White Matters in Rett Syndrome:

Differential expression of myelin genes in the MeCP2 null mouse

Raena Mina

Supervisor: Dr. Emma Frost

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Department of Biology

The University of Winnipeg
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ABSTRACT

Rett syndrome (RTT) is a neurodevelopmental, X linked disorder, which is one of the leading causes of mental retardation in female children. After 6 -18 months of normal development, patients regress mentally and physically, and develop autistic behaviour. Most cases commonly show mutations within the global transcriptional repressor, methyl-CpG-binding protein 2, MeCP2. Research has focused primarily on neuronal affects of MeCP2 depletion. Since neurons and oligodendrocytes are interdependent for proper maturation and nerve conductance, and neurons are abnormal in the RTT brain, the aim was to investigate MecP2 depletion on myelin. In this study immunocytochemistry and western blots identified MeCP2 expression in oligodendrocytes, the myelinating cells of the central nervous system. The major structural components of myelin are Myelin basic protein (MBP), Proteolipid protein (PLP) and Myelin-associated glycoprotein (MAG). qRT-PCR revealed an increased MBP and MAG expression, as well as a decrease in NG2 and PLP expression in the MeCP2 null mouse. RT-PCR showed no apparent differential expression of myelin gene isoform ratios, suggesting that cell proliferation and maturation are not the causes of the differences in myelin gene expression in the MeCP2 null mouse. These data provide insight in determining the role of MeCP2 regulation in myelination and may lead to potential therapeutic strategies for Rett patients.

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Introduction

Rett Syndrome

Rett syndrome (RTT) was originally described by Viennese physician, Dr. Andreas Rett in 1966 (Reviewed by Charour and Zoghbi, 2007). As an X-linked neurodevelopmental disorder occurring almost entirely in females it is considered lethal in hemizygous males with a single X gene (Colantuoni *et al*., 2001). Studies have mapped RTT to the gene rich locus Xq28 (Webb *et al.,* 1998), which is known to be associated with mental and neurological syndromes and symptoms, such as autism and Angelman syndrome (Hogart *et al*., 2007).

RTT is one of the leading causes of mental retardation in females, with prevalence rates estimated at about 1:10,000 to 1:15,000 births (Reviewed by Bienvenu *et al.*, 2006). There are four clinical stages and manifestations in RTT, beginning with early stagnation in development, followed by regression, and then late motor deterioration (Reviewed by Jedele, 2007). The disorder is typically characterized by handwringing, and developmental regression of higher brain and motor functions around 6 – 18 months of age (Horike *et al.*, 2005). Clinically, the stereotypic handwringing can be one of the earliest signs for diagnosis (Reviewed by Jedele, 2007). Classical RTT patients also lose purposeful hand use, coordinated movements, and develop intermittent hyperventilation (Amir *et al.*, 1999). By year two head growth is decelerated, resulting in microcephaly, where the head becomes abnormally small in size (Reviewed by Chahrour and Zoghbi, 2007). Walking and talking milestones can be reached, but are then lost as the syndrome progresses (Reviewed by Chahrour and Zoghbi, 2007). Patients can survive into adulthood, however by 15-20 years of age 80% of them will be wheelchair dependent and

severely mentally retarded (Lekman *et al.*, 1991). An analysis of early behaviour characteristics of RTT was reported to be sleep disturbances and autistic features (Nomura, 2005). However it is suggested that with age, autistic features become less evident due to the severe overlying mental retardation (Nomura, 2005). Most females develop breathing complications and seizures (Reviewed by Chahrour and Zoghbi, 2007). Over 80% of RTT patients suffer from seizures (Jian *et al.*, 2006) however as adults it may lessen or disappear during the late stages of the disease (Reviewed by Jedele, 2007). Females have a random pattern in X inactivation, leading to variability in phenotype (Young and Zoghbi, 2004). Males typically develop encephalopathy, the degeneration of the brain and death within the first year; however surviving males with RTT usually have a less severe form of the disease as they are XXY and have the additional X chromosome to alleviate the symptoms (Reviewed by Chahrour and Zoghbi, 2007).

Magnetic resonance spectroscopy has shown RTT patients have focal reductions of white matter in the frontal lobe, putamen, hippocampus and parietal and temporal lobes of the brain (Khong *et al.*, 2002). RTT brains are characteristically reduced in size, which are a result of smaller, immature neurons (Kishi and Macklis, 2004). Cerebral lipid membrane composition analysis in the RTT brain revealed loss of myelin-associated lipids as well as dysmyelination in the temporal lobe (Lekman and Hagberg, 1991). This may be confirmed by the reduction found in the myelin marker, galacto-cerebroside and the increase in axonal gangliosides in the temporal lobe of RTT patients (Lekman and Hagberg, 1991). These findings are seen in degenerative demyelinating disorders, supporting slight demyelination in the RTT brain (Lekman and Hagberg, 1991).

Volumetric analyses also suggest that the reductions in RTT brain volume may be due to the reduction in the gray and white matter (Naidu *et al*., 2001).

Methyl-CpG binding Protein 2

Studies have shown 70% of classical RTT patients had mutations in the X-linked gene, methyl-CpG binding protein 2 (MeCP2) (Vacca *et al.*, 2000; Bourdon *et al*., 2001). MeCP2 has also been found to play a role in neuronal maturation and maintenance, including dendritic aborization (Kishi and Macklis, 2004). The disease is often a result of mosaic mutations, where half of cells express the mutant allele (Reviewed by Chahrour and Zoghbi, 2007). Mutations are usually sporadic and de novo in 95% of RTT (Reviewed by Chahrour and Zoghbi, 2007). The variability in mutations includes missense, nonsense and frameshift (Reviewed by Chahrour and Zoghbi, 2007; Bourdon *et al.*, 2001).

MeCP2 contains an 85 amino acid methyl-CpG binding domain (MBD), and a 104 amino acid transcriptional repression domain (TRD) that interacts with histone deacetylase and the transcriptional corepressor Sin3A (Amir *et al*., 1999) Mutations can disrupt the function of either domain (Amir *et al.*, 1999).

