CHAPTER 5

TO STUDY THE EFFECT OF ASTROCYTIC MeCP2 ON AXO-GLIAL INTERACTIONS AND EVENTS OF MYELINATION

5.1 Introduction

Neurons and myelinating glia form intimate connections with each other and interact bi-directionally throughout life (Klaus-Armin Nave, 2010). While neuron-derived signals regulate the differentiation and proliferation of oligodendrocytes (Simons and Trajkovic, 2006); signals from oligodendrocytes define the molecular domains in neurons (Nave and Trapp 2008). In addition, the neuronal signals also regulate the thickness of the myelin sheath, myelin wrapping machinery of oligodendrocytes and myelin integrity (Bozzali and Wrabetz, 2004; Nave and Trapp 2008; Taveggia et al., 2010). Axo-glial communication also has major implications in regulating the pathogenesis of many neurological disorders (Piaton 2010). Thus, myelin biogenesis as well as myelin repair, both require reciprocal communication between neurons and oligodendrocytes (Ahrendsen and Macklin, 2013; Shimizu et al., 2018). The ability of myelinated axons to conduct saltatory conductions depends on the distribution of numerous molecular components into discrete domains- the internode, juxtaparanode, paranode and the node of Ranvier. These specific domains are also formed as the result of specific interactions between axons and oligodendrocytes (Devaux and Faivre-Sarrailh, 2013).

A distinct class of astrocytes, called perinodal astrocytes, approaches the node through the nodal gap and becomes associated with the initial axo-glial interactions (Perez-Alvarez and Araque, 2013) where they secrete the cytotactin, an extracellular matrix protein which functions in the electrical coupling of extracellular ions (Serwanski et al., 2017) and buffering the perinodal space (Gutnick et al., 1981; Masa and Mugnaini, 1982). This association of perinodal astrocytes and nodal membrane is similar to that by perinodal Schwann cell processes which suggests similar functional properties for both. Thus, similar to PNS assembly, presence of perinodal astrocytes may also be indirectly related to the assembly and/or maintenance of nodal membrane in CNS. Similarly, cortical astrocytes have also been shown to occupy non-overlapping domains, enwrap neuronal cell bodies, hundreds of dendrites and participates in synaptic transmission (Halassa et al., 2007, Bolton and Eroglu, 2009).

Astrocytes are also important in assisting CNS myelination by secreting factors that promote or inhibit myelination (Barnett and Linington, 2013). They are also a source of growth factors, neurotrophins and many trophic factors which influence the

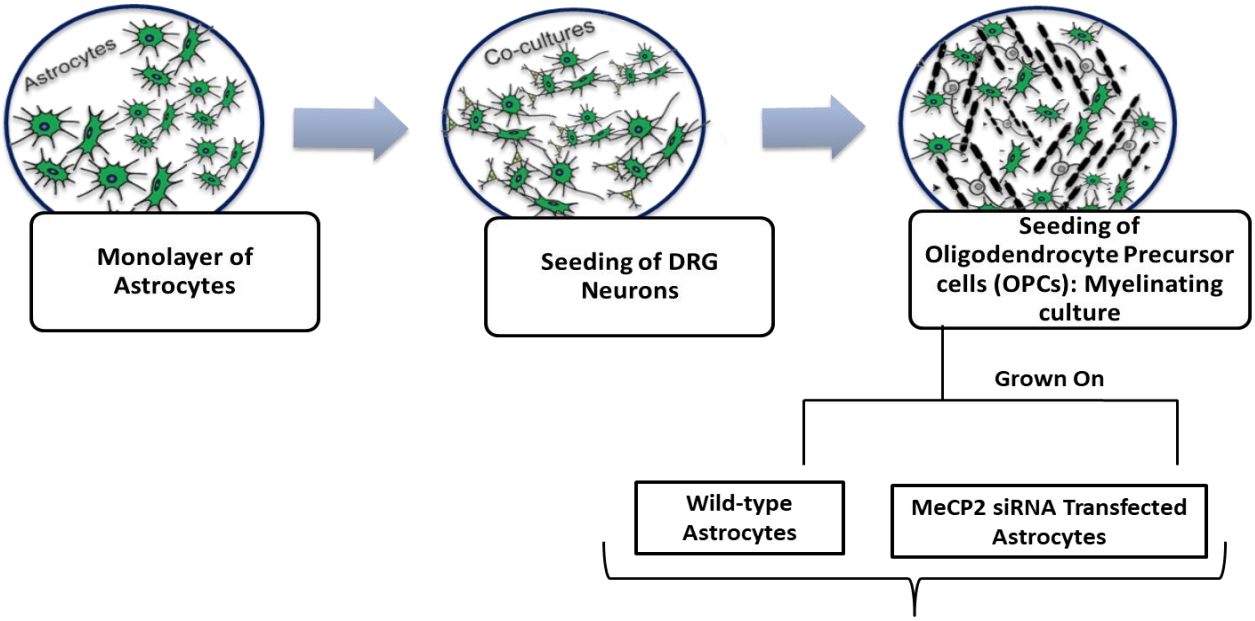
myelination event. The presence of astrocytes in myelinating cultures increases the speed of myelin wrapping around axons (Watkins et al., 2008). The significant role of astrocytes in myelination can be understood by the failure to mimic the complex myelination environment in their absence (Talbott et al., 2005).

