functions including synthesis of extracellular matrix proteins thereby promoting or inhibiting neurite outgrowth, in controlling neuronal maturation and synaptogenesis, in angiogenesis and BBB induction and maintenance, regulating extracellular ion buffering, providing metabolic support to neurons and their detoxification and immune functions along with participation in synaptic transmission (Wang and Bordey, 2008).

1.1.2 Microglia

Microglia are the resident macrophages of the CNS and play an important role during development by eliminating the neural apoptotic debris (Linnartz-Gerlach et al., 2014). In addition microglia are also involved in neuronal pruning during development. They secrete a number of growth and trophic factors such as basic fibroblast growth factor, hepatocyte growth factor as well as neurotrophins which aid the neuronal development and function (Streit et al., 1988). Most importantly, activation and proliferation of microglia is increased in response to CNS damage, during trauma, stroke, inflammation, or autoimmune attack. Microglia play an essential role in pathogenesis of diseases such as amyotrophic lateral sclerosis, Alzheimer's disease, Huntington's disease, multiple sclerosis, Rett syndrome, X-linked adrenoleukodystrophy (X-ALD) and Lysosomal storage diseases (LSD) (Cartier et al., 2014).

1.1.3 Oligodendrocytes

Oligodendrocytes are the myelinating cells of the CNS which produce the myelin sheath around the axons and provide rapid conduction of nerve impulses. A single oligodendrocyte can myelinate 30-40 axons. Oligodendrocytes undergo a pre-defined lineage of development during which they arise from neural precursors located within discrete regions in the CNS as Oligodendrocyte Precursor Cells (OPCs) which proliferate and migrate long distances and populate the entire CNS. The OPCs then differentiate into immature oligodendrocytes and then finally to mature myelinating oligodendrocytes which form the myelin sheath around axons. Each stage of development involves drastic change in its morphology from bipolar to extensive branching in mature oligodendrocytes along with the expression of characteristic stage specific cell surface antigens (Baumann and Pham-Dinh, 2001; Fields, 2008).

1.2 CNS myelination

Neuronal signal propagation or action potential in vertebrates is sped up by the electrical insulation of axons with an ensheathing, specialized glial plasma membrane: myelin sheath (Bunge et al., 1968). Myelination of axons increases their transverse resistance and insulation attributed to the high lipid molecular composition (Baumann and Pham-Dinh, 2001; Jahn et al., 2009). Myelination occurs caudorostrally in the brain and rostrocaudally in the spinal cord. In rodents, is achieved in all regions 45-60 days postnatally whereas in humans it starts in the second half of gestation and continues until 20 years of age (Giedd, 2004; Minkowski, 1967). In the CNS, myelination is carried out by specialized cells called oligodendrocytes whereas in the PNS, Schwann cells majorly drive the myelination event. These cells have a remarkable capacity to synthesize membranes at a given time, specific for a species and regions; the timing being so precise and characteristic that the age of human foetuses can be determined accurately simply by assessing which pathways have been myelinated (Schwab and Schnell, 1989).

1.2.1 Myelin: Structure and composition

The myelin sheath has a unique composition compared to normal membrane composition with a low water content of 40%, a high proportion of lipids with 70-85% (highly enriched in glycosphingolipids and cholesterol), which contributes to its ability to be a good insulator and 15-30% of protein of the dry mass (Snaidero et al., 2014; Waxman et al., 1995).

Myelin proteins

Though the myelin protein content is only 30%, these proteins are very specific and essential for the function of the myelin sheath. Major proteins in myelin are the proteolipid protein (PLP) and its splicing isoform DM20 (together 30-45%), myelin basic protein (MBP, 22-35%), and 2′,3′-Cyclic nucleotide 3′-phospho-diesterase (CNP, 4-15%). The remaining is composed of other myelin proteins like the myelin oligodendrocyte glycoprotein (MOG), myelin oligodendrocyte basic protein (MOBP), the isoforms of the myelin associated glycoprotein (MAG) and Claudin11.

- Proteolipid protein (PLP)

PLP is an integral membrane protein with a molecular mass of 30 kDa. An isoform of PLP, known as DM20 (molecular mass 26 kDa) is formed as a result of alternative mRNA splicing of PLP (Nave et al., 1987). PLP expression is usually seen in late postnatal development whereas DM20 is expressed early in the oligodendrocyte lineage. The primary structure of PLP is highly conserved between mouse and man, which suggests that the protein might be engaging in multiple protein-protein interactions. Chief function of PLP is in the compaction of myelin sheath by stabilizing the intraperiod line; mutant PLP causes abnormal myelin compaction and in many cases causes premature death of oligodendrocytes (Klugmann et al., 1997). PLP mutations cause Pelizaeus-Merzbacher disease (PMD) which is associated with a substantial decrease in oligodendrocyte survival, oligodendrocyte apoptosis caused by accumulation of PLP product in the endoplasmic reticulum and myelin sheath formation impairment (Boison and Stoffel, 1989; Knapp et al., 1986).

- Myelin Basic Proteins (MBP)

Several MBP isoforms- 14, 17, 17.2, 18.5 and 21.5 kDa have been identified which are formed by the alternative splicing of a single *mbp* primary transcript. Interestingly all isoforms do not behave in the same way when individually expressed. 14 and 18.5 kDa isoforms have a plasma membrane distribution similar to the proteins that participate in membrane compaction while the 17 and 21.5 kDa isoforms distribute diffusely through the cytoplasm. MBP is highly basic; which allows it's binding to negatively charged lipids bringing about the opposing cytoplasmic surfaces of the myelin membrane closely together and thus leading to proper compaction of the myelin sheath (Roach et al., 1983). During this it squeezes out the cytoplasm in a zipper like fashion leading the MBP molecules to interact with each other to form a meshwork that hinders other proteins from entering into compacted regions (Aggarwal et al., 2013). MBP mRNA is synthesized in the cell perikaryon and transported as mRNA granules along processes to myelin (Wake et al., 2011). Oligodendrocytes may have developed this mechanism of local synthesis of MBP at the plasma membrane to ensure that MBP exerts its adhesive action at the appropriate place in the cell. This process has been described to be regulated by the signaling events resulted from axon–glia interaction. Axonal signals such as laminins and action potentials induce MBP mRNA translation through integrins and F3/contactin signaling pathways (Laursen and Chan, 2011). Another mechanism to regulate the physicochemical properties of MBP is post-translational modification including methylation, phosphorylation, and N-terminal acylation which regulate the interaction of MBP to the membrane (Harauz et al., 2004). In addition, MBP also influences the lipid composition in myelin (Fitzner et al., 2006). Shiverer mutant mice which carry a mutation in the MBP gene and lack the MBP isoforms exhibit abnormal myelin compaction, suffer from convulsions and die at a very young age (Chernoff, 1981).

- Myelin-associated glycoprotein (MAG)

MAG is also a transmembrane protein which shows significant homology to the neural cell adhesion molecule (NCAM). MAG is an adhesion molecule present in the periaxonal layer of non-compact myelin membrane facing the axon. Based on the fact that its early expression correlates with the initiation of myelination (Quarles, 2007),

MAG is thought to mediate interactions between the axons and oligodendrocytes (Lopez, 2014). Early stage loss of MAG causes mild clinical and pathological alterations in both CNS and PNS, delayed CNS myelination and redundant or disrupted compact myelin. On the other hand, at a later stage additional alterations are observed including degeneration of axons and myelin (Nguyen et al., 2009) and dying-back oligodendrogliopathy, which possibly represents initial stages of a slow demyelinating process.

- Myelin-oligodendrocyte glycoprotein (MOG)

MOG was first identified as an antigen responsible for the demyelination in animals (Birling et al., 1993). MOG is expressed on the surface of oligodendrocytes in the later stage of their differentiation and on the outermost lamellae of myelin sheath. MOG is thought to have adhesive functions although the exact functions are not well characterised. Mice deficient in MOG were healthy and indicated no pathological abnormalities (Delarasse et al., 2003).

Myelin Lipids:

The abundant amount of lipids present in brain has simplified its biochemical purification with an enhanced purity and yield (Norton and Poduslo, 1973). Lipids help in the tight organization and protection of molecules within the membrane and for the long term maintenance of myelin (Schmitt et al., 2015). Reports suggest that the overall ratio of proteins to lipids is 1:186 in myelin (O'Brien and Sampson, 1965). All the lipids found in the brain are found in the myelin and there are no lipids exclusive to the non-myelin areas; although regional differences do occur- spinal cord has a higher lipid-to-protein ratio than brain myelin. PNS and CNS lipids are qualitatively similar but they do differ quantitatively. The most noteworthy lipids in myelin are cholesterol, galactosylceramide and ethanolamine.