Housekeeping genes code for proteins necessary for normal cell function and contain cytosine (C) and guanine (G) (connected via a phosphodiester bond) areas at their promoter regions, (Preuss, 2004). When methylated, MeCP2 binds to those CG dinucleotide islands, interacting with histone deactylase components (HDAC) and Sin3A to repress transcription and silence expression (Preuss, 2004). Hyperactylation was found in studies using MeCP2 knockout (KO) mice, indicating abnormal chromatin architecture and gene dysregulation (Shahbazian *et al.*, 2002a). RTT is thus the first

epigenetic disease, linking the defects with protein involved in DNA methylation (Willard and Hendrich, 1999).

Since MeCP2 is present in many tissues and throughout the genome, it was thought to be a global transcription repressor (Mnatzakanian *et al.*, 2004). However initial microarray analyses, revealed no changes in transcription levels in a RTT mouse model (Horike *et al.*, 2004). It was not until candidate neuron targets were tested, that some neuronal genes were shown to be affected in the absence of MeCP2, such as Dlx5/6 and Brain Derived Neurotrophic Factor (Reviewed by Charour and Zoghbi, 2007).

Despite the presence of MeCP2 in various tissues throughout the body, it is found in greater abundance within the brain, specifically postmigratory neurons (Reviewed by Chahrour and Zoghbi, 2007). MeCP2 expression increases postnatally during the development and maturation of neurons (Reviewed by Chahrour and Zoghbi, 2007). While most published studies imply that only neuronal cells express MeCP2 in the brain (Shahbazian *et al.*, 2002b), there is some published evidence to the contrary (Colantuoni *et al.* 2001; Mnatzakanian *et al*., 2004).

Exactly how gene mutations in MeCP2 results in a neurological disorder, still remains a mystery. To date RTT research has been focused on identifying targets of MeCP2 in neurons.

Myelination and MeCP2 in the brain

While the brain is composed of 10% neurons, up to 90% of the remaining cells are made up of the three types of glial cells. Oligodendrocytes (OL) are the glial cells that produce the myelin sheaths that insulate axons against neurotransmission loss in the

central nervous system (CNS) (Horike *et al.*, 2005), and are essential to facilitate nerve conduction (Armstrong, 1998). Myelin formation is known to occur in a rostral to caudal direction during development (Power *et al.*, 2002). With myelin loss, the inner axon shrinks, affecting the propagation of the action potential (Dyer, 2002). The myelin sheath acts to pump fluid into the axon, as well as cleaning out excess ions from the periaxonal space, which is important for normal action potential transmission (Dyer, 2002).

Furthermore, from the neural stem cell to the process of myelin formation, oligodendroglia and neurons form a close association (Bozzali and Wrabetz, 2004). Studies have shown that reciprocal intercellular signals regulate the proliferation, survival, and differentiation of neurons and oligodendroglia, as well as the initial process of myelination (Bozzali and Wrabetz, 2004). Axonal maturation needs to reach a critical stage before OLs wrap and compact the axons with the myelin sheaths (Windebank *et al.*, 1985). A balance of proliferation of progenitor cells and programmed cell death determine the final number of mature OLs (Bozzali and Wrabetz, 2004). After the oligodendrocyte precursor cells (OPC) stop dividing to differentiate, it is no longer sensitive to the survival signal of platelet-derived growth factor (PDGF), and unless it has contacted a non-myelinated axon within 2-3 days, the cells will die (Bozzali and Wrabetz, 2004). Research has shown that in the absence of OLs, neurons do not function optimally while in the absence of neurons, OLs simply die (Frost *et al*., 1999). These critical interactions indicate the important interdependency of neurons and OLs for proper myelin formation. Gaps between the axon and myelin, and the separation of lamellae are characteristic of myelin diseases (Dyer, 2002), such as Age related Cognitive Decline (ARCD), which results in loose and redundant myelin sheaths (Hinman and

Abraham, 2007). In light of this and the crucial interaction and interdependency between oligodendrocytes (OL) and neurons, it remains necessary to investigate the affects of MeCP2 depletion on OLs and myelination in RTT syndrome.

Proteins important in Myelination

As there are neuronal deficits within the RTT brain, and neurons and OLs are interdependent for proper structure and function, the hypothesis is that myelin formation is also affected in the RTT brain. By studying the expression of major structural components which are crucial for proper myelin development, it can be determined if the white matter is detrimentally affected by the MeCP2 mutation.

In addition to its repressor role, MeCP2 has also been found to interact with Y box-binding protein 1, a protein involved in transcriptional and translational regulation which suggests a possible role in regulating RNA splicing (Young *et al.*, 2005). In MeCP2 null mice, several genes were found to be abnormally spliced (Young *et al.*, 2005). Alternative splicing is a common mechanism where two or more proteins are produced by a single gene (Karp, 2005). The primary transcripts can thus follow two or more pathways for processing, acting as an intron sequence through one pathway and an exon in another (Karp, 2005). This study will therefore verify alternative splicing of myelin genes to investigate whether myelin protein isoforms are differentially expressed in the brain within the MeCP2 null mouse.

- Myelin Basic Protein -

Myelin Basic Protein (MBP) is expressed in OLs and the Schwann cells, the myelinating cells of the peripheral nervous system (PNS) (Mikoshiba *et al.*, 1991). MBP represents 30% of myelin proteins, and are major structural proteins in the CNS

(Mikoshiba *et al*., 1991). MBP has a regulatory role in myelination and are required for myelin membrane growth and membrane adhesion (Dyer, 2002). Specifically MBP represents peripheral cytoplasmic membrane proteins which act as intracellular adhesion molecules, fusing and stabilizing the leaflets of the myelin sheaths, maintaining structure (Mikoshiba *et al*., 1991). In OLs, MBP is believed to fuse the cytoplasmic surface, forming the major dense lines and compacting the myelin lamellae (**Figure. 1**) (Mikoshiba *et al.*, 1991).