MeCP2, a genome-wide transcriptional modulator is reported to be the key molecule involved in the neurodevelopmental disease, Rett syndrome (Amir et al., 1999). Until now, MeCP2 was studied specifically in neurons, however many recent reports have confirmed its presence in glial cells as well, where it performs a wide variety of functions. RTT brains are typically reduced in size due to smaller and immature neurons (Kishi and Macklis, 2004) and have reduced white matter (Khong et al., 2002) which may be attributed to loss of myelin associated lipids along with dysmyelination in in RTT brains (Lekman and Hagberg, 1991). Moreover, syndromic and idiopathic autism spectrum disorders commonly exhibit defects in myelination (Phan et al., 2017). Also as explained in earlier chapters, MeCP2 is a major regulator of BDNF, depletion of which aggravates RTT-like symptoms in the brain (Chang et al., 2006). MeCP2 is reported to interact with some master transcriptional regulators such as REST and coREST (Ballas et al., 2005) which have diverse functions in modulating several signalling pathways in cell-cell interactions (Abrajano et al., 2009). Recently, findings from our laboratory have indicated the significance of oligodendroglial MeCP2 in regulating myelin genes and transcriptional regulator YY1 (Sharma et al., 2015). Similarly, presence of MeCP2 is also reported in astrocytes which contribute to RTT neuropathology (Ballas et al., 2009; Lioy et al., 2011) and the alteration of astrocytic processes also influence the neuronal networks in the RTT brain (Nguyen et al., 2012; Cuddapah et al., 2015). A recent transcriptomic study in MeCP2-deficient mice revealed dysregulation in several astrocytic and oligodendroglial genes along with disruption of pathways related to axo-glial interactions, myelination and demyelination (Pacheco et al., 2017).

These studies led to hypothesize the possible role of astrocytic MeCP2 in regulating certain axo-glial interaction molecules and myelination. Present study was undertaken to understand the involvement of oligodendroglial, neuronal and astrocytic MeCP2 in modulating selected axo-glial interaction molecules present on membranes of the cells participating directly in myelination- both oligodendrocytes and neurons. Further, effect of astrocytic MeCP2 was also studied in regulating myelin genes and proteins.

A triple culture of astrocytes-DRGN-oligodendrocytes was established (section 2.3) and characterized on which the above mentioned hypothesis was tested.

5.2 Strategy of work



5.3 Results

5.3.1 Regulation of axo-glial interaction genes by MeCP2 in oligodendrocytes

MeCP2 expression was suppressed in oligodendrocytes using rat-specific siRNA. Oligodendrocytes transfected with MeCP2 siRNA exhibited 30-35% reduction in transcript levels compared to cells treated with negative control siRNA. NF155 and Notch are axo-glial molecules present on the oligodendroglial cell membrane when an oligodendrocyte contacts an axon. Both NF155 and Notch are expressed during the initial phases of myelination (Tait et al., 2000, Hu et al., 2004). Significant down-regulation was observed in NF155 transcript levels in MeCP2 knockdown

oligodendrocytes compared to control while Notch was up-regulated but not significantly (Figure 5.1).

5.3.2 Regulation of axo-glial interaction genes by MeCP2 in DRGN

Similar to oligodendroglial membrane expressed molecules, role of neuronal MeCP2 was investigated in the regulation of genes expressed on the neuronal membrane. DRGN were grown *in vitro* for 6 days to achieve 80% confluency followed by transfection with rat-specific MeCP2 siRNA. Transfected cells exhibited 40-50% reduction in MeCP2 mRNA compared with the cells treated with negative control siRNA. Transcript levels of axo-glial molecules- Caspr and Nrg1 were analysed. Both of these proteins are expressed on the neuronal membrane during axo-glial interactions (Einheber et al., 1997; Menegoz et al., 1997; Michailov et al., 2004). mRNA levels of Caspr and Nrg1 were 3-fold up-regulated in MeCP2 knockdown DRGN compared to control (Figure 5.2).

5.3.3Establishment of Astrocyte-DRGN-Oligodendrocyte triple cultures

Cortical mixed glial cell cultures containing oligodendrocytes and astrocytes and DRGN cultures were prepared as per the protocols described in materials and methods section.

Initially, co-cultures of OPCs and DRGNs were established which were observed and monitored under inverted phase contrast microscope. In the initial days of co-culture (DIV 3), the two cells begin to interact with each other which is apparent by the increased length of neuronal processes extending towards immature oligodendrocytes which are still observed as clusters. Around DIV 5-6, the co-cultures are observed to gradually develop and mature oligodendrocytes now evidently start to make contact with DRGN, thus myelinating the latter (Figure 5.3 A, B). Co-cultures were immune-characterized using cell specific markers for mature oligodendrocytes (MBP) and DRGN (Neurofilament- NF) to affirm the initiation and establishment of myelination. Oligodendrocytes are observed to ensheath DRGN with a MBP positive membrane

and myelin segments, represented by MBP/ NF double-labelled lines, were found in the co-culture (Figure 5.3 C).

In order to demonstrate the role of astrocytes in myelination, triple cultures of astrocytes-DRGN-oligodendrocytes (ADO) were established (as described in section 2.3). Cortical astrocytes were transfected with specific MeCP2 siRNA duplexes or with a universal negative control siRNA. DRGN cultures were plated on this astrocytic monolayer followed by gently plating OPCs without disrupting the astrocyte-DRGN bed. These triple cultures (ADO) were employed to elucidate the involvement of astrocytic MeCP2 in regulating axo-glial interactions and also in myelination.

5.3.4 Effect of astrocytic MeCP2 on axo-glial interaction genes in ADO co-culture

In the preceding observations, the involvement of oligodendroglial and neuronal MeCP2 in regulation of axo-glial interaction molecules was established; the study was further extended to assess the effect of astrocytic MeCP2 on axo-glial interaction genes using triple cultures of ADO wherein transcript levels of NF155, Notch, Caspr and Nrg1 were analysed by real time PCR. While oligodendrocyte specific genes- NF155 and Notch did not show any change in response to astrocytic MeCP2; both neuronal specific axo-glial genes- Caspr and Nrg1 exhibited significant up-regulation by astrocytic MeCP2 compared to control (Figure 5.4).