- Cholesterol

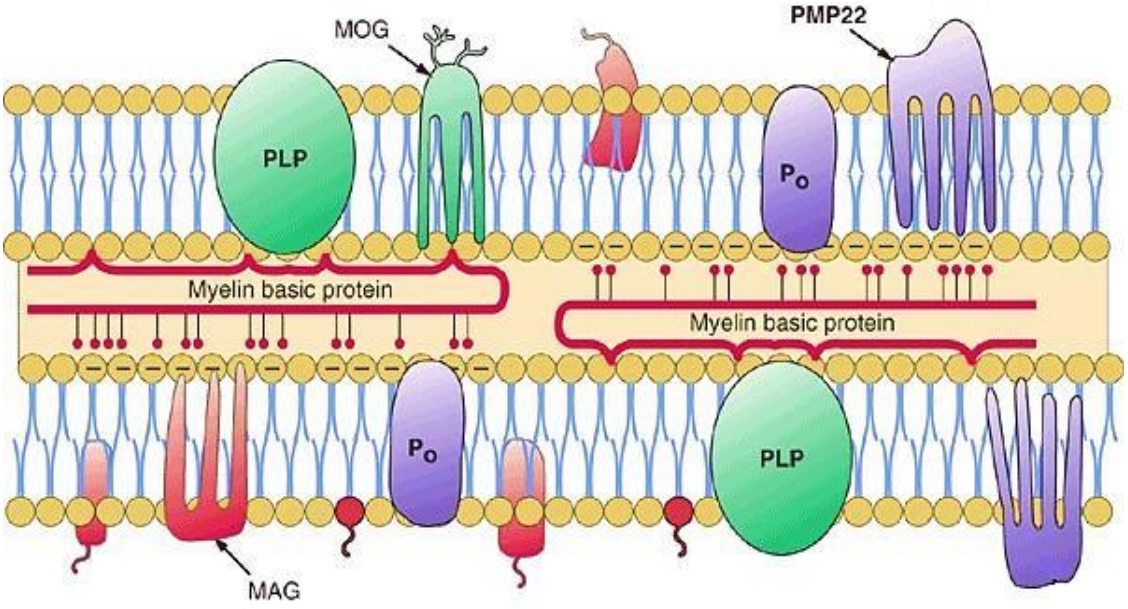
Both astrocytes and oligodendrocytes are producers of cholesterol in the brain and they do so through a series of complex interactions with each other (Dietschy and Turley, 2004). The rate of myelination is combined and determined by cholesterol uptake; mutation or problem with any of the cholesterol synthesis, mobilization or utilization steps results in drastic reduction in number of myelinated axons, reduced number of mature oligodendrocytes and severe hypomyelination in PNS with number of uncompacted myelin sheaths (German et al., 2002). Cholesterol also coordinates the myelin assembly possibly by regulating signalling pathways of energy supply in myelinating glia or by directly influencing the transcription factors (Emery et al., 2009). According to a recent report, PMD phenotypes can be corrected by feeding mice with a diet rich in cholesterol thus indicating the role of cholesterol in influencing cases with deficient or loss of myelination (Saher et al., 2012).

- Galactosylceramides/sulfatides

Galactosylceramide is the most typical cerebroside of the myelin. Concentration of cerebroside in brain is directly proportional to the amount of myelin present. A small proportion of total galactolipid is sulphated and occurs as sulfatide. It was initially thought that galactocerebroside in oligodendrocytes and myelin was essential for oligodendroglia differentiation and for the structure and function of myelin. This dogma was contradicted when in a knockout mouse of galactosylceramide, the myelin formed was normal although myelin abnormalities like reduced myelin thickness, redundant myelin out-foldings and differences in axon conduction velocity were observed along with a vacuolation of myelin in the spinal cord (Bosio et al., 1996). However, the axoglial contacts at the paranodal junctions were significantly disordered in galactosylceramide-sulfatide null mice with low levels of Caspr and contactin and absence of glial Neurafascin-155 (NF-155) (Schafer et al., 2004). Thus, these cerebrosides are not crucial for the synthesis of myelin but as essential for the maintenance and stability of myelin, more so in adults and old animals.

- Phosphoinositides

Phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2) is the chief phosphoinositide in most plasma membranes including myelin (Deshmukh et al., 1980) wherein it regulates endocytosis, actin cytoskeleton remodelling and cell growth. Phosphoinositides are abundantly present at the leading edge of the growing myelin sheath (Goebbels et al., 2010) and its downregulation leads to disintegration of non-compact myelin (Snaidero et al., 2014). This is hypothesized to be because of their interaction with MBP leading to its self-polymerize and resulting in myelin membrane compaction (Aggarwal et al., 2013; Bakhti et al., 2014).



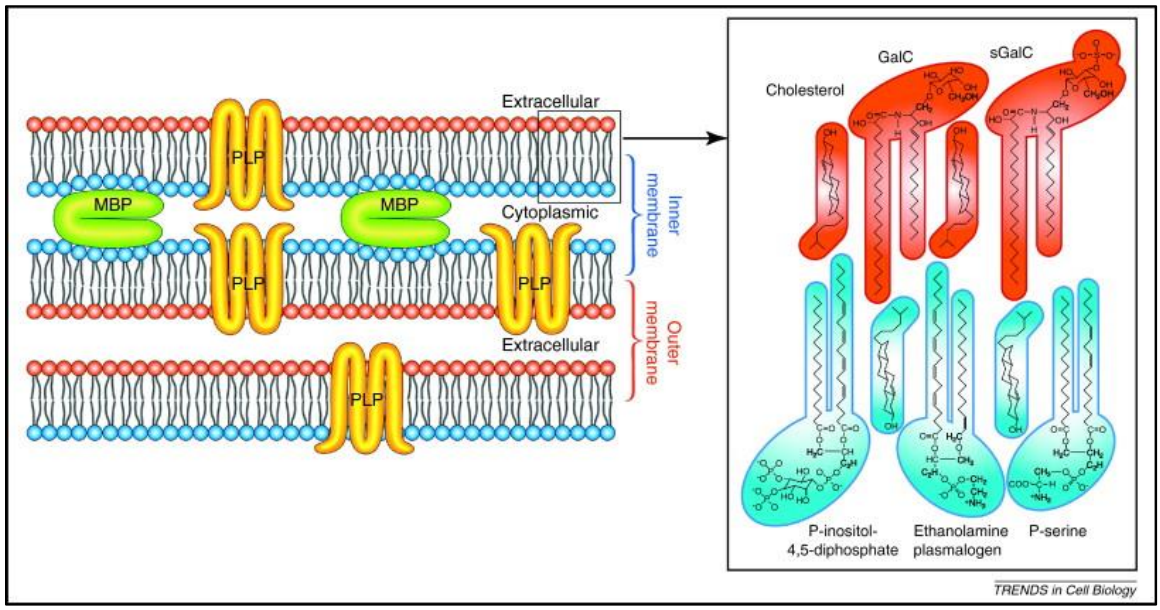


Figure 1.1: Myelin composition: Proteins- PLP, MBP, MAG and MOG. Lipids- Cholesterol, Galactosylceramides (on the extracellular membrane) and Phosphoinositides, Ethanolamine (on the inner membrane). Lipids interact with myelin proteins resulting in myelin membrane formation, maintenance and compaction. (Aggarwal et al., 2011).

1.2.2 Mechanism of myelination:

Myelination occurs through a number of sequential steps. It proceeds with the proliferation and migration of OPCs, outgrowth and retraction of oligodendrocyte cell processes, target axon recognition, adhesion of oligodendrocyte process to the axon, stabilization of cellular contacts, rapid biosynthesis and trafficking of lipid and protein constituents of the myelin membrane, spiralling of the myelin membrane, and its organization as a multi-layered compacted structure around the axon (Beirowski, 2013; Sherman and Brophy, 2005).

1) Migration of oligodendrocytes to axons that are to be myelinated; the OPCs migrate away from the neuroepithelium of the ventricular/subventricular zone of the brain into the developing white matter tracts. Some OPCs remain in a precursor state while others differentiate into myelin-forming oligodendrocytes. A large number of locally (cytokine CXCL1, Netrin-1 etc.) and transiently expressed (Platelet derived growth factor- PDGF) molecules along with soluble signalling proteins and extracellular matrix proteins are involved in regulating the migration of OPCs to their destination (Canoll et al., 1996; Frost et al., 2009; Milner et al., 1997; Patel et al., 2010; Tripathi et al., 2017).

2) Adhesion of the oligodendrocyte process to the axon; occurs by the dual communication of axons with the oligodendrocyte. The relationship between myelin thickness and axon diameter indicates the active role of axonal signals during myelination. For oligodendrocytes, the threshold of axonal diameter is variable (0.4-1.2μm) indicating that the myelinating cells ‘measure’ the caliber of axons and create myelin layers appropriate for the axon diameter. Different molecular mechanisms also regulate myelination in PNS versus CNS; in addition it may vary between different regions of the CNS thus reflecting distinct neuronal types (Fruttiger et al., 1999; Kim

et al., 2006; Taveggia et al., 2010). A number or pathways, membrane associated soluble factors and extracellular matrix proteins are involved in the regulation of oligodendrocyte development which works hand in hand with neuronal components. These factors are Nrg1 mediated pathway, laminins, growth factors (IGF, FGF, BDNF), Notch ligands, PSA-NCAM and Lingo-1 (Tomassy et al., 2016). Electrical activity of neurons also influences the expression of cell adhesion molecules and stimulates the release of synaptic vesicles which stimulate the synthesis of myelin protein by oligodendrocytes (Wake et al., 2011). In response to these action potentials astrocytes release Leukaemia inhibitory factor (LIF) which further induces myelination by mature oligodendrocytes (Ishibashi et al., 2006).

Oligodendrocytes interact with neurons, particularly with axons in a very specific manner for myelination to take place. The importance of myelination can be understood from the observations that demyelinated axons need more energy than myelinated ones and thus loss of myelin leads to major decrease in the overall energy balance of the axon. Absence of myelination also isolates the underlying axons thus conferring protection from the surrounding environment and providing fundamental support. Axonal loss is a common feature of many myelin diseases such as multiple sclerosis and Pelizaeus–Merzbacher (Waxman, 2006). Oligodendrocytes also support neuronal survival and metabolism through the release of lactate which myelinated axons use for as their energy sources (Fünfschilling et al., 2012; Lee et al., 2012). An excess of oligodendrocytes are produced in the CNS to match the number of axons to be myelinated. Oligodendrocytes that succeed in contacting axons survive while those who do not are eventually eliminated by apoptosis (Barres and Raff, 1994; Simons and Nave, 2016; Trapp et al., 1997). These series of interactions between axons and the myelinating glial cells are called axo-glial interactions.