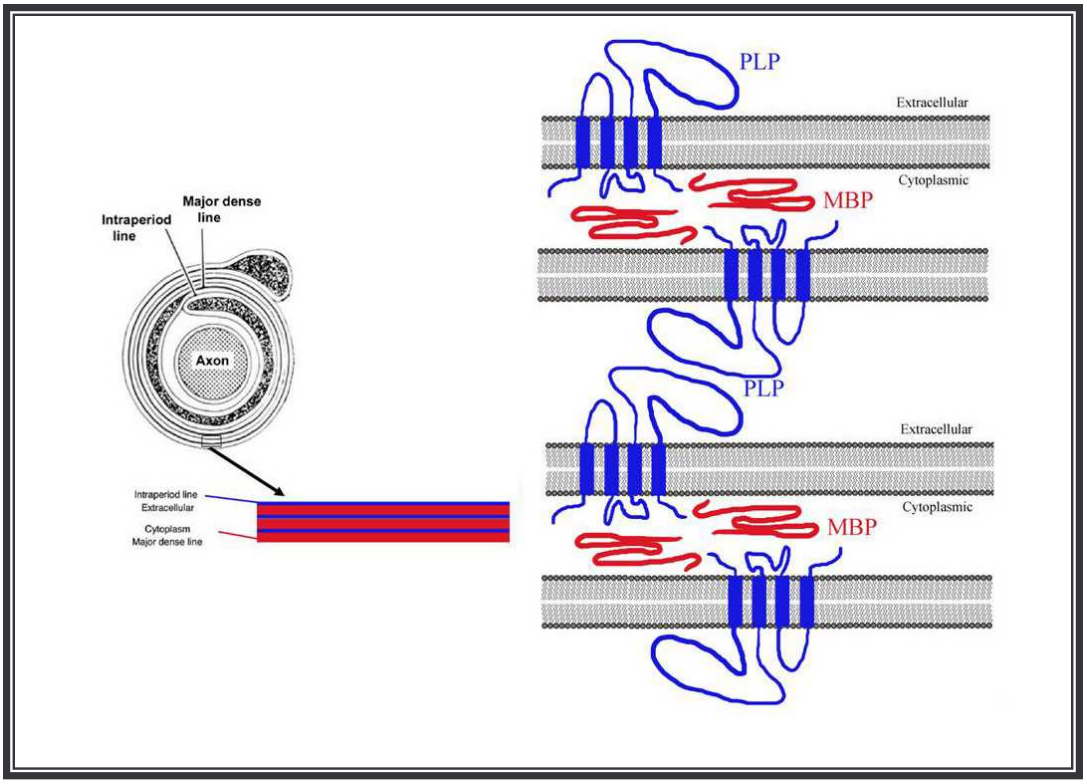

Figure 1. Role of Myelin Basic Protein (MBP) and Proteolipid Protein (PLP) in the membrane of myelin sheaths. MBP forms the major dense line (red) located in the cytoplasm between the lipid bilayers, with PLP forming the intraperiod line (blue) located on the extracellular surface of the lipid bilayer of a compacted myelin sheath around the axon.

MBP mutants also have abnormal myelin wrapping. MBP deletion in mice results in *Shiverer* mice, showing loosely wrapped myelin sheaths and little layer formation (Kimura *et al.*, 1997). Alternative splicing is responsible for four classic

isoforms. The five widely known MBP genes, 354, 422, 442, 479 and 574 bp are represented by the four bands at 14, 17 (422 and 442 bp), 18.5 and 21.5 kDa respectively (Mikoshiba *et al.*, 1991). During the early stages of myelin wrapping, 21.5 and 17 kDa MBP forms are more abundant, suggesting their important role at that stage (Mikoshiba *et al.*, 1991).

- Proteolipid Protein -

Proteolipid protein (PLP) is another major myelin protein, representing 50% of myelin membrane proteins in the CNS (Mikoshiba et al, 1991). PLP is a transmembrane protein (Campagnoni and Skoff, 2001), formed by OLs (Mikoshiba *et al.*, 1991). The gene PLP is found on the X chromosome and is alternatively spliced, resulting in 2 major mRNA products encoding a PLP and DM-20 polypeptide (Griffiths *et al.*, 1998), with polypeptides sized at 30 kDa for PLP and 25 kDa for DM-20 (Campagnoni and Skoff, 2001). PLP is necessary for the fusion of extracellular membranes of myelin lamellae to form an intraperiod line (Duncan *et al*., 1987). PLP and MBP form part of the putative adherens junction, connecting outer myelin sheaths with the inner sheaths (**Figure. 1**) (Dyer, 2002). It is also speculated that PLP may be important in OL maturation (Mikoshiba *et al.*, 1991). Expression of PLP/DM-20 is increased when myelin is actively formed (Mikoshiba *et al.*, 1991), with DM-20 the dominant form during early development and PLP mRNA as the dominant isoform within adult mice (Griffiths *et al.*, 1998; Ye *et al.*, 2003). During mid-embryonic development, it is suggested that PLP/DM-20 transcripts assist in migrating OL progenitors to the future white and grey matter brain and spinal cord zones, from the ventricular zone (Campagnoni and Skoff, 2001). PLP/DM-20 may also be important in regulating OL death, during which

PLP/DM-20 has high gene expression (Campagnoni and Skoff, 2001). Neuron survival is also affected by PLP/DM-20 ablation and overexpression, exhibiting axonal swellings and neuronal degeneration and axonal disintegration respectively (Campagnoni and Skoff, 2001).

The PLP mutant mouse, *Jimpy* is characterized by OL cell death and dysmyelination (Campagnoni and Skoff, 2001; Mikoshiba *et al*., 1991). *Jimpy* mice are considered to be a model of Pelizaeus-Merzbacher disease (PMD), where mental and motor coordination are disrupted (Mikoshiba *et al.*, 1991). PMD is caused by PLP gene duplications, point mutations and deletions (Campagnoni and Skoff, 2001). The mice are completely myelin deficient which is thought to be due to an activation of the caspase cascade, which induces apoptosis via the cleavage of many protein substrates (Southwood and Gow, 2001).

- Myelin-associated Glycoprotein -

Myelin-associated glycoprotein (MAG) is also important in myelination, acting as an integral membrane glycoprotein within the CNS and PNS (Fujita et al, 1989). MAG spans the periaxonal space, contacting the axon, and may be responsible for pore opening and closure in the myelin sheaths (Dyer, 2002). Myelin and MAG synthesis are tightly coupled and MAG is considered to play a role in the recognition and adhesion of OLs to the axolemma (Mikoshiba *et al.*, 1991; Li *et al*., 1998). Alternative splicing of exon 12 results in two polypeptides, large MAG (L-MAG) at 72 kDa and short MAG (S-MAG), at 67 kDa (Fujita, *et al.*) In the CNS, isoform expression is regulated during postnatal development (Mikoshiba et al, 1991). L-MAG is dominant during early development until myelin formation (myelinogenesis), where the expression level declines as S-MAG

becomes more increasingly expressed within the adult brain (Mikoshiba *et al.*, 1991). Isoform expression is dysregulated in the myelin synthesis mutant mouse, *Quaking* (Mikoshiba *et al.*, 1991). MAG mutant mice also show redundant myelin, typical of early myelinogenesis and immature myelinated fibres, suggesting a delay in myelin maturation (Li *et al*., 1998).