5.3.5 Effect of astrocytic MeCP2 on myelin genes in ADO co-culture

Triple cultures of ADO were grown 6 days *in vitro*, on negative control astrocytes and MeCP2 knockdown astrocytes, after which transcript levels of myelin genes were analysed by real time PCR. Transcript levels of myelin genes- MBP, PLP, MAG and MOG were significantly decreased in MeCP2 knockdown astrocytes as compared to control (Figure 5.5). In contrast, previously our lab had demonstrated significant induction in myelin genes (MBP, PLP, MOG, and MOBP) mRNA expression in

MeCP2 deficient oligodendrocytes suggesting negative regulation of oligodendroglial MeCP2 (Sharma et al., 2015).

5.3.6 Effect of astrocytic MeCP2 on myelin proteins in ADO co-culture

Triple cultures were established using DRGN and oligodendrocytes on monolayers of negative control and MeCP2 knockdown astrocytes (section 2.3). Protein levels of myelin protein, MBP and PLP, were analysed by western blot in these ADO cultures at DIV 8 by which majority of oligodendrocytes have matured and started myelination. In agreement to transcript level expression of myelin genes, expression of myelin proteins were also observed to be down-regulated in MeCP2 knockdown astrocytes compared to negative control (Figure 5.6 A,B,C). Myelin was extracted from triple cultures by myelin extraction protocol (Thomson et al., 2008) (section 2.11). This corresponds to the total myelin in these cultures due to the presence of both neurons and oligodendrocytes as compared to a myelin-like fraction from a pure culture of oligodendrocyte with no neurons around which myelin sheaths can form. Decreased levels of total myelin were found in MeCP2 knockdown ADO cultures compared to control, although not very significantly (Figure 5.6 D).

5.4 Discussion

In the mammalian CNS, myelin sheath is composed of regularly spaces small unmyelinated segment through which the action potential passes. These gaps constitute the nodes of Ranvier which is an essential specialization of myelinated fibers in both CNS and PNS. In the CNS, nodes are present between the opposing terminal paranodal loops of oligodendrocytes. In the optic nerve and spinal cord, the exposed axon is enclosed by astrocytic processes to some extent (Hildebrand and Waxman, 1984; Black and Waxman, 1984; Raine, 1984). This indicates that the assembly as well as maintenance of nodal and internodal junctions involve complex interactions not only between neurons and oligodendrocytes but also between astrocytes and neurons as well as astrocytes and oligodendrocytes.

A number of cell-cell interaction molecules, secreted factors, adhesion molecules as well as ECM proteins are involved in the coordination of the formation of the myelin membrane (Laursen and Ffrench-constant, 2007). Some of these are PSA-NCAM (Charles et al., 2000; Coman et al., 2005), Netrins (Rajasekharan et al., 2009), Lingo-1 (Mi et al., 2005, Mi et al., 2007), Notch (Wang et al., 1998; Genoud et al., 2002), integrins and secreted proteins. While these have been widely studied on their role in axo-glial interactions, there are hardly any reports on their link with MeCP2 in regulating these specific associations. NF155 and Notch (molecules specific to oligodendroglia) and Caspr and Nrg1 (specific to neuronal cell membrane) were studied with respect to MeCP2.

NF155 is a glial specific isoform of the cell adhesion molecule Neurofascin which is expressed on the myelinating glial cell membrane and functions in the formation and organization of the paranodal domain while the other isoform is NF186 which is a key component of the node. Studies have shown that mice deficient for total neurofascin, lacking both NF155 and NF186 isoforms die around postnatal day 7 (P7) which is the active period of saltatory conduction initiation (Sherman et al., 2005). These knockouts however have normal levels of myelination (Zonta et al., 2008). Loss of NF155 results is axons that lack paranodes and fail to segregate nodal sodium channels from the adjacent juxtaparanodal voltage gated potassium channels. These animals display severe loss of axo-glial junctions and die around P17 (Pillai et al., 2009; Thaxton et al., 2011; Taylor et al., 2017). Dysfunctional organization of the nodal domains and cell-adhesion pathways has been observed in pathogenesis of autism spectrum disorders (Betancur et al., 2009). Moreover, FXYD1 (trans-membrane modulator of Na+/K+ ATPase activity) (Deng V et al., 2007), SCN2A (sodium channel type II) (Lunyak et al., 2002), E-cadherins (Darwanto et al., 2003) and protocadherin PCDH7 (Miyake et al., 2011) have been reported as MeCP2 targets which suggests the potential role of this epigenetic regulator in cell adhesion and synaptogenesis. MeCP2 has never been studied so far with respect to neurofascins, and the down-regulated levels of NF155 in response to MeCP2 guides towards a possible contribution of oligodendroglial MeCP2 in the organization of the nodal assembly. However, NF155 levels were not altered in response to astrocytic MeCP2 deficiency which suggests differential role of the epigenetic protein in different cell types, possibly by interacting with different co-repressors or activators.