3) Spiralling of the process around the axon; once a particular axon has been engaged by a myelinating oligodendrocyte process, dramatic changes in plasma membrane architecture are induced and converted into flat sheets that spread and wind along the axons to generate a multi-layered stack of membranes. A predetermined number of myelin sheaths are programmed to spiral around the axon while regular presence of sodium channels in the spaced gaps in myelin at the nodes of Ranvier separate the nodes from the myelinated axon regions. This molecular specialization enables the regeneration of the action potential at the node of Ranvier (Eshed-Eisenbach and

Peles, 2013). The most accepted theory of myelin wrapping and thickening is by the addition of new myelin layers on top of the inner ones in a coiling or corkscrew motion. After contact with the axon, the myelin growth occurs with a single glial process spiralling circling the future internode. On achieving the required number of turns, the individual membrane layers grow laterally and glide over each other (Pedraza et al., 2009; Snaidero et al., 2014; Sobottka et al., 2011). The cytoplasmic rich membrane pockets of the myelin layer are closely in contact with the axonal surface which then move towards the prospective nodal region where they align and position as paranodal loops (Butt and Berry, 2000; Sobottka et al., 2011). The glial membrane, during this process, is faithfully attached to the axon by the formation of axoglial adhesion complex of CNTN1 on the axon and NF155 on the glial membrane (Pedraza et al., 2009; Zonta et al., 2008), thus suggesting that the axoglial adhesion complex is not only essential for the node formation, but also for stimulating the lateral extension of myelin layers.

4) Compaction of the myelin sheath: starts early in development, after a few myelin wraps (Readhead et al., 1987) in the outermost layers and progresses inwards. Compaction requires special adhesive proteins like MBP and the removal of molecules that prevent compaction (Nave and Werner, 2014). MBP is synthesized in the innermost tongue and regulated by axonal signals (Wake et al., 2011) followed by its outward diffusion where compaction initiation is occurring. MBP present on the two adjacent cytoplasmic surfaces rapidly polymerise into a network like structure that provides the basis for unidirectional membrane compaction (Aggarwal et al., 2013; Aggarwal et al., 2011); while the extracellular leaflets are attached by relatively weaker forces (Bakhti et al., 2013) probably because the inner layer tends to wind multiple times around the axon and thus weaker connections would support the slippage of myelin layers (Hirano and Dembitzer, 1967). The compact myelin is composed of fully mature oligodendrocytes which lack most of the glycoproteins and complex glycolipids. Thus there is also a transformation of oligodendrocyte lineage cells from repulsive OPCs that layer the entire CNS to sticky oligodendrocytes which ensheath axons with a multi-layered stack of self-associating membranes (Simons and Nave, 2016).

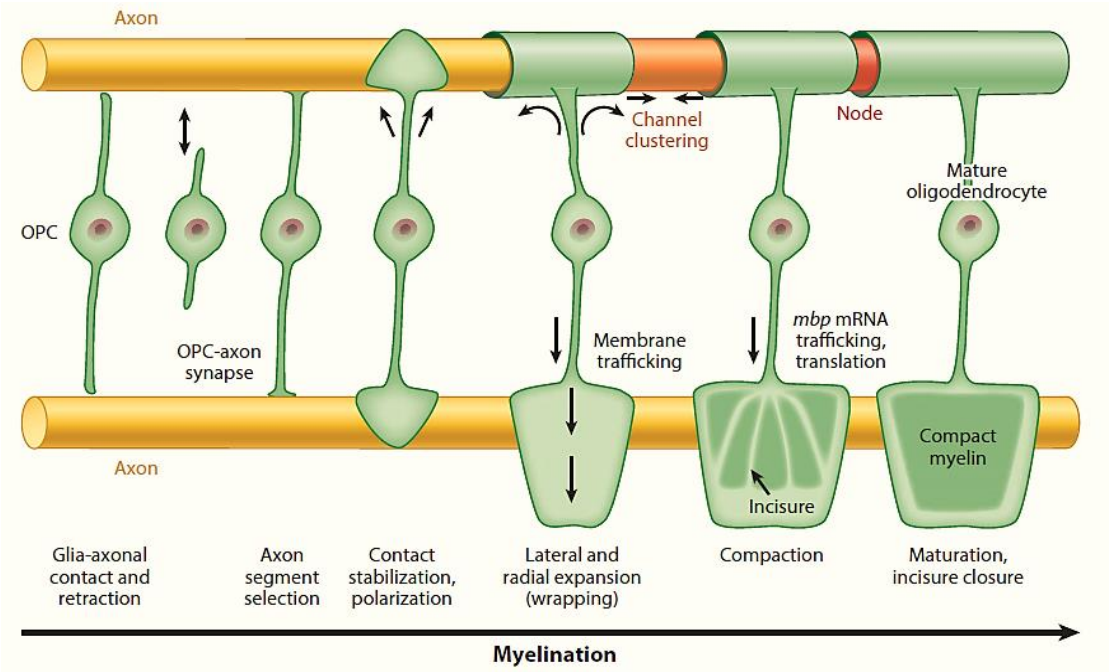


Figure 1.2: Schematic model of the differentiation of a committed OPC initiating interaction with an axon into a mature myelinating oligodendrocyte. Maturation of the nodes of Ranvier involves the glia-dependent clustering of axonal voltage-gated ion channels. (Nave and Werner, 2014)

1.2.3 Myelin Function

The primary function of myelin is the faster conduction of an action potential along the myelinated fiber by saltatory conduction. Myelin also decreases the capacitance and increases the electrical resistance across the axolemma thus preventing the electric current from leaving the axon. Along unmyelinated fibers, impulses are propagated by local ion currents that flow throughout the axon in a continuous sequential fashion. On the other hand, in myelinated axons the excitable axonal membrane is exposed to the extracellular space only at the nodes which is also the location of sodium and potassium channels. The node when excited generates a local circuit which flows out and depolarizes the membrane at the next node which increases the speed of local current spreading. The action potential thus jumps from node to node and is termed as a saltatory conduction. In addition, because the nodes are the only regions which are excited during conduction, the sodium flux into the nerves is significantly less than in unmyelinated nerves wherein the entire membrane

is involved thus resulting in impressive energy and space conservation. The secondary role of myelin and myelination is increased temporal precision, efficient feedback loop mechanisms, decreased reaction time to stimuli, ability to recognize and react to external environment changes and economy of space thus enabling a more compact nervous system and faster communication between distant body parts. These advantages provide clear indications for its evolutionary preference (Siegel et al., 1994; Zalc, 2006; Zalc et al., 2008).

1.3 Axo-glial interactions

In axons, action potentials are delimited to regularly spaced small segments which are unmyelinated and highly enriched in ion channels. These intervals along the axons are known as nodes of Ranvier, named after the discoverer French anatomist Louis-Antoine Ranvier. These complex structures allow ion influx into the axon to generate the action potential and thus the faster conduction of nerve impulse. While in PNS microvilli surround the nodal axolemma and Schwann cells, in CNS, nodes are contacted by perinodal astrocytes (Black and Waxman, 1988; Butt et al., 1994; Butt et al., 2002) whose exact function is still not well understood. Nodes of Ranvier are flanked on side by highly specific domains- the paranodal junctions (PNJ), juxtaparanodes (JXP), and internodes which are formed as the result of specific interactions between axons and myelinating cells (Devaux and Faivre-Sarrailh, 2013).

Nodes of Ranvier: These regions are formed of clusters of Na+ and K+ ions which regulate the rapid de and repolarization of the axolemma thus monitoring the conduction of the action potential. Nodes are composed of different kinds of voltage gated Na+ channels like Nav1.1, Nav1.2, Nav1.6, Nav1.7, Nav1.8, and Nav1.9 (Black et al., 1999; Boiko et al., 2003; Duflocq et al., 2008; Fjell et al., 2000) which function as pore-forming protein subunits that maintain the ion flux across the membrane. The nodes are also composed of different K+ channels including Kv3.1b, KCNQ2, and KCNQ3 (Devaux et al., 2003; Devaux et al., 2004), which regulate neuronal excitability (Battefeld et al., 2014). Apart from ion channels, nodes of Ranvier are also rich in cytoskeletal and scaffolding proteins ankyrin G and bIV spectrin which link the Na+ and K+ channels to the underlying cytoskeleton (Kordeli et al., 1995)

and to the glial interactions through the CAMs- NF186 and NCAM (Davis et al., 1996).

Paranodal junction (PNJ): It is a specialized axoglial contact formed between the axolemma and the paranodal loops of the oligodendrocyte cells which border the nodes of Ranvier. Oligodendrocyte cytoplasmic loops are spirally wrapped around the axons at a gap of 2.5-3mm to form septate-like junctions which are the largest of the vertebrate intercellular junctions. The paranodal loops are attached to the underlying axon via the axoglial junction which is in turn bordered by a tight junction network between the glial loops and assist in the attachment of myelin sheath to the axon thus distinguishing the electrical activity of the nodal region from the internodal region and operating as a boundary for the lateral diffusion of membrane proteins (Rosenbluth, 1976, 2009). PNJ consist of CAM complex made of glial NF155 and an axonal component of glycosylphosphatidylinositol (GPI) anchored contactin and Caspr (Charles et al., 2002; Sherman and Brophy, 2005) which are important for the formation of septate-like junction (Bhat et al., 2001; Boyle et al., 2001; Gollan et al., 2002; Rosenbluth, 2009). In addition, cytoskeletal proteins like ankyrinB, αII spectrin, βII spectrin are also found in this region. Faulty assembly of paranodal junctions leads to significant reduction in motor nerve conduction velocities (Laquérriere et al., 2013).

Juxtaparanodal junction (JXP): is located underneath the compact myelin, at the boundary between the PNJ and the internode. They are composed of clusters of K+ channels which help in maintenance of the internodal resting potential during myelination. JXP demonstrate high-density clustering of K+ channels consisting of Kv1.1, Kv1.2, Kv1.4, and Kvβ2 subunits (Rasband et al., 2001). JXP also shows presence of a CAM complex homologous to that of paranodes. This complex consists of GPI-anchored TAG-1 (also known as contactin-2) on the oligodendroglial membrane which interacts with Caspr2 and neuron-glial-related nodal axonal cell adhesion molecules and are required for the proper clustering of K+ channels (Poliak and Peles, 2003).