- NG2 chondroitin sulfate proteoglycan -

NG2 is a cell surface proteoglycan found in the CNS of developing mice and humans (Ye *et al.*, 2003). NG2 is required for OPC proliferation and couples with PDGF receptor to regulate cell numbers (Nishiyama *et al*., 1996). Experimentally, NG2 expression is commonly used to indicate presence of oligodendrocyte precursors (Ye *et al.*, 2003). NG2 is normally downregulated with the onset of myelination (Nishiyama *et al.*, 1996). Thus NG2 serves as a marker in the OL lineage (**Appendix A**).

Overall hypothesis: ***MeCP2 directly regulates the formation of myelin in the CNS.***

So far there has been little to no importance given to the role of glial cells in the pathology of RTT, overlooking their role within RTT and their crucial partnership with neurons during development. Therefore this study provides significant information on the affects of myelination in RTT, as well as provides some information on possible therapeutic strategies.

In order to investigate the hypothesis that *expression patterns of myelin genes are altered in the MeCP2null mouse* quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) was used.

The hypothesis was tested by comparing changes in expression of myelin genes in the MeCP2 null mouse with wild type control mice.

Further, after densitometry Reverse-Transcriptase PCR (RT-PCR) determined the alternative splicing patterns of myelin genes in the MeCP2 null mouse, and thus whether OL maturation was affected.

In order to investigate the MeCP2 *protein expression patterns in OLs,* Western blotting (WB) was used. In addition, developmental changes of MeCP2 expression in rat OLs from day 0 – 10 *in vitro* were investigated to determine correlation of MeCP2 expression with OL maturation using WB and Immunocytochemistry (ICC).

Methods

Mouse Model

There are two known available RTT mice models (MeCP2 deficient). The Zoghbi transgenic mouse (Shahbazian et al., 2002a) was chosen in this project because they demonstrate a phenotype similar to the human disease, with abnormal involuntary movement, tremors and impaired coordination, with surviving males 100% affected, and females 62% affected in heterozygotes (Shahbazian et al., 2002a). The Zoghbi homozygous females breed with hemizygous males to produce a full litter of truncated MeCP2 null mice, negating the need for genotyping each mouse, and the possibility of loss of null status.

This project compares the RNA of MeCP2 null females with that isolated from age matched wild-type control mice, a swiss black 6 strain.

RNA extractions

Total RNA was prepared from brain tissue from two, 42 day-old female Zoghbi mice from The Jackson Laboratory. Brains were dissected into 2mm slices from brain stem to olfactory bulb and placed into RNALater™ (RNA stabilization reagent; Qiagen) in preparation for RNA extractions. Mice were sacrificed and dissected by the licensed PI in accordance with local animal care and committee approved protocol.

Total RNA was isolated using The RNeasy Lipid Tissue Minikit (Qiagen, cat no.74804) according to manufacturer protocols and stored at -20°C. Spectrophotometric absorbance verified RNA purity, by measuring the absorbance ratio (A_{260}/A_{280}).

One-Step PCR

PCR was performed on isolated total brain RNA, with 3 replicates for each gene at each of 3 different areas of the brain (Forebrain, Middle brain and Hindbrain) using an ABI 7500 thermal cycler and a One-Step RT-PCR Kit (Qiagen).

PCR amplification was performed according to kit protocol, to a final volume (25 µl) with 5X PCR buffer (KCl, Tris-CL, 12.5 mM MgCl2), dNTPs (400 µlM each), *Taq* polymerase and primers (0.6 µM each). PCR conditions were: RT activation at 50°C for 30 min, initial denaturation at 95 °C for 15 min followed by 35 cycles of denaturation at 95 °C, annealing at (Tm) and extension at 72 °C for 1 min each. The Tm was 54°C and 56 °C for PLP and MBP, MAG, NG2, GAPdh respectively. GAPdh served as a control to ensure PCR reaction was successful. The amplified products were electrophoresed at 120 volts for one hour on 1.5 % agarose gels. Densitometry of isoform ratios was analyzed using the Flurochem program, with White as 0. Analysis compared isoform ratios of WT to MeCP2 null mouse.

Two-Step PCR

cDNA was prepared from 350 ng – 500 ng mRNA using a Cloned AMV first strand synthesis kit (Invitrogen), and stored as a stock in -20°C until PCR is needed.

Using a Taq Polymerase kit (Invitrogen) PCR was performed on the ABI 7500 thermal cycler in the same manner as One-Step PCR and products were evaluated on 1.5% agarose gel electrophoresis with ethidium bromide staining.

qRT-PCR

To quantify relative gene expression qRT-PCR was performed on isolated brain RNA with 7 replicates for each target gene at each of 3 different areas of the brain (Forebrain, Middle brain and Hindbrain) using an ABI 7500 thermal cycler and a qRT-PCR Kit (Qiagen). GAPdh served as a control to create Cycle threshold (Ct) values in order to obtain ratios of expression between the WT and MecP2 null mouse:

$$\textbf{Ratio} = E_{TARGET}^{\Delta Ct\ (Target)\wedge(Control-Sample)} / E_{REFERNCE}^{\Delta Ct\ (Reference)\wedge(Control-Sample)}$$

Where E = PCR efficiency + 1 or $10^{-(1/slope)}$
Control is the WT RNA
Sample is the MeCP2 null RNA
Target is the myelin primer used
Reference is the GAPdh control

PCR amplification was performed to a final volume (25 µl) with 5X PCR buffer (KCl, Tris-CL, 12.5 mM MgCl2), dNTPs (400 µM each), *Taq* polymerase and primers (0.6 µM each). PCR conditions were: RT activation at 50°C for 30 min, initial denaturation at 95 °C for 15 min followed by 35 cycles of denaturation at 95 °C, annealing at (Tm) and extension at 72 °C for 1 min each. The Tm was 54°C and 56 °C for PLP and MBP, MAG, NG2, GAPdh respectively. The amplified products were electrophoresed at 120 volts for one hour on 1.5% agarose gels with ethidium bromide staining.

Results of the output from the ABI 7500 thermocycler will be analyzed using the GraphPad Prism 6.0 program, using a t-test to test for statistical significance between the experimental means, with the control means.