Notch1 is a type-I transmembrane molecule which presents itself on the oligodendroglial cell surface as a heterodimer, while the axons express Jagged1, a ligand of Notch1 receptor. The timing of oligodendrocyte differentiation and myelination is controlled by the Notch signaling pathway (Park and Appel 2003). Overexpression of Notch1 in OPCs leads to a delayed maturation which is consistent with the negative role of Notch1 in the later stages of differentiation (Zhou et al., 2001; Zhang et al., 2009). Increased Notch signalling may also mediate myelin decompaction by downregulation of gap junction proteins that are involved in myelin compaction (Alejandro López-Juárez et al., 2017). On the other hand, mice heterozygous for null Notch1 allele reported increased myelination (Givogri et al., 2002). Interestingly, notch signalling is involved in the proliferation and differentiation of adult neuroprogenitor cells by influencing phosphorylation of MeCP2 S421 (Li et al., 2014) whereas Notch1 is repressed in RTT-iPSCs with distinct mutations in different MeCP2 domains (Tanaka et al., 2013). Notch 1 transcript levels were up-regulated in MeCP2 knockdown oligodendrocytes although not significantly; however the same was not observed in response to astrocytic MeCP2 again indicating differential role of MeCP2 in different cells.

Contactin associated protein (Caspr) was the first axoglial protein to be identified present on the axonal side at the paranodes (Einheber et al., 1997; Menegoz et al., 1997) which interacts with NF155 on the glial cell surface (Tait et al., 2000). These specific interactions are important in stabilization of the axoglial contact and the restriction of sodium channels to the nodal regions (Bhat et al., 2001; Pedraza et al., 2001). Caspr plays a decisive role in the cell fate decision by suppressing Notch signalling during cortical development (Wu et al., 2017). Caspr knock-out (shm mutants) show complete or partial absence of paranodal junctions thus impairing nerve impulse conduction (Sun et al., 2009). Mutations in CNTNAP1, gene encoding Caspr, cause severe hypomyelination and neuropathy (Hengel et al., 2017) while increased levels or overexpression of Caspr leads to decreased expression of APP (Amyloid precursor protein), a synaptic regulator and key molecule in Alzheimer's disorder (Fan et al., 2013). Novel functions of Caspr family have been identified as risk factors in autism spectrum disorders (ASDs) (Anderson et al., 2012; Karayannis et al., 2014). Up-regulated transcript levels of Caspr (3 fold) were observed in MeCP2 knockdown DRGN compared to control. Interestingly, Caspr levels were highly

elevated in MeCP2 deficient astrocytes in the triple cultures (7 fold). The up-regulated transcript levels of Caspr in both, DRGN and astrocytes knockdown MeCP2, can be suggested as a possible approach to repair damaged axo-glial interactions and myelination.

Neuregulin (NRG) family encompasses >15 membrane-associated as well as secreted proteins (Esper et al., 2006) generated by multiple transcription sites and extensive RNA splicing (Law et al., 2006). In PNS, type III neuregulin-1, expressed on axons, is necessary to govern myelination and myelin sheath thickness (Michailov et al., 2004, Taveggia et al., 2005) whereas in CNS, it only promotes myelination in selected areas (Taveggia et al., 2008). In CNS, Nrg1 interacts with specific Erb receptors present on oligodendrocytes (Sussman et al., 2005, Chen et al., 2006). Surprisingly, double mutants lacking both Nrg1 and ErbB receptors exhibit normal myelination, however transgenic over-expression of Nrg1 leads to hypermyelination (Brinkmann et al., 2008). Nrg1 transcript levels were up-regulated 3-fold in MeCP2 knockdown DRGN and similar results, 3-fold up-regulation, was observed in MeCP2 deficient astrocytes in the triple cultures. These findings suggest that increased Nrg1 expression in response to MeCP2 may rescue hypomyelination.

One of the crucial functions of astrocytes is their role in myelination. A number of reports suggest the indirect/direct involvement of astrocytes in CNS myelination (Talbott et al., 2005; Watkins et al., 2008). As discussed in earlier chapters of the present study, astrocytes also influence the key participating cells of myelination in CNS- neurons and oligodendrocytes thus indirectly affecting myelination. They are a viable source of growth factors and neurotrophins which impact the myelination milieu. A recent study from our laboratory has shown the negative regulation of oligodendroglial MeCP2 on myelin genes and proteins by directly binding to their promoter regions in oligodendrocytes (Sharma et al., 2015). However, in the present study, knockdown of MeCP2 in astrocytes down-regulates the expression of myelin genes (MBP, PLP, MAG, MOG) and myelin proteins (MBP and PLP). Myelin was extracted from triple cultures which correspond to the total myelin present in the cultures which was down-regulated in MeCP2 knockdown groups compared to control. Reduced expression of PLP and MBP protein levels have been studied to cause abnormal myelin sheath formation and compaction (Boison and Stoffel, 1989; Knapp et al., 1986, Fitzner et al., 2006), thus leading to the speculation that astrocytic

MeCP2 might also be involved in the further events of myelination such as myelin wrapping and compaction which remains to be tested.

5.5 Conclusion

The present study thus focused on the role of astrocytic MeCP2 in regulation of axo-glial interaction molecules- NF155, Notch, Caspr and Nrg1. Astrocytic MeCP2 was found to negatively regulate neuronal axo-glial molecules- Caspr and Nrg1. On the other hand, MeCP2 deficiency in astrocytes showed no alteration in oligodendroglial axo-glial molecules- NF155 and Notch. Further, MeCP2 deficiency in astrocytes positively regulates major myelin genes (MBP, PLP, MAG and MOG) and myelin proteins (MBP and PLP).