Internode: The intermodal axolemma is just under the compact myelin and consists of distinct membrane proteins. The membrane consists of two rows of juxtaparanodal type intramembranous proteins and exists as a line of alternating JXP-PNJ-JXP

proteins. The contact sites of axon and myelin sheath share molecular features of JXP and PNJ. Internodes are enriched in CAMs like Cadm3 and Cadm2, which forms a ligand pair with internodal Cadm4 on the adaxonal surface of oligodendrocytes (Spiegel et al., 2007). Another cytoskeletal adaptor protein 4.1G is also associated with Cadm4 especially in Schwann cells (Ivanovic et al., 2012) and the entire assembly together is a highly organized membrane protein distribution found along myelinated axons.

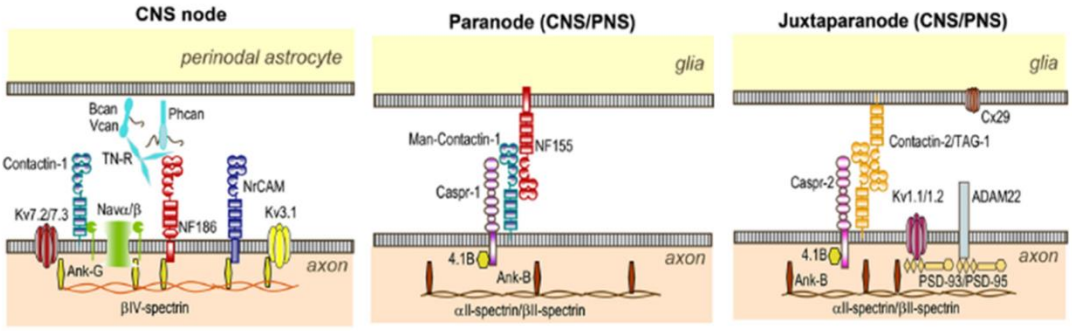


Figure 1.3: Molecular organization of the axonal domains of myelinated fibers (Devaux and Faivre-Sarrailh, 2013).

A detailed glial mechanism contributes to the formation of node of Ranvier. In CNS a combination of both secreted factors and contact-dependant interactions are important for the nodal assembly (Susuki et al., 2013). A soluble factor secreted by oligodendrocytes promotes the Na+ channel clustering in axons (Kaplan et al., 1997) while some reports suggest that oligodendrocyte contact initiates the formation of node. A sequence of contact-dependant events occurs in the CNS for the node formation. Formation of PNJ and myelination start off as parallel events (Rasband et al., 1999; Susuki et al., 2013). PNJ acts as a barrier to prevent the flow of membrane proteins between neighbouring myelin segments. The initial events are the axoglial interactions which take place at the PNJ. Na+ channels along with NF186, ankG and βIV spectrin start to gather at the edges of the growing myelin sheath. The nodal ECM proteins then bind to NF186 and stabilize the protein complex. These three mechanisms- PNJ-barrier mechanism, NF186-ECM mechanism and ankG-βIV spectrin clustering mechanism work independent and overlapping manners and form the node of Ranvier. Loss or disruption in any one of them results in mild damage to

channel clustering but not disrupt the node formation due to the compensation by the other two mechanisms. However, disruptions in more than one of them simultaneously impaired new conduction and cause lethargy in mice (Susuki et al., 2013). These interpretations suggest that all the three mechanisms work hand in hand in the assembly of node of Ranvier.

1.4 Astrocytes

Until recently, astrocytes were considered to be merely supportive cell components in the neural tissue which when reactive were markers of diseased tissue. The idea that pathological changes in CNS tissue could also be due to dysfunctional or reactive astrocytes was generally not considered. Increasing interest in the study of the biology and pathology of astrocytes resulted in changing of this viewpoint and it is now understood that astrocytes are responsible for a variety of essential and complex functions right from maintaining normal homeostasis to information processing to synaptic transmission in the CNS. Moreover, there are increasing evidences that lead towards the potential for loss or gain of astrocytic functions playing primary roles in disease processes and contribute to clinical and pathological mechanisms (Sofroniew and Vinters, 2010; Wang and Bordey, 2008).

1.4.1 Morphology and distribution in the CNS

Astrocytes tile the entire CNS in a systematic, well organized and non-overlapping manner and none of the CNS regions are devoid of astrocytes. They are a very heterogeneous population of cells with distinct morphologies and properties. Earlier they were divided into two main subtypes- protoplasmic astrocytes and fibrous astrocytes. However, Emsley and Macklis 2006, divided astrocytes into 9 classes based on three complementary astrocyte labelling methods- GFAP–GFP expressing mice, GFAP expression and S100β immunostaining. They termed these cell populations tanycytes, ‘radial’ cells, Bergmann glia, protoplasmic astrocytes, fibrous astrocytes velate glia, marginal glia, perivascular glia, and ependymal glia. Several types of astrocyte populations can co-exist within a given brain region along with

variations in their densities (Emsley and Macklis, 2006). Astrocyte morphology differs in different brain regions. Fibrous astrocytes are found in the white matter oriented along fiber tracts and exhibit morphology of many long fiber-like processes whereas protoplasmic astrocytes are found throughout in gray matter and exhibit morphology of a dense network of finely branching processes (Ramón y Cajal, 1909, Molofsky et al., 2012). Studies have indicated that astrocytes make extensive contacts with blood vessels; protoplasmic astrocytes envelop synapses and those of fibrous astrocytes contact nodes of Ranvier and both types form gap junctions with surrounding astrocytes (Peters and Palay, 1991).

Glial fibrillary acid protein (GFAP) is generally used as a prototypical marker for immunohistochemical identification of astrocytes. GFAP belongs to the family of intermediate proteins which are principally cyto-architectural in nature. It was originally isolated in abundant amounts in demyelinated plaques from multiple sclerosis patients and was then found to be immunohistochemically associated with astrocytes in such plaques (Eng et al., 1970). GFAP expression is thus considered as a sensitive and reliable marker of reactive astrocytes, however it may or may not be detectable in non-reactive astrocytes. It is also expressed by neural stem cells of the brain (Doetsch et al., 1999, Molofsky and Deneen 2015), radial glial cells (Kriegstein and Alvarez-Buylla, 2009), enteric glia of the enteric nervous system (Rühl et al., 2005) and in non-neural cell types like mesenchymal stellate cells that share structural and functional similarities with astrocytes in many organs (Lim et al., 2008). Aldh1L1 is also a specific antigenic marker for astrocytes with a considerably wider array of expression than GFAP (Cahoy et al, 2008).

Despite the diverse anatomical characteristics of astrocytes, they share a common set of physiological and functional properties. Astrocytes do not propagate action potentials like neurons although they do express potassium and sodium channels which evoke inward currents in compliance with regulated increase in intracellular calcium concentration (Cornell-Bell et al., 1990) that represent a form of astrocyte excitability which is of functional significance in astrocyte-astrocyte as well as astrocyte-neuron intercellular communication. Astrocytes also couple to adjacent astrocytes through gap junctions formed by connexins and these interactions play an important role in both normal function and in CNS disorders (Seifert et al., 2006).

1.4.2 Astrocytic functions

Astrocytes perform a variety of diverse functions right from embryonic development stage to the adult stage. Astrocytes are essential for the formation and function of developing synapses and synaptic pruning by releasing certain molecular signals (Barres, 2008), along with their involvement in development of white matter where dysfunction of astrocyte connexins and gap junctions leads to dysmyelination (Lutz et al., 2009). Astrocytes have many bidirectional communications with blood vessels including regulation of local blood flow and in the formation and maintenance of the BBB (Abbott et al., 2006). They also have their processes in contact with both blood vessels and synapses via which they titrate blood flow in response to synaptic activity (Schummers et al., 2008; Wolf and Kirchhoff, 2008). Astrocytes exert essential functions in maintaining the fluid, ion, pH, and transmitter homeostasis of the synaptic interstitial fluid and synaptic space by regulating and clearing certain molecules and neurotransmitters like glutamate, GABA and glycine which is important for healthy synaptic transmission (Barres, 2008; Seifert et al., 2006). They are also found in close association with the synaptic junctions as tripartite synapses which suggest direct and interactive roles with neurons during synaptic activity essential for neural circuits (Araque et al., 1999; Halassa et al., 2007; Perea et al., 2009). A large amount of literature now indicates that astrocytes make important contributions to CNS metabolism. They are principal storage sites of glycogen which are modulated by glutamate neurotransmitter and during hypoglycaemia these glycogen reserves break down to lactate and are transferred to neural elements where it is used as fuel (Brown and Ransom, 2007; Brown et al., 2004).

Astrocytes are also the major source of ECM proteins, adhesion molecules and trophic factors like laminin, N-cadherin, NCAM, fibronectin, growth factors like nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), fibroblast growth factor-2 (FGF-2) and Ciliary neurotophic factor (CNTF) all of which regulate neurite outgrowth, neuronal maturation and survival. They are also involved in extracellular ion buffering particularly of sodium and potassium that also regulates neuronal activity and behaviour. Astrocytes serve as a bridge between the CNS and immune system by phagocytosing cells and also producing a wide array of

chemokines and cytokines that act as immune mediators in cooperation with those produced by microglia (Sofroniew and Vinters, 2010; Wang and Bordey, 2008).