Primers

Some of the primers for the myelin genes PCR analysis were commercially available (**Table 1**); others will be those used in previous studies.

Table 1: Primers for PCR analysis of gene expression

Gene	Forward	Reverse	Reference
MeCP2	5'-aacggggtagaaagcctg-3'	5'-atgctccagactgccttg-3'	Jackson Laboratories RT-PCR protocol
GAPdh	PPM02946E		SuperArray
MAG	5'-ctctatggcacccagagcct-3	5'-tgtccttggtgggtcgtttt-3'	Ye et. al (2003)
MBP	5'-atggcatcacagaagagacc-3'	5'-catgggagatccagagcggc-3'	Ye et. al (2003)
PLP	5'-gaaaagctaattgagaccta-3'	5'-gagcagggaaactagtgtgg-3'	Ye et. al (2003)
NG2	5'-gaacgcatcagccaccgtaa-3'	5'-ggacgcttcttcctggtttc-3'	Ye et. al (2003)

Oligodendrocyte Isolation

Pups were decapitated at one day of age, and cerebral cortices dissected into minimal essential medium, RT-MEM-Hepes. A 22 gauge needle was used for trituration to break larger clumps and the cell suspension was strained through a 70 um cell strainer. Cells were then washed with complete medium DFG (Dulbecco's Modified Eagle Medium supplemented with 5000U penicillin and streptomycin, 4mM L-Glutamine and 10% calf serum), then centrifuged at 130 G and resuspended in complete medium. The cells were then plated into Falcon 75 ml tissue culture flasks which were coated with poly-D-lysine (PDL). Medium was changed every third day.

After 10-14 days, oligodendrocyte precursor cells (OPCs) were removed by shaking the flasks at a force of 260 revolutions per minute (rpm) at 37ºC for 16 hours, after an initial two hour shake to remove microglia. Medium was replaced with DFG.

After a further 16-20 hours, the supernatant was removed and transferred to a non-tissue culture plastic petri dish for 20-25 minutes at 37ºC with 7.5% CO_2 for differential cell adhesion to remove remaining microglia. The supernatant was then centrifuged at a force of 1000-1200 rpm for 5 minutes. To create a single cell suspension, the pellet was resuspended in Sato medium and triturated for 30 seconds with a pipette, 3 times. 10 µl of a 1:3 Trypan Blue dilution was added to a 10 µl cell suspension and cells were counted with a haemocytometer. OLs were prepared by the licensed lab technician following the same local animal care and committee approved protocol.

Western Blot

Cells were fixed to the bottom of culture plates using PDL, and stored in Sato medium. OLs from one day old Rat pups cortex were collected in Lamellae buffer by scraping and fixed from Day 0-7. Cells were washed in Phosphate Buffered Saline (PBS), then lysed in 2X sample buffer and boiled (at 95º-100ºC) for 5 minutes. Samples were stored at -20ºC until analyzed. Protein concentration was verified using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA), and samples were diluted to give equal protein loads per sample. Aliquots of each lysate are resolved by SDS-PAGE, where equal quantities of protein, at least 10-30 µg, were loaded into an 8% acrylamide gel and electrophoresed at 100 volts for 1.5 hours.

The resolved proteins are then transferred to PVDF membranes (GE Health Care) using a Panther Semidry electroblotter (Owl Separation Systems). Blots were rinsed in double distilled water for one minute then blocked with 5% skim milk, in Tris-buffered saline (TBS) containing 0.5% Tween-20 (TBS-T) for one hour at room temperature. The blot is incubated with a 1:500 dilution of the primary monoclonal MeCP2 antibody

(SantaCruz) in TBS-T plus 5% skim milk at 4ºC for two days. Protein bands are visualized by incubating the blot in secondary antibody (1:5000) in TBS-T with 5% skim milk for one hour. Detection of the HRP-conjugated secondary antibody is done using a luminol-HRP-chemiluminescence reaction using the Chemilucent ECL Detection System (Chemicon) and exposure to X-ray film. Protein expression is normalized to GAPdh (Genway Biotech Inc.) as a loading control.

Immunocytochemistry

OLs were obtained as described above. OPCs were plated on PDL coated 12 mm glass coverslips, and fixed at various stages of differentiation from Day 0-7, with 3 replicates per time point. This was done by removing the coverslips from Sato filled wells, and submerged in PBS, formalin, then 0.1 % TritonX-100 solution. After blocking the cells in 20% Normal Goat serum, the cells were then incubated with primary rabbit polyclonal anti-MeCp2 antibody (UpState Biologicals) to assess MeCP2 immunoreactivity in the developing brain, 1:100 dilution overnight at 4°C, followed by the secondary goat anti-rabbit Fluorescein isothiocyanate (FITC) Conjugated antibody (Upstate), 1:100 dilution for 30 minutes. Coverslips were also stained with DAPI fluorescent staining at a 1:500 dilution, which binds to DNA. Coverslips were then mounted onto slides.

Images were then collected using an Inverted Olympus IX51 with c-mounted RETIGA 2000RV monochrome camera with EXFO X-Cite metal halide fluorescence system. Images are captured in Image Pro 7.0 and saved to DVD prior to colorization in Image Pro 7.0.

Results

Initial MeCP2 expression

The western blot analysis showed that OLs express MeCP2 *in vitro*. Strong bands are found at 75kDa in the OL preparations grown for 10 days *in vitro* (DIV) (**Figure. 2.a**). In addition, cortex lysates including neurons, astrocytes and OLs show the expected expression of MeCP2 (**Figure. 2.a**). These antibodies were also used for the immunoreactivity studies and similarly, MeCP2 was expressed in the OLs cultured at 0 DIV (**Figure. 3**) and 10 DIV (**Figure. 4**). Cells stained with Upstate Biologicals anti-MeCP2 antibody (green) counter stained with DAPI (blue) clearly show the association of the protein expression within the nuclei of the OLs (**Figure 3** and **4**).