Figure 5.1

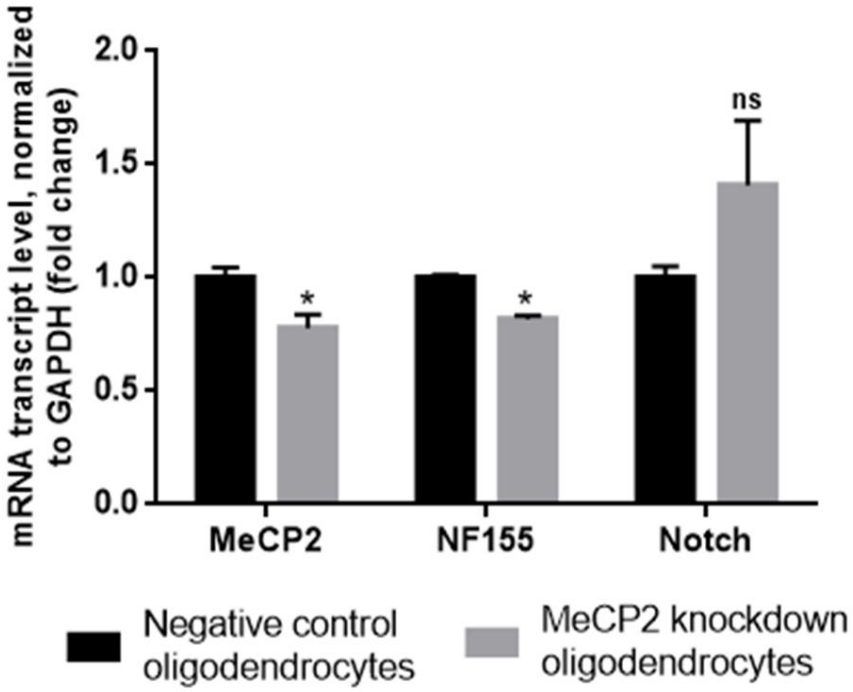


Figure 5.1: Effect of oligodendrocyte MeCP2 on axo-glial interaction genes: The data reflect the transcript levels of MeCP2, NF155 and Notch genes in oligodendrocytes, normalized to GAPDH and relative to control. Results are represented as mean ± SEM of 3-4 independent experiments and evaluated using Student's t-test (*P<0.05; **P<0.01; ***P<0.001).

Figure 5.2

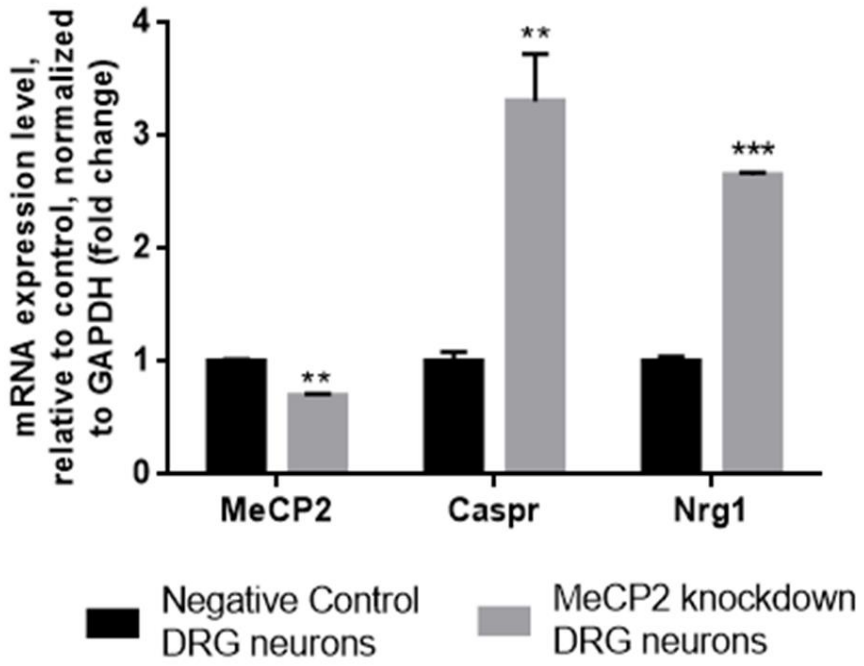


Figure 5.2: Effect of DRGN MeCP2 on axo-glial interaction genes: DRGN transfected with MeCP2 siRNA showed 30-40% reduction in MeCP2 transcript levels. The data reflect transcript levels of Caspr and Nrg1 in negative control neurons and MeCP2 knockdown neurons, normalized to GAPDH and relative to control. Results are represented as mean ± SEM of 3-4 independent experiments and evaluated using Student's t-test (*P<0.05; **P<0.01; ***P<0.001).