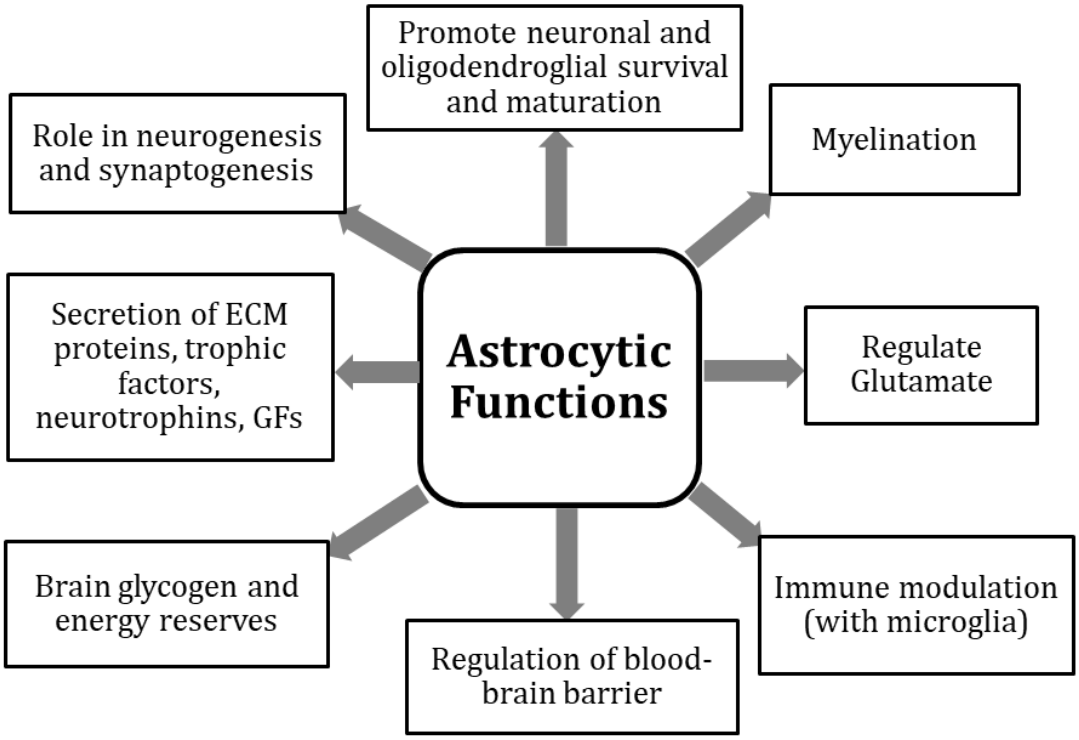


Figure1.4: Functions of astrocytes in the brain

1.5 Astrocytes in myelination

Astrocytes have for long been considered secondary and as merely supporting cells to neurons. Recent research, however, has demonstrated that role of astrocytes extend over a wide range of brain function beyond providing basic support as discussed above (Sofroniew and Vinters, 2010). The first hypothesis on the role of astrocytes in myelination came in 1904 by Műller who remarked that the cause of multiple sclerosis was ingrained in astrocytic dysfunction (Williams et al., 2007). Since then many reports, both in vivo and in vitro studies, have supported this notion that astrocytes could be important in regulating myelination and remyelination as well (Barnett and Linington, 2013; Moore et al., 2011; Sofroniew and Vinters, 2010; Williams et al., 2007, Kiray et al., 2016).

Astrocytes have been long known support and promote neuronal survival through secretion of various neurotrophic factors in the neuronal surroundings including NGF, BDNF, LIF, FGF and CNTF (Albrecht et al., 2007; Messersmith et al., 2000; Schwartz and Nishiyama, 1994). Cytokine activated astrocytes can also stimulate the differentiation of neural stem cells (NSCs) vested in the subventricular zone and dentate gyrus in adult animals and thus promote neurogenesis. These multipotent NSCs can then migrate beyond their sites of origin and differentiate into oligodendrocytes and neurons and have the capacity to promote recovery from CNS injury and disease (Liberto et al., 2004). CNTF treated astrocytes have been shown to support the survival of a number of ventral spinal motor neurons and promote neurite outgrowth compared to untreated astrocytes (Albrecht et al., 2003). These activated astrocytes also promote the percentage of myelinated fibres in rat cultures (Nash et al., 2011). Moreover, mice infected with mouse hepatitis virus (MHV-A59), an animal model for MS (Jordan et al., 1989; Messersmith et al., 2000), have been shown to secrete increased levels of CNTF by astrocytic cells during remyelination (Albrecht et al., 2003). Increase in IL-1β levels at early stages of CNS pathology also induces production of CNTF (Liberto et al., 2004; Stöckli et al., 1991) possibly through FGF-2 signalling, which in turn is already known to enhance OPC proliferation thus substantiating for remyelination (Albrecht et al., 2003; Messersmith et al., 2000). LIF production is in turn stimulated by IL-1β; LIF is known to promote survival and differentiation of oligodendrocytes (Kahn and De Vellis, 1994; Mayer et al., 1994) thus indicating towards a positive role on myelination (Bugga et al., 1998).

Although the brain is only 2% of the body's weight, it utilizes around 20% of the total oxygen and 20% of the total energy consumption (Sokoloff, 1991); a major chunk of which is demanded by myelination. The principle energy source of brain is glucose which enters the brain via the BBB which is in close contact with astrocytes which biochemically convert glucose into glycogen (Pellegri et al., 1996; Pfeiffer and Guglielmi et al., 2003). Also in low glucose concentrations, astrocytes can degrade stored glycogen and convert it into lactate which is directed towards the node of Ranvier and provide energy (Tekkök et al., 2005). The production of lactate is also sometimes considered to be a direct indication of the neuronal activity. Oligodendrocytes are also major consumers of lactate and myelination via oligodendrocytes is enhanced when lactate is supplied (Rinholm and Bergersen, 2012)

which indicates towards the possible contribution of astrocytic lactate production to myelinating oligodendrocytes (Rinholm et al., 2011; Sánchez and Abarca et al., 2001). Cholesterol is another important link between energy and cellular membrane composition- both of which are important factors in myelination. It is a precursor to many of the important signalling molecules like steroid hormones and is a major structural component of the myelin sheath. Dietary cholesterol cannot cross the BBB which means the cholesterol must be derived by de novo synthesis in the CNS (Orth and Bellosta, 2012). Astrocytes are supposed to be one of the primary sources of cholesterol (Pfrieger and Ungerer, 2011) and along with oligodendrocytes, conduct a horizontal transfer of cholesterol with neurons to maintain normal myelin sheath formation (Boyles et al., 1985; Kurumada et al., 2007). Presence of cholesterol is an essential rate limiting step to myelin production (Liu et al., 2010) and thus regulation of its production and maintenance by astrocytes is a crucial step for myelination.

Astrocytic effects on OPC survival, proliferation and maturation (Noble and Murray, 1984; Noble et al., 1988; Raff et al., 1988) and the rate of oligodendrocyte axonal ensheathment (Watkins et al., 2008). Astrocytes are the primary source of many secreted growth factors like CNTF (Cao et al., 2010; Stöckli et al., 1991), PDGF and FGF-2 (Bögler et al., 1990; Pringle et al., 1989) which are important for both neuronal and oligodendroglial proliferation and survival. Other in vitro studies have shown that neuronal cells when incubated with conditioned medium collected from primary astrocyte monolayers, showed enhanced neuronal survival, proliferation of OPCs and protection of oligodendrocytes from stress (Arai and Lo, 2010; Noble and Murray, 1984; Yamamuro et al., 2003). Another function of astrocyte with respect to myelination is the elimination of excitotoxic molecules from the extracellular space thus favouring neuronal survival. They convert excitotoxic glutamate and convert it into glutamine thus preventing neuronal death (Eng et al., 1997; Krum et al., 2002). They are also present at synaptic junction where they secrete regulatory molecules to modulate synaptic neurotransmission; tripartite synapse (Araque et al., 1999; Halassa et al., 2007). Synaptic signal transmission can also trigger astrocytes to secrete LIF which increases the number of myelinated axons in DRG-oligodendrocyte co-cultures (Ishibashi et al., 2006).

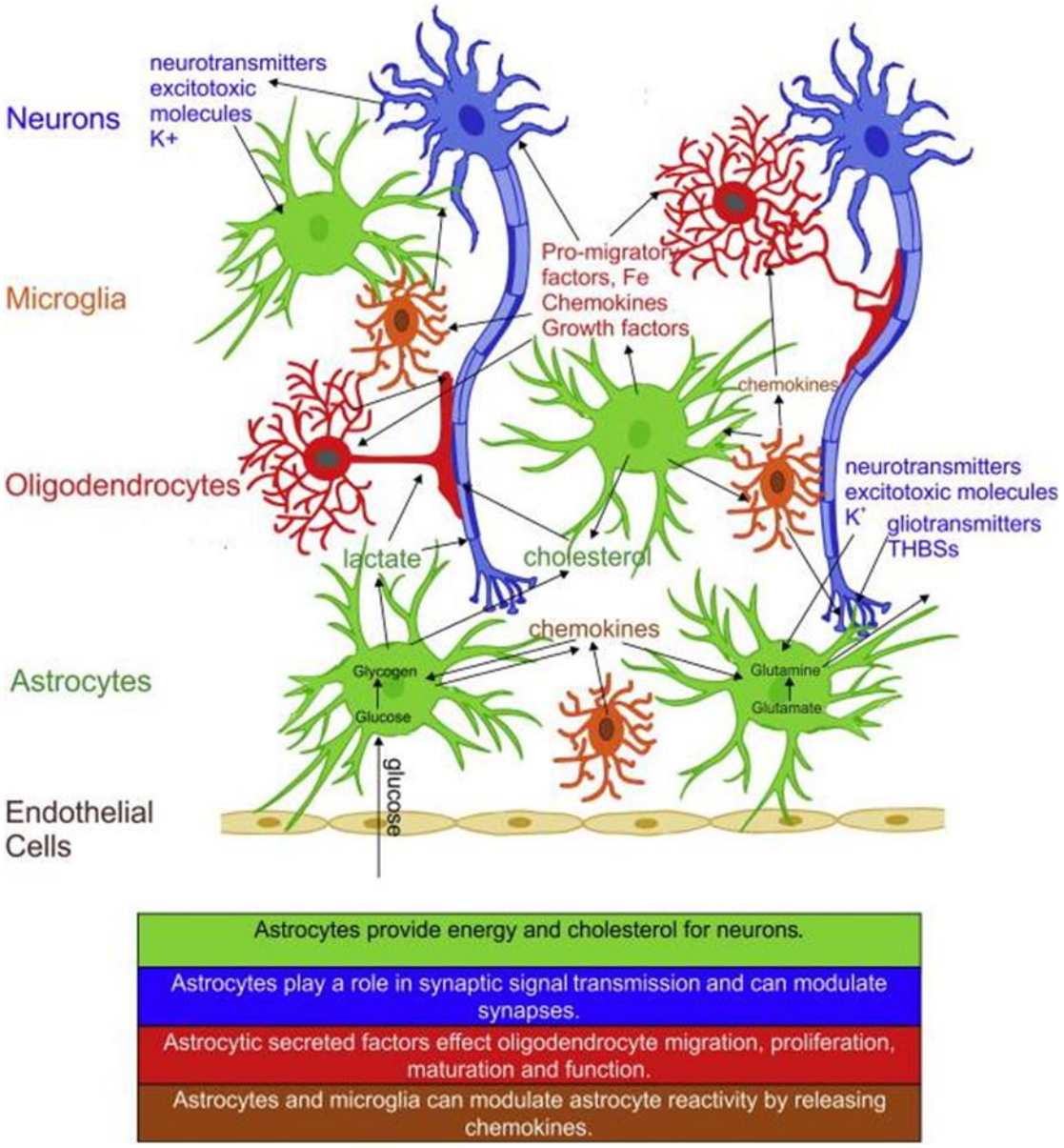


Figure 1.5: Effect of astrocytes in myelination (Kıray et al. 2016).