MeCP2 protein expression during OL maturation

After three attempts at the western blot with OLs cultured cells (Day 0-7), no clear bands were obtained. Fresh buffers and reagents were made. The western blot was repeated again to increase concentration of cells. A double thick gel was made, so 40 ul of OL lysate preparations could be run in the wells. No clear bands were present and no marker seen. The experiment was attempted again with OLs preparation collected in 200 uL lamallae buffer, to ensure the higher concentration of cells in order to show bands on the blots. There was around 1.4 μg/μl in each preparation, therefore 21 μg of protein was loaded in each well. Results were positive, showing increasing and decreasing MeCP2 levels. The glyceraldehyde 3-phosphate dehydrogenase (GAPdh) loading comparison did not work, however. This was repeated again, adding both the MeCP2

antibody and GAPdh antibody at the same time, however the blot was inconclusive and time restrictions prevented completion of the experiment.

Immunofluorescence of MeCP2 expression during OL maturation

OLs were isolated and fixed from the same preparation as corresponding western blots. OLs were only found on Day 0,1,2 and 4, and do not appear in the best quality, as the cells are not expressing MeCP2 according to staining. This was repeated with a new preparation of OLs. Cells were present in Day 4-7, however based on shape and processes, the OLs do not appear healthy, which may explain the irregular pattern of MeCP2 expression. This could not be continued within the timeframe of the project.

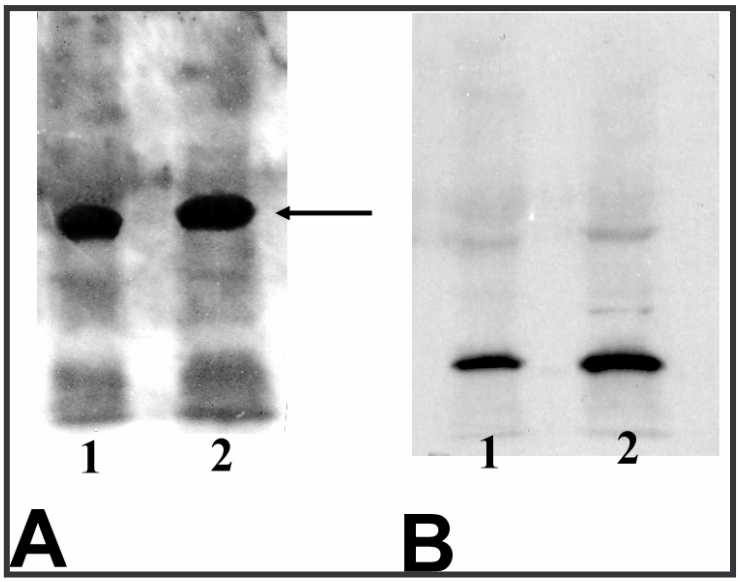

Figure 2. Western blot analysis showing oligodendrocyte expression of MeCP2.
a) Lane1 – Cortex tissue lysates (Control) and Lane 2 – Oligodendrocyte lysates expressing MeCP2 at approximately 75kDa Mm
b)) Lane1 – Cortex tissue lysates (Control) and Lane 2 – Oligodendrocyte lysates showing the protein loading control of GAPdh

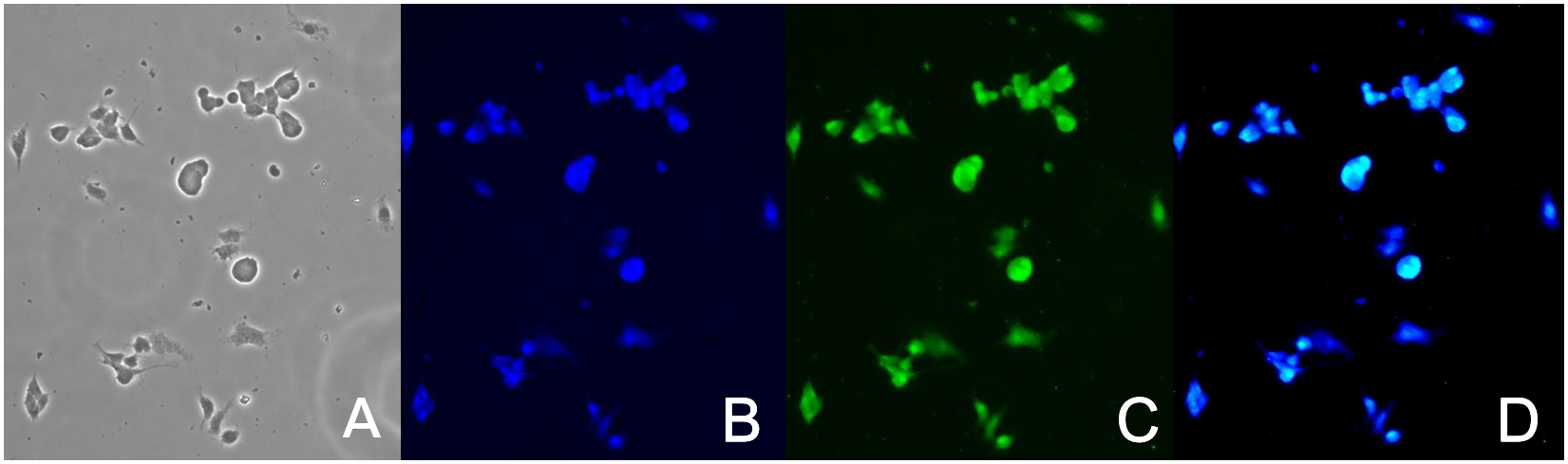

Figure 3. Immunocytochemistry analysis showing MeCP2 expression in oligodendrocytes grown for 0 days *in vitro* at 40 X magnification. The same image is captured in various channels to show different fluorescent staining.
a) Phase contrast image
b) Blue channel showing DAPI staining
c) Green channel showing FITC staining, indicating location of cells stained with anti-MeCP2 antibody
d) Cells stained with anti-MeCP2 antibody (green) counter stained with DAPI (blue); coexpression showing a cyan colour localized to the nucleus