Figure 5.3 (A) Co-culture DIV 3

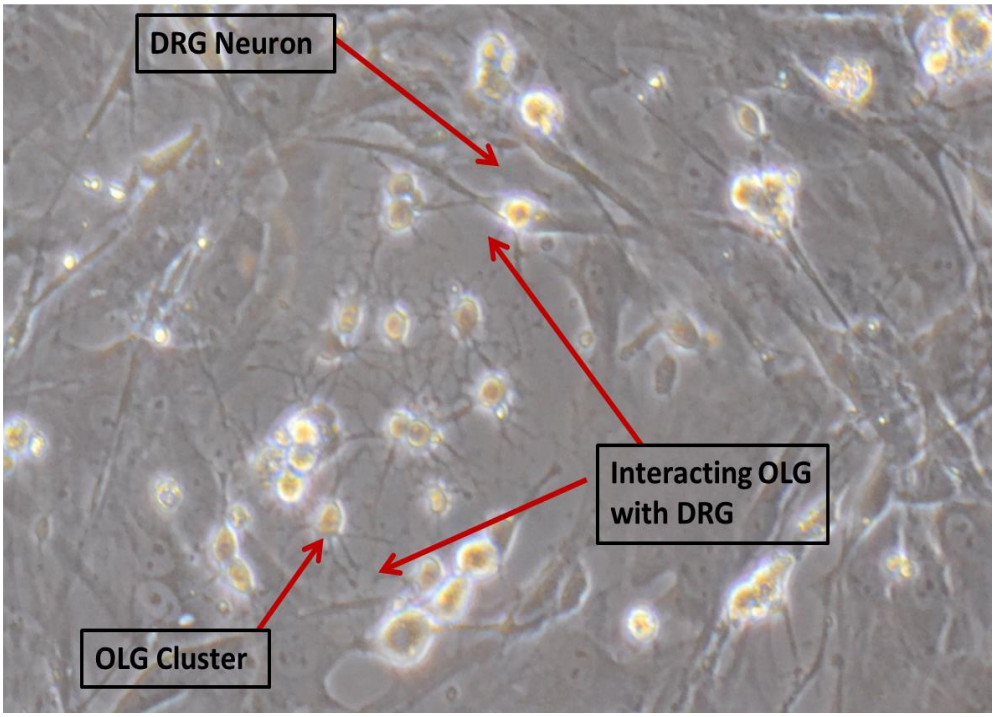


Figure 5.3 (B) Co-culture DIV 5

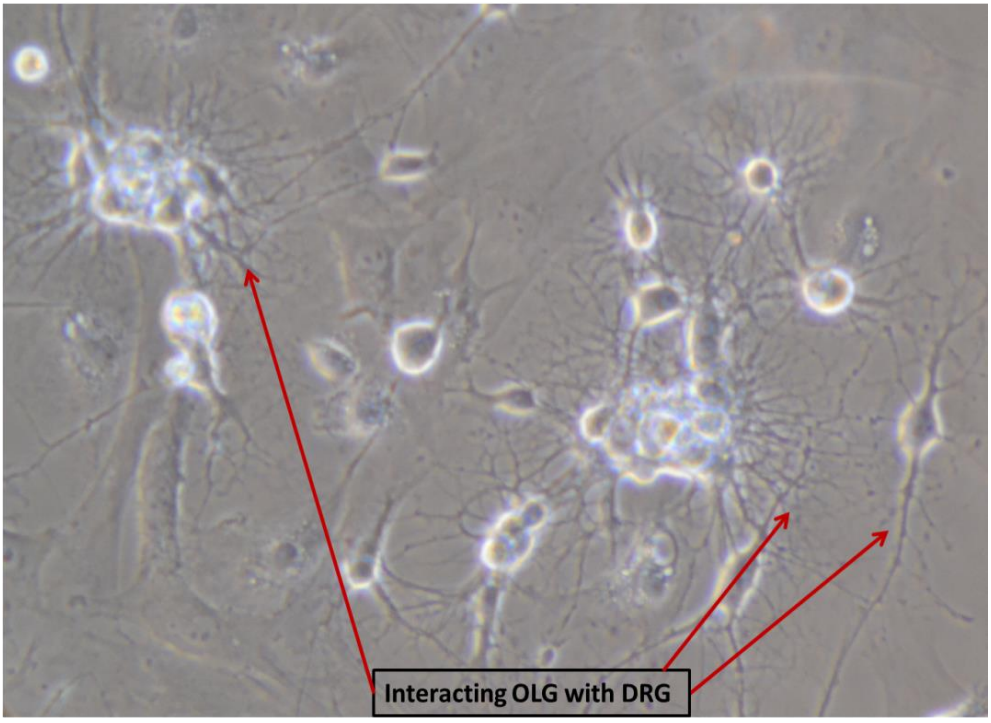


Figure 5.3 C

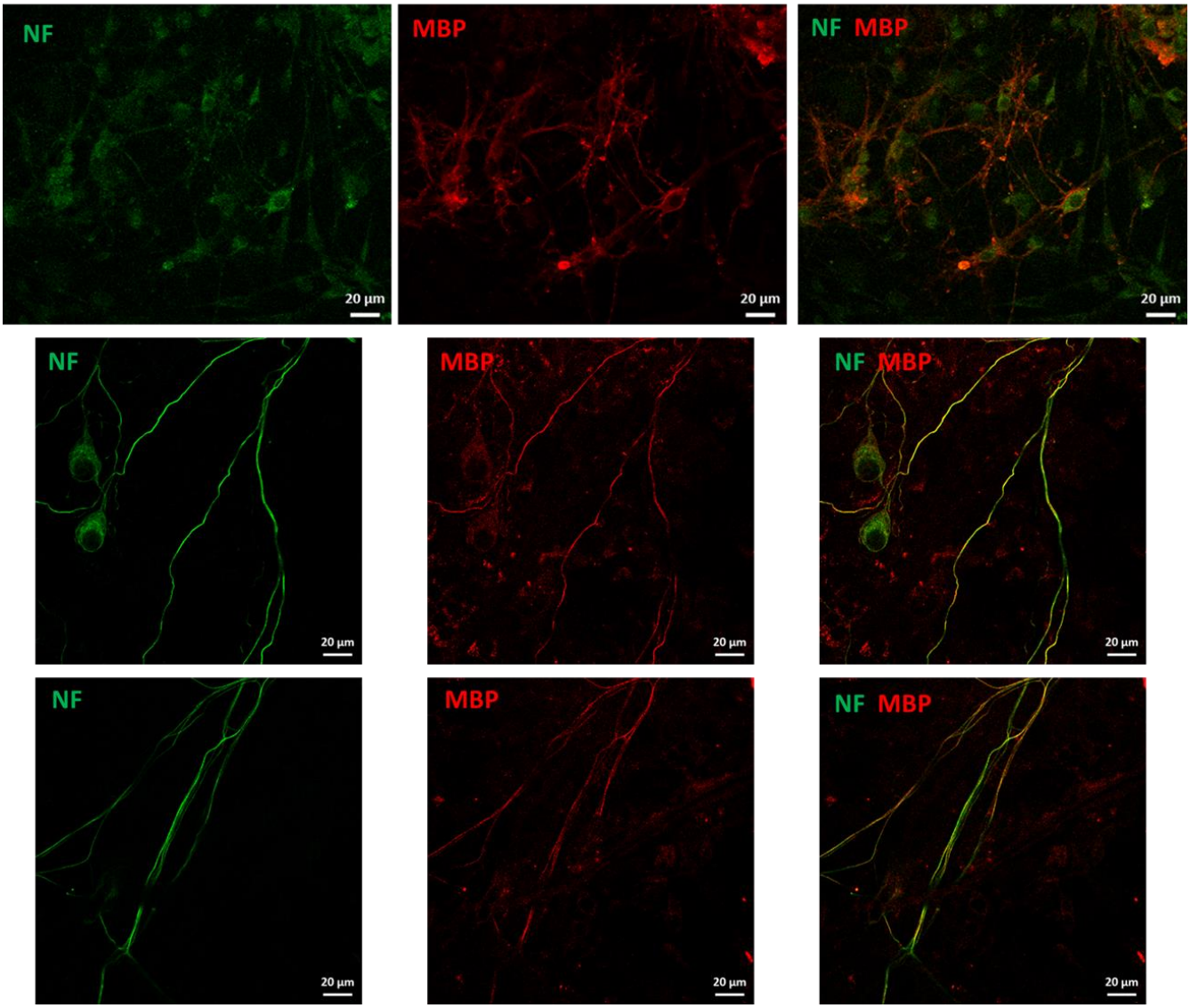


Figure 5.3: Morphological and Immunocytochemical characterization of Oligodendrocyte and DRGN co-culture: A and B) Phase Contrast images of co-culture at DIV 3 and DIV 5. Clusters of highly branched oligodendrocytes are seen to interact with long branches of neurons. Arrows indicate oligodendrocytes interacting with DRGN. C) DRGN and oligodendrocytes were identified using cell specific