1.6 Methyl CpG binding Protein 2 (MeCP2)

DNA methylation in vertebrates is a covalent modification of DNA characterized by DNA methyltransferase enzymes (DNMTs) (Bogdanović and Veenstra, 2009). Methylation of DNA occurs favourably on dinucleotides of cytosine residues followed by guanine residues – also known as CpGs islands predominantly located in regulatory regions. These islands are found in approximately 60% of all mammalian genes and mostly occur in an unmethylated state (Díaz de León Guerrero et al., 2011).

A nuclear protein that specifically binds to methylated DNA was first defined in 1989 and was named MeCP (Methyl CpG binding Protein) (Meehan et al., 1989). MeCP2 was later distinguished as two different proteins- MeCP1 and MeCP2 with the latter being 100 times more abundant in adult somatic nuclei than MeCP1 (R. MEEHAN et al., 1992). MeCP2 normally binds to methylated DNA with respect to chromatin thus leading to long term transcriptional repression. Thus the first key in the study of MeCP2 was methylated DNA binding preference and it also earned the isolation of the first functional domain- the methyl CpG binding domain (MBD) (Nan et al., 1993). The second domain to be identified and characterised was the transcription repression domain (TRD) principally responsible for binding to the corepressor mSin3A and further recruit histone deacetylases (Jones et al., 1998) thus supporting the post-transitional modifications functions of MeCP2 by causing changes in chromatin structure. At present a combinatorial refined model of MeCP2 working is understood wherein MeCP2 functions as a methyl-DNA gene silencer that recruits corepressors and histone deacetylases (R. MEEHAN et al., 1992).

1.6.1 MeCP2 and Rett Syndrome

Rett syndrome (RTT) (OMIM 312750), first identified and named after Dr. Andreas Rett (Rett, 1966), is a postnatal progressive neurodevelopmental disorder that expresses in girls during early childhood characterized by normal development for the first 6-18 months of age followed by deceleration of head growth, microcephaly, general growth retardation, mental retardation, and autism and respiratory dsyfunctions. MeCP2 mutations were identified with Rett syndrome (Amir et al., 1999) and these encouraged new studies to determine how this protein functions on a global genomic scale. The location on the X chromosome supported the increased female occurrence of RTT (Quaderi et al., 1994) because females heterozygous for the mutant MeCP2 allel are able to survive due to X chromosome inactivation as compared to males who are hemizygous for MeCP2 mutations and have a drastic short lifespan of around 2 years (Ravn et al., 2003; Villard et al., 2000). Further studies have identifies 218 mutations linked to RTT (Miltenberger-Miltenyi and Laccone, 2003); almost in all domains indicating equal significance of each structural domain of MeCP2. Eight of these MeCP2 mutations have been commonly found in

RTT patients and occur in MBD and TRD domains. A much recent study also links MeCP2 to mRNA splicing which is concurrent with the aberrant alternative splicing patterns observed in a mouse model of RTT (Young et al., 2005). Studies demonstrated that MeCP2 could directly compact chromatin without DNA methylation or other proteins such as mSin3A (Georgel et al., 2003). This paved a new way of understanding MeCP2 as a complex multifunctional nuclear protein with a central role in regulating global chromatin architecture.

1.6.2 MeCP2 Structure

MeCP2 exists in two isoforms (MeCP2_E1 and MeCP2_E2) formed by alternative splicing both of which differ only in 21 amino acids at the N terminus. MeCP2_E1 is expressed 10 times more in brain compared to MeCP2_E2 (Mnatzakanian et al., 2004) especially in primary neurons as compared to primary astrocytes (Zachariah and Rastegar, 2012) while MeCP2_E2 is essential for embryo viability and placenta development. MeCP2_E1 is sufficient to cause Rett syndrome (Itoh et al., 2012). MeCP2 protein has five structural domains namely- N-terminal domain (NTD), methyl binding domain (MBD), the intervening domain (ID), the transcription repression domain (TRD) and the C-terminal domain (CTD); among which the MBD domain is linked with most of the disease causing mutations while TRD domain is required for the association with co-repressor proteins for transcription repression (Bedogni et al., 2014; Hansen et al., 2010).

MeCP2 mechanisms are mediated by several post-translational modifications (Bedogni et al., 2014). Among the many post-transcriptional modifications, MeCP2 widely undergoes phosphorylation, majorly at three sites serine 80 (S80), serine 421 (S421) and threonine 308 (T308). S80 is phosphorylated in neurons under resting conditions and bound to the heterochromatin genome; neuronal activity causes dephosphorylation and disrupts its chromatin association and causes activation of genes which were previously repressed (Tao et al., 2009). Similarly, phosphorylation at T308 site inhibits the interaction with NCoP co-repressor complex thus supressing the MeCP2 mediated transcriptional repression (Ebert et al., 2013; Lyst et al., 2013). On the other hand, MeCP2 S421 phosphorylation functions as a histone like factor and regulates genome wide response of chromatin to neuronal activity (Cohen et al.,

2011). Neuronal activity causes phosphorylation of MeCP2 at serine 421 (S421) which induces transcription of BDNF (Zhou et al., 2006). MeCP2 also binds to other macromolecules and confers multiple protein-protein interactions; which includes co-repressors like mSin3A (Nan et al. 1998), transcription factor YY1 (Forlani et al., 2010), NcoR (nuclear receptor co-repressor), Dnmt1 (DNA methyl transferases) (Kokura et al., 2001), CoREST (corepressor of RE1 silencing transcription factor) (Ballas et al., 2005) and many more. This multi-protein associations and post-translational modifications confers MeCP2 its multifunctional property.

1.6.3 MeCP2 and Neurons

MeCP2 is primarily expressed in the brain and especially neuronal cells; although studies have reported its expression in other cell types. It is crucial in the neuronal maintenance and maturation, synapse formation and dendritic arborisation. This is evident from the fact that MeCP2 expression correlates with neuronal maturation (Kishi and Macklis, 2004; Shahbazian et al., 2002; Shahbazian, Young, et al., 2002), regulating nuclear size and RNA synthesis in neurons during maturation (Yazdani et al., 2012) and RTT patient's reduced brain size because of smaller and immature neurons (Miyake and Nagai, 2007; Nagai et al., 2005). RTT affected brain also shows the presence of reduced spine density, smaller somas and decreased dendritic arborisation (Chapleau et al., 2009; Zhou et al., 2006). Loss of MeCP2 in neurons results in change in chromatin organization mediated by increase in histone acetylation and doubling of histone H1 (Skene et al., 2010). While mutation and deletion of MeCP2 is harmful as evident by RTT patients, MeCP2 duplication syndrome is an equally detrimental in effect. MeCP2 duplication or over expression causes MeCP2 duplication syndrome which is more evident in males than females. Long term effect of MeCP2 duplication is clinically more severe and different from RTT symptoms (Ramocki et al., 2010). Both MBD and TRD are responsible for toxicity in MeCP2 duplication syndrome (Heckman et al., 2014).

Gene silencing through recruitment of co-repressor like HDAC and mSin3, is the conventional function of MeCP2 (Nan et al., 1998). BDNF is one the most widely studied MeCP2 target gene shown to be involved in neuronal survival, differentiation and synaptic plasticity. MeCP2 represses the expression of BDNF but neuronal

membrane depolarization phosphorylates MeCP2, causes it to dissociate from BDNF promoter and de-represses it (Chen et al., 2003; Martinowich et al., 2003; Zhou et al., 2006). BDNF protein levels also decrease in MeCP2 knockout brains (Chang et al., 2006; Li et al., 2012) and corresponding increase in MeCP2 overexpressing brains thus signifying the importance of BDNF on RTT disease progression. While role of MeCP2 as a transcriptional repressor is very well documented, some reports also suggest the transcriptional activator role in certain genes of the hypothalamus and cerebellum (Chahrour et al., 2008). MeCP2 is associated with transcriptional activator CREB1 (cAMP responsive element binding protein 1) at the promoter region of activated targets but not at repressed targets (Ben-Shachar et al., 2009; Chahrour et al., 2008).

1.7 Glia and MeCP2

Studies till now have reported presence of MeCP2 only in neurons while it was shown to be absent in glial cells (Jung et al., 2003; Kishi and Macklis, 2004; Shahbazian et al., 2002 (a); Shahbazian et al., 2002 (b)). However recently several reports from other laboratories and our own observations have shown the expression of MeCP2 in all glial cells- astrocytes, oligodendrocytes and microglia (Ballas et al., 2009; Maezawa and Jin, 2010; Sharma et al., 2015; Tochiki et al., 2012; Vora et al., 2010). Loss of glial MeCP2 also has RTT syndrome pathophysiology and thus the studies needs due consideration (Jin et al., 2017).