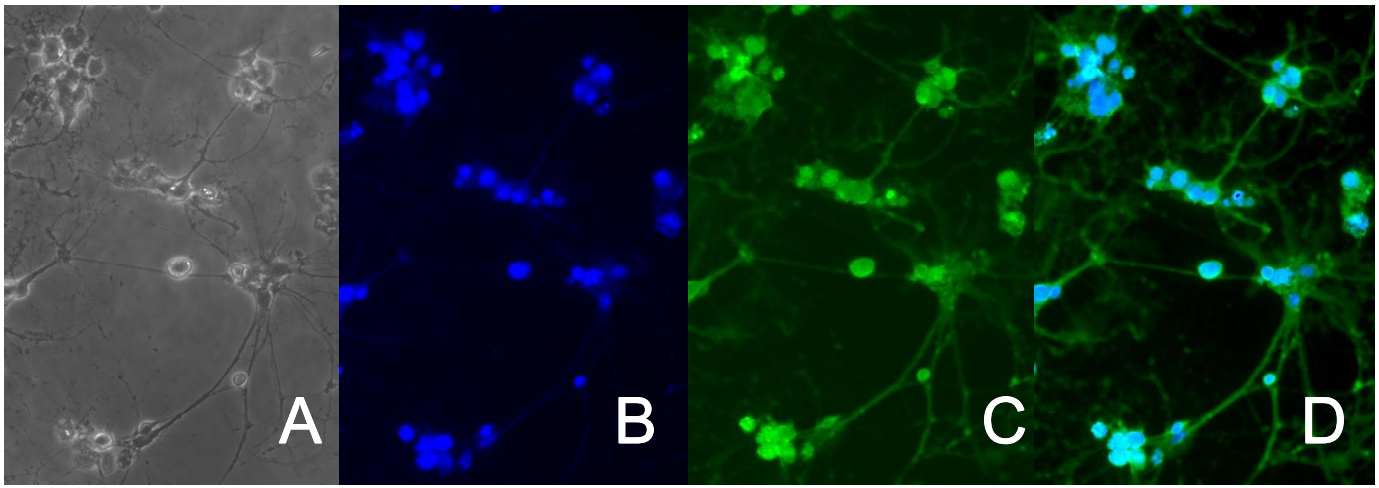

Figure 4. Immunocytochemistry analysis showing MeCP2 expression in oligodendrocytes grown for 10 days *in vitro* at 40 X magnification. The same image is captured in various channels to show different fluorescent staining.
a) Phase contrast image
b) Blue channel showing DAPI staining
c) Green channel showing FITC staining, indicating location of cells stained with anti-MeCP2 antibody
d) Cells stained with anti-MeCP2 antibody (green) counter stained with DAPI (blue); coexpression showing a cyan colour localized to the nucleus

Two Step PCR

A Two-Step kit was used after initial One-Step kit was exhausted. The kits were switched due to the Two-Step kit capable of more experiments. Instead of cDNA production and amplification occurring in one process by the enzymes and thermocycler, a Two-Step kit requires the formation of cDNA separately, after which it was be amplified in another step. After preparing 4 cDNA stocks and running PCR with MBP primers, no bands were seen.

PCR using GAPdh was attempted to see if the cDNA template was causing the problem. After running it out on the agarose gel, the GAPdh bands were seen, suggesting a problem with the PCR process.

The original cDNA was run again using the primers MP5/6, MBP (Superarray), MBP (Operon) and GAPdh to see if it is the primers or the PCR that is the problem. The primers appeared normal except for the one consistently used, MBP (O), which was likely degraded.

PCR was repeated with freshly made MBP, NG2 and MAG primers, as well as GAPdh to ensure the reaction ran smoothly. The isoform bands were not that bright, but did show a positive result, suggesting a low yield of amplified DNA. After using double the amount of cDNA in the mix, some MeCP2 null bands were bright whereas the WT were light again.

It appears that the Cloned AMV first strand cDNA synthesis kit is not as efficient as a One-Step kit, making less cDNA, resulting in lower amplification.

Primers were run using the Two-Step kit with the remaining tests, using 4 μL and 50 cycles, as well as using 500 ng of RNA to start cDNA synthesis, as opposed to 350 ng.

Nonetheless agarose gel photos reveal that the kit is not optimal, showing no bands/very very faint bands. The original One-Step kit on the remaining primers completed the experiment and other brain sections, as time allowed.

RT-PCR

Consistent with the primers from Ye et al. (2003) MBP isoforms 470 bp, 422-442 bp (found as a single band) and 354 bp (18.5, 17, 14 kDa respectively) were detected. 14 kDa was most abundant, followed by 17 kDa and 18.5 kDa (**Figure. 5** and **Table 2**) in both the WT and MeCP2 null mouse. 14 kDa is the isoform predominantly found within the adult mouse CNS (Ye *et al*., 2003). Based on the ratios of isoform expression there were no apparent differences between the WT middle brain RNA and the MeCP2 null RNA, with relatively small standard deviation (**Table 2**), however only two replicates were completed. The 18.5 kDa isoform to 14 kDa isoform ratio was 0.0760 ± 0.0041 (n = 2) in the WT, compared to 0.0924 ± 0.0018 (n = 2) in the null mouse. The ratio for the 17 kDa isoform to 14 kDa isoform was 0.2460 ± 0.0080 (n = 2) in the WT compared to 0.2583 ± 0.0156 (n = 2) in the null mouse. The 14 kDa isoform to 14 kDa isoform ratio was 1.0000 ± 0.0000 (n = 2) in the WT compared to 1.0000 ± 0.0000 (n = 2) in the null mouse. However more replicates will need to be completed to determine statistical significance.

L-MAG (388 bp) and S-MAG (343 bp) isoforms were detected (Ye *et al*., 2003) (**Figure. 6**). The L-MAG isoform to S-MAG isoform ratio was 2.9169 ± 0.6143 (n = 2) in the WT compared to 3.8891 ± 2.3979 (n = 2) in the null mouse. The S-MAG isoform to S-MAG isoform, the ratio was 1.0000 ± 0.0000 (n = 2) in the WT compared to 1.0000 ± 0.0000 (n = 2) in the null mouse. There was no apparent difference in the expression

patterns however more replicates will need to be completed to determine statistical significance.

For the *PLP* primers, PLP isoform (687 bp) was most abundant, compared to DM-20 (585 bp) (**Figure. 7**). Within the adult mouse CNS, PLP is predominantly expressed (Ye *et al*., 2003). There was no apparent difference in the expression patterns. The PLP isoform to DM-20 isoform ratio was 3.5662 ± 1.4957 (n = 3) in the WT, compared to 3.5974 ± 0.8293 (n = 3) in the null mouse. The DM-20 isoform to DM-20 isoform ratio was 1.0000 ± 0.0000 (n = 2) in the WT compared to 1.0000 ± 0.0000 (n = 2) in the null mouse. More replicates will need to be completed to determine statistical significance.