markers, Neurofilament (Anti-NF; green) and Myelin Basic Protein (Anti-MBP; red), respectively. Scale bar= 20µm.

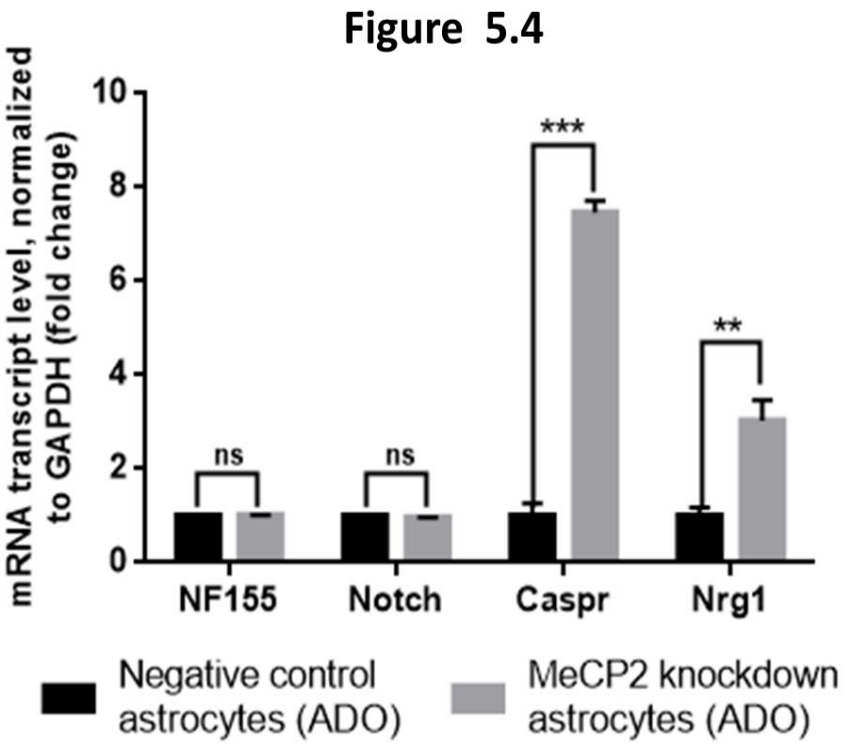


Figure 5.4: Effect of astrocytic MeCP2 on axo-glial interaction genes: The data reflect observations of transcript expression levels of axo-glial interactions genes (NF155, Notch, Caspr and Nrg1) in ADO triple cultures grown on control and MeCP2 knockdown astrocytes; normalized to GAPDH and relative to control. Results are represented as mean ± SEM of 3-4 independent experiments and evaluated using Student's t-test (*P<0.05; **P<0.01; ***P<0.001).