1.7.1 Astrocytes and MeCP2

A group of studies have shown a non-cell autonomous effect of astrocytic MeCP2 on neuronal cells and the subsequent contribution in RTT pathogenesis. Astrocytes from RTT mouse model fail to support normal neuronal growth and result in dendritic aberrations. Mutant MeCP2 astrocytes cause abnormal dendritic arborisation of wild type neurons in co-culture (Williams et al., 2014) while condition media from MeCP2 null astrocytes also leads to neuronal damage (Ballas et al., 2009) thus indicating towards a crucial role of astrocytic MeCP2 in RTT pathogenesis. Mutant astrocytes

differentiated from induced pluripotent stem cell (iPSC) lines from human RTT patients also affect the morphology of wild type neurons possibly by secreted factors (Williams et al., 2014). MeCP2 also regulates astrocytic immune response by altering the expression of pro-inflammatory cytokines (TNF –alpha, IL-1β, and IL-6) and p38 MAPK kinase (Maezawa et al., 2009). Interestingly MeCP2 expression in wild type astrocytes was found to be reduced when cultured with MeCP2 deficient astrocytes, and this non-cell autonomous effect was mediated by gap junctions. These interpretations suggest that MeCP2 deficiency spreads across cells in a non-cell autonomous transfer possibly via negative regulators such as Ca2+, inositol trisphosphate, glutamate, or small regulatory miRNAs (Klein et al., 2007; Maezawa et al., 2009).

MeCP2 deficient astrocytes show altered microtubule assembly and dynamics due to down expression of stathmin-like 2 (STMN2) protein which in astrocytes promotes microtubule disassembly. Disrupted microtubule dynamics in MeCP2 mutant astrocytes is believed to be the reason behind faulty dendritic outgrowth in RTT brain (Nectoux et al., 2012; Slezak and Pfrieger, 2003). Altered microtubules dynamics and vesicular transport was also reported from astrocytes derived from iPSC from a RTT patient with MECP2p.Arg294*mutation which were restored on treatment with EpothiloneD (EpoD) which is a microtubule stabilizing agent (Delépine et al., 2015).

An exhaustive gene expression microarray and corresponding MeCP2 ChIP-sequencing in astrocytes led to a unique set of genes in astrocytes which are responsive to MeCP2. These genes are involved in a number of astrocytic signalling functions, neuronal support and other cellular functions; loss of which may contribute to Rett syndrome phenotype. MeCP2 deficiency in astrocytes affects the expression of a variety of genes ranging from number of functions from cell adhesion molecules, glutamate regulatory molecules, neuronal related proteins and immune modulatory proteins. MeCP2 also regulates Cntn1 (Contactin 1), Syn2 (Synapsin 2), Gabrg1, and Gria1 which are present at the synaptic junction where astrocytes make functional crosstalk with neurons in a structural unit called a tripartite synapse; thus influencing the ability of astrocytes to sense neuronal activity and modulate synaptic transmission. Some other genes such as Apoc2 (apolipoprotein C-II), Cdon (cell adhesion molecule-related/down regulated by oncogenesis) have elevated expression in MeCP2 deficient mice while Nrep (neuronal regeneration-related protein) has reduced expression

(Yasui et al., 2013). Several other glial genes such as GFAP, glial excitatory amino acid transporter 1 (EAAT1) and α-crystallin were upregulated in RTT brain (Colantuoni et al., 2001). Certain other genes encoding secreted protein, CHGB (Chromogranin B) and LCN2 (Lipocalin 2) were dysregulated in MeCP2 negative astrocytes. LCN2 is involved in the regulation of neuronal excitability and spine morphology (Ferreira et al., 2013), while Chromogranin B is found in secretory granule cargoes involved in BDNF secretion (Sadakata et al., 2004). Astrocytic mGluR3 and Sphingosine 1-phosphate receptor 1 (S1pr1/S1P1), both known to mediate calcium signals in astrocytes were found to be down-regulated in MeCP2 deficient brains, alongwith disruption in proteomic or transcriptomic levels of PTEN and the glutamate aspartate transporter (GLAST) (Pacheco et al., 2017). MeCP2 is also involved in glutamate clearance through regulation of glutamate transporters and glutamine synthetase (Okabe et al., 2012). These aberrant changes and interactions could be a contributing factor to the astrocytic defects in RTT (Nguyen et al., 2012).

MeCP2 deficiency in medullary astrocytes was studied to be associated with abnormal production of lactate and reduced ability to sense changes in pCO2/[H+] (Turovsky et al., 2015). It also depresses hypercapnic ventilator response in mice further indicating towards role of astrocytic MeCP2 in respiratory abnormalities observed in Rett syndrome (Garg et al., 2015). In human embryonal carcinoma cell line, NTera-2 were induced to differentiate into astrocytes wherein MeCP2 in association with Sin3A inhibits the differentiation of NTera-2 into astrocytes by inhibiting GFAP expression. Upon differentiation, the promoter undergoes a conformational change triggered by STAT3 binding, which causes release of Sin3A/MeCP2 complex and GFAP gene activation. Also, MeCP2 deficiency in RTT-hiPSC line (human induced pluripotent cells) derived neural stem cells resulted in an increase in the number of differentiated astrocytes (Andoh-Noda et al., 2015).

Interestingly, MeCP2 re-expression in astrocytes restores the RTT phenotypes like locomotion, anxiety levels, prolonged lifespan and respiratory abnormalities to a normal pattern thus indicating that glia are an integral part of RTT neuropathology (Lioy et al., 2011).

1.7.2 Oligodendrocytes and MeCP2

Oligodendrocytes significantly contribute towards Rett syndrome pathology as evident by a number of recent studies. Mice oligodendrocytes lacking MeCP2 show severe hind limb clasping phenotype which is significantly restored when MeCP2 was specifically restored in oligodendrocytes. Loss of oligodendroglial MeCP2 also alters some of the myelin proteins. MBP expression was reduced while PLP protein level was increased in Mecp2Stop/y mice brain. MBP level was partially restored upon re-expression of MeCP2 in oligodendrocytes while level of PLP remain unchanged, suggesting that in addition to role of oligodendrocyte MeCP2, there is possibility of non-cell autonomous effect of other cells on expression of myelin proteins (Nguyen et al., 2013). MBP and MAG have also been found to be increased in the corpus callosum of Mecp2 mouse brain (Vora et al., 2010). Our laboratory has also carried out studies that show that MeCP2 knock down in cultured rat oligodendrocytes leads to up-regulation of several myelin genes expression including MBP, PLP, MOG, MOBP, BDNF and transcriptional regulator YY1 thus suggesting the negative regulation of myelin gene expression by MeCP2 (Sharma et al., 2015). Similarly, MOBP was found to be up regulated in Mecp2- null mice by direct binding of MeCP2 through its promoter (Urdinguio et al., 2008).

These studies are supported by brain magnetic resonance study in Mecp2-/y mice which recorded a significant reduction in the thickness of the corpus callosum (Saywell et al., 2006) while diffusion tensor imaging (DTI) in Rett syndrome patients found significant reduction in fractional anisotropy (FA) in corpus callosum (Mahmood et al., 2010), which suggests white matter impairment in Rett syndrome patients. Some of the neurological signs in RTT patients overlap with X- linked adrenoleukodystrophy and supports the white matter damage in RTT (Durand et al., 2012).

1.7.3 Microglia and MeCP2

Mecp2 null microglia conditioned media shows presence of excess glutamate which affects the neurotoxic activity and wild type neurons when treated with this condition media shows abnormal dendritic morphology, microtubule disruption and damage of

postsynaptic glutamatergic components. The increased levels of glutaminase and connexin 32 in Mecp2-null microglia are responsible for increased glutamate production and release, respectively (Maezawa and Jin, 2010). MeCP2 functions as a repressor of major glutamine transporter SNAT1 in microglia, down-regulation of which leads to mitochondrial dysfunction and neurotoxicity (Jin et al., 2015). Population of monocytes and peripheral macrophage were found to be depleted in MeCP2 null mice. MeCP2 also regulates glucocorticoid and hypoxia-induced transcripts in Mecp2 deficient microglia and macrophages. Furthermore, MeCP2 modulate the inflammatory genes transcript in response to TNF stimulation to microglia (Cronk et al., 2015; Derecki et al., 2012). Restoration of MeCP2 in microglia mitigates the symptoms of RTT in mice; increased lifespan, increase in body weight, normalized breathing patterns and improved locomotor activity. Mecp2+/− females also exhibited significant improvements as a result of wild type microglial engraftment. Postnatal re-expression of Mecp2 using Cx3cr1creER extends the lifespan of Mecp2 null mice (Derecki et al., 2012).

1.8 Involvement of Astrocytes in disease

Defects in the myelin sheath cause neurological disorders causing demyelination, dysmyelination or degeneration in general. These defects are caused by damaged or dysfunctional brain cells. Some of the major myelin disorders are discussed below.

1.8.1 Multiple Sclerosis (MS)

MS is an inflammatory disease causing demyelination and axonal injury. Although its etiology remains elusive, the most accepted theory is that it is an autoimmune disorder which leads to inflammation, demyelination and finally oligodendrocyte death (McFarland and Martin, 2007). Most patients initially represent a relapsing clinical course and the pattern becomes progressive after 10-15 years during which clinical symptoms cause gradual and constant deterioration over time (Lublin and Reingold, 1996). The inability to repair the areas where myelin has eroded, results in astrocytic scar formation, further myelin degeneration and eventual axonal loss (Adams et al.,

1989; Melanson et al., 2009; Prineas and Raine, 1976; Ransohoff, 1999). The immunopathological events can be divided into: (i) an initial T cell priming, (ii) activation phase in the periphery (i.e., thymus, lymph nodes), (iii) migration of the pro-inflammatory T cells and monocytes across the BBB, (iv) amplification of local inflammation and activation of resident antigen presenting cells (APCs), such as microglia, and finally (v) effector phase of the disease: attack on CNS parenchyma causing damage of oligodendrocytes, myelin sheath and axons. The consequential myelin damage disrupt the normal propagation of electrical impulses through the axon which causes a number of symptoms like weakness in limbs, pain insensitivity, ataxia and gradually progress to more severe physiological functions such as fatigue, memory loss, slower information processing, cognitive defects, impaired attention (Melanson et al., 2010; Roxburgh et al., 2005).

Normal and reactive astrocytes are not only prominent features of multiple sclerosis, but they also have central roles towards the key pathogenic disease mechanisms. Most commonly, plaques of demyelination are intermingled and surrounded by reactive astrocytes of varying intensities throughout white matter and in some regions of grey matter (Kuhlmann et al., 2008; Love, 2006). Astrocytes also exhibit unusual nuclear and cytological features such as multiple nuclei also referred to as Creutzfeldt astrocytes which may be fragmented (Kuhlmann et al., 2008; Nishie et al., 2004). They may also exhibit the phenomenon of emperipolesis i.e an astrocyte engulfing oligodendrocytes (Ghatak, 1992) or lymphocytes. Both these features, multinucleation and emperipolesis, also occurs in certain tumors and in spongiform encephalopathy (Colodner et al., 2005; Ghatak, 1992). Astrocytes also produce a number of pro-inflammatory molecules (Dong and Benveniste, 2001; Sofroniew 2009) and exert suppressive effects on inflammatory cells (Kostianovsky et al., 2008). Reactive astrocytes participate in attracting inflammatory cells to specific sites and also in restricting spreading of inflammatory cells to adjacent healthy CNS parenchyma (Sofroniew, 2009). This suggests that loss of normal functions in astrocytes or gain of abnormal functions in reactive astrocytes contributes to disease mechanisms in multiple sclerosis and other autoimmune inflammatory condition. In another CNS inflammatory/demyelinating disease, neuromyelitisoptica, which is also considered as a variant of multiple sclerosis have autoantibodies to aquaporin-4 which is expressed only on astrocyte foot processes (Lennon et al., 2005; Takahashi et al., 2007). The

potential involvement of astrocytes in other autoimmune inflammatory conditions, such as systemic lupus erythematosus, has been suggested but not been extensively investigated (Tomita et al., 2004).

1.8.2 Leukodystrophies

Leukodystrophies are group of genetic disorders characterized by demyelination/dysmyelination in the CNS and PNS caused by an inherited biochemical defect in the metabolism of myelin-related proteins or lipids. Leukodystrophies fall into three categories: (1) hypomyelination, in which there is absent or diminished myelin production; (2) dysmyelination, in which there is abnormal myelin development; and (3) demyelination, in which there is loss and/or destruction of previously established myelin. However, Rett syndrome and Batten disease can have white matter changes and clinical features that would initially suggest a leukodystrophy before subsequent testing leads to their ultimate diagnosis thus falling in none of the above mentioned categories (Gordon et al., 2014).

- Alexander disease is a leukodystrophy characterised by the genetic disorder of astrocytes cause by gain-of-function mutation of the gene encoding GFAP (Brenner et al., 2001) wherein astrocytes exhibit numerous Rosenthal fibres that are aggregates of GFAP which are immunoreactive. Patients exhibit macrocephaly, seizures, psychomotor disturbances and premature death and overexpression of GFAP causes a fatal encephalopathy (Messing et al., 1998).
- Vanishing white matter disease (VWM) is an uncommon autosomal recessive leukoencephalopathy caused by mutations in translation initiation factor 2B (eIF2B). Mutations in the eIF2B genes impair the ability of cells to regulate protein synthesis. VWM lesions in vivo lack GFAP expressing astrocytes possibly contributing to loss of white matter (Dietrich et al., 2005; van Kollenburg et al., 2006).
- Megaloencephalicleukoencephalopathy with subcortical cysts (MLC): In CNS, MLC1 protein is specifically expressed in astroglial processes in perivascular, subependymal and subpial regions with possible functions of transport across blood-brain and brain-cerebrospinal fluid barriers. MLC is an autosomal recessive,

progressive white matter disease in children characterized by myelin splitting and intramyelin vacuole formation (Boor et al., 2005; Schmitt et al., 2003).

1.8.3 Alzheimer's disease (AD)

A well-known feature of Alzheimer's disease is reactive astrogliosis which tends to be focal and intimately associated with amyloid plaques and surround them with dense layers of processes appearing like miniature scares around them and acting as neuroprotective barriers. Reactive astrocytes contain significant amounts of different forms of amyloid beta, including amyloid beta 1-42 (Ab42) (Nagele et al., 2004; Thal et al., 2000) and they can also take up and degrade extracellular deposits of Ab42 (Wyss-Coray et al., 2003) suggesting that functions and dysfunctions of reactive astrocytes could play a role in the progression and severity of AD.

1.8.4 Amyotrophic lateral sclerosis (ALS)

ALS is characterized by loss of or dysfunction of astrocyte glutamate transporters in spinal cord and cortical areas that exhibit loss of lower and upper motor neurons (Fray et al., 1998; Maragakis and Rothstein, 2006; Rattray and Bendotti, 2006; Rothstein et al., 1992) indicating that elevated levels of glutamate excitotoxicity contributes to motor neuron death. Transplantation of astrocytes may be a potential therapeutic strategy as indicated by focal grafts of healthy astrocytes (Lepore et al., 2008). ALS may also cause missense mutations of the gene encoding superoxide dismutase (SOD); which when occurs in astrocytes leads to production of soluble molecules by astrocytes that are selectively toxic to motor neurons but not to spinal cord neurons (Di Giorgio et al., 2007; Nagai et al., 2007).

1.9 Rationale of the Present Study:

Astrocytes are unarguably the most abundant cells in the central nervous system. While various functions of astrocytes including regulation of BBB, immune modulation, glutamate uptake have been well studied and documented, few studies have been carried out with respect to their role in myelination. There are evidences to suggest that astrocytes contribute to myelination by secreting some of the major growth factors, ECM molecules and neurotrophins, by providing the right conditions for neurons to myelinate and by regulating the oligodendrocyte physiology. The role of astrocytes in myelination is also evident by the failure to reproduce the multifaceted cellular environment of myelination in absence of astrocytes. Presence of perinodal astrocytes at nodes of Ranvier and at the tripartite synapses favours their involvement in axo-glial interactions. Astrocytes are also found in close association with neurons and oligodendrocytes, both of which are key participants in the myelination event.

MeCP2 is a global transcription regulator, mutation of which causes neurodevelopmental disorder, Rett syndrome (Amir et al., 1999). Although wide number of studies till date have majorly emphasized on the involvement of MeCP2 in neurons, their functions in the glial populations is gradually gaining interest. Deficiency of MeCP2 in astrocytes has been linked to various dysfunctions like reduced dendritic outgrowth (Ballas et al., 2009), dysregulation of genes of tripartite synapse (Yasui et al., 2013), while re-expression of MeCP2 in astrocytes significantly improves many of the disease pathologies of RTT (Kifayathullah et al., 2010; Lioy et al., 2011; Zachariah et al., 2012).

However, nothing much is reported in regards to involvement of MeCP2 in astrocytic regulation of CNS myelination.

1.10 Hypothesis

In view of the above reports, we hypothesized that astrocytic MeCP2 have a pivotal role in the regulation of axo-glial interactions and myelination events. In addition, MeCP2 is hypothesized to modulate certain essential astrocytic secreted factors which in turn are involved in regulating oligodendrocyte and neuronal physiology.

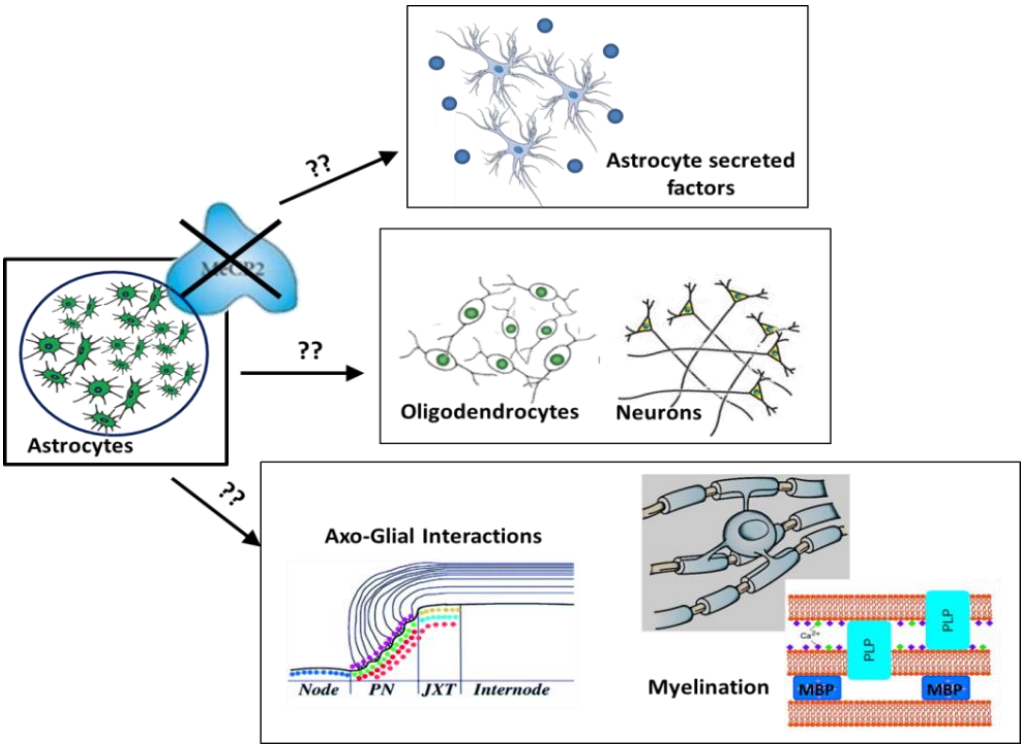


Figure 1.6: Hypothesis of study.

1.11 Objectives

Based on the above hypothesis, following were the objectives of the present study:

Objective 1: To determine the role of MeCP2 on astrocyte secreted factors regulating myelination.

Objective 2: To determine the effect of MeCP2 deficient astrocytes on oligodendrocytes and neuronal survival, proliferation and differentiation.

Objective 3: To study the effect of astrocytic MeCP2 on axo-glial interactions and myelination.