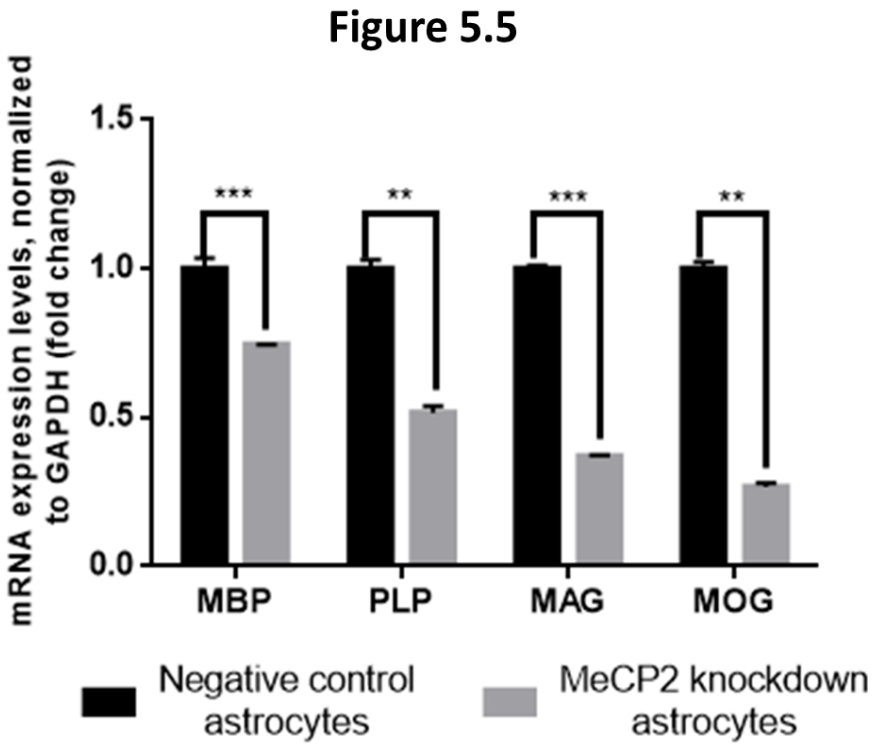


Figure 5.5: Effect of astrocytic MeCP2 on myelin genes in ADO co-cultures: Expression levels of myelin genes- MBP, PLP, MAG and MOG were assessed in DRGN and Oligodendrocyte co-cultures grown on control and MeCP2 knockdown astrocytes; normalized to GAPDH and relative to control. Results are represented as mean ± SEM of 3–4 independent experiments and evaluated using Student's t-test (*P<0.05; **P<0.01; ***P<0.001).

Figure 5.6 (A)

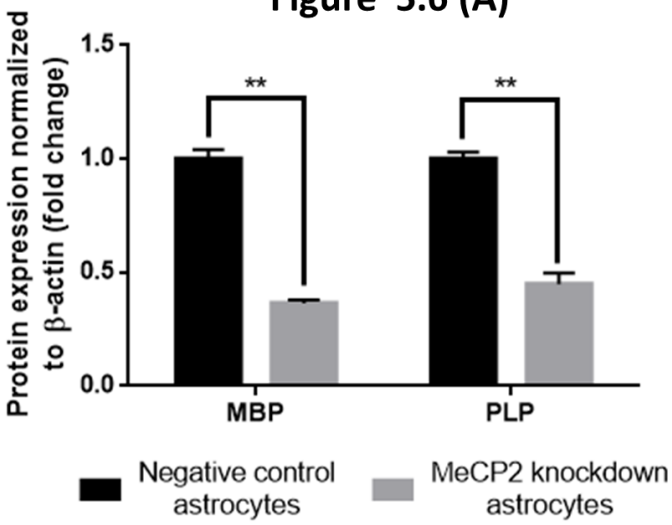
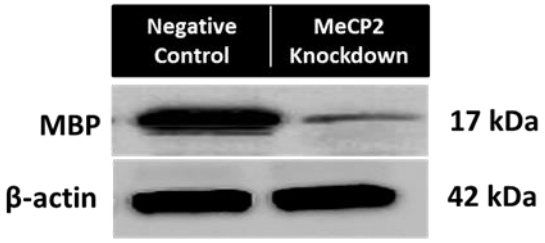


Figure 5.6 (B)



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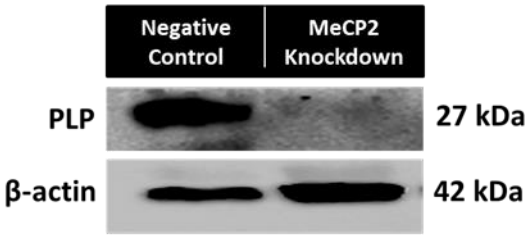

Figure 5.6 (D)

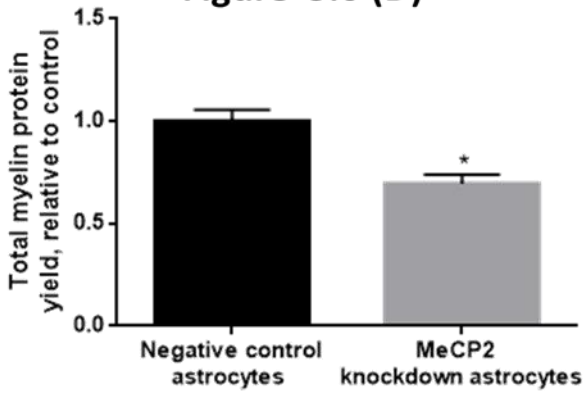

Figure 5.6: Effect of astrocytic MeCP2 on myelin proteins in ADO co-cultures: Myelin proteins- MBP and PLP assessed in DRGN and Oligodendrocyte co-cultures grown on control and MeCP2 knockdown astrocytes (A) Relative change in MBP and PLP protein expression (B and C) Representative western blot image of MBP and PLP respectively, normalized to β-actin and relative to control. (D) Total myelin protein from confluent ADO cultures isolated by myelin extraction, relative to

control. Results are represented as mean ± SEM of 2-3 independent experiments and evaluated using Student's t-test (*P<0.05; **P<0.01; ***P<0.001).