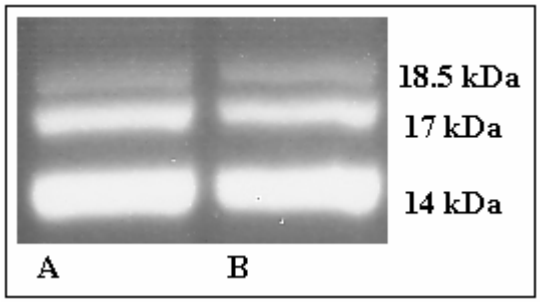

Figure 5. RT-PCR results of Myelin Basic Protein on the middle brain from agarose gel. There are no apparent differences in isoform ratio expression between the midbrain region of the MeCP2 null mouse (B) and the wild type mouse (A). Bands at 14, 17, 18.5 kDa are represented by 354, 422 and 442, and 479 bp respectively.

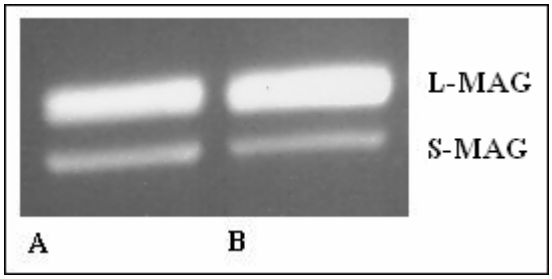

Figure 6. RT-PCR results of Myelin-associated glycoprotein on the middle brain from agarose gel. There are no apparent differences in isoform ratio expression between the midbrain region of the MeCP2 null mouse (B) and the wild type mouse (A)

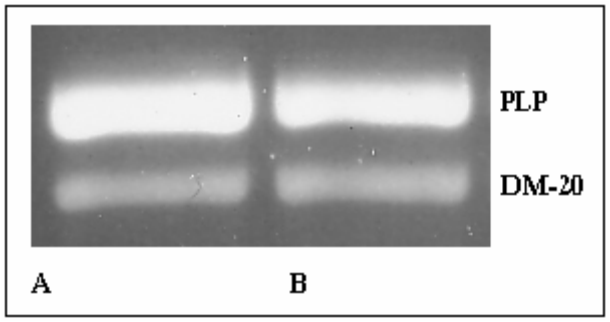

Figure 7. RT-PCR results of Proteolipid Protein on the middle brain. There are no apparent differences in isoform ratio expression between the midbrain region of the MeCP2 null mouse (B) and the wild type mouse (A)

Table 2. Average (Avg) myelin gene isoform expression and Standard Deviation (S.D.) in the Wild Type mouse (WT) compared to the MeCP2 null mouse (Null) based on densitometry analysis from middle brain of RT-PCR on agarose gels (n = sample number)

PRIMERS		**Avg WT ± S.D.**	**Avg Null ± S.D.**
	Isoform		
MBP	*18.5 kDa*	0.0760 ± 0.0041 (n = 2)	0.0924 ± 0.0018 (n = 2)
	17 kDa	0.2460 ± 0.0080 (n = 2)	0.2583 ± 0.0156 (n = 2)
	14 kDa	1.0000 ± 0.0000 (n = 2)	1.0000 ± 0.0000 (n = 2)
PLP	*PLP*	3.5662 ± 1.4957 (n = 3)	3.5974 ± 0.8293 (n = 3)
	DM-20	1.0000 ± 0.0000 (n = 3)	1.0000 ± 0.0000 (n = 3)
MAG	*S-MAG*	2.9169 ± 0.6143 (n = 2)	3.8891 ± 2.3979 (n = 2)
	L-MAG	1.0000 ± 0.0000 (n = 2)	1.0000 ± 0.0000 (n = 2)

Preliminary data show similar ratios of intensity in expression in the forebrain and hindbrain between the MeCP2 null mouse and WT mouse for MBP, MAG and PLP myelin genes (**Table 3**). For PLP in the forebrain, the PLP isoform to DM-20 isoform ratio was 1.9159 to 1 in the MeCP2 null mouse, while it was 1.5460 to 1 in the WT mouse (n=1) (**Figure. 8.a**). In the hindbrain, PLP isoform to DM-20 isoform was 3.2072 to 1 in the MeCP2 null mouse, while it was 2.6229 to 1 in the WT mouse (n=1) (**Figure. 8.b**). More replicates will need to be completed for statistical significance. For *MAG* in the forebrain, the L-MAG isoform to S-MAG isoform ratio was 1.9145 to 1 in the MeCP2 null mouse, while it was 2.2328 to 1 in the WT mouse (n=1) (**Figure. 9**). In the

hindbrain, L-MAG isoform to S-MAG isoform was 3.5579 to 1 in the MeCP2 null mouse, while it was 4.5110 to 1 in the WT mouse (n=1) (**Figure. 9**). More replicates will need to be completed for statistical significance. For MBP, only the hindbrain was completed, with the 18.5 kDa isoform to 17 kDa isoform to 14 kDa isoform ratio being 0.0758 to 0.2874 to 1 in the MeCP2 null mouse respectively (n=1) (**Figure. 10**). In the WT mouse, the corresponding ratio was 0.0486 to 0.2151 to 1 (n=1) (**Figure. 10**). More replicates will need to be completed for statistical significance.

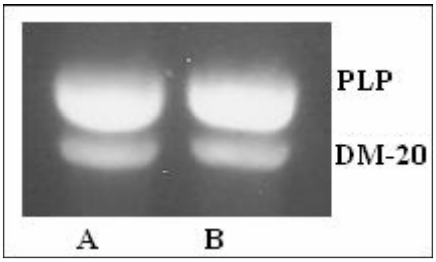

Figure 8.a) Preliminary RT-PCR results of Proteolipid Protein gene on the forebrain. There are no apparent differences in isoform ratio expression between the forebrain region of the MeCP2 null mouse (A) and the wild type mouse (B)

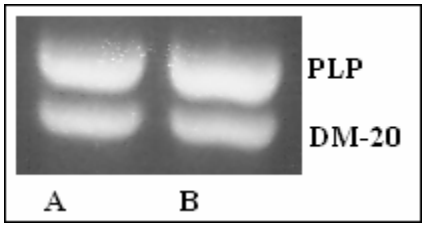

Figure 8.b) Preliminary RT-PCR results of Proteolipid Protein gene on the hindbrain. There are no apparent differences in isoform ratio expression between the hindbrain region of the MeCP2 null mouse (A) and the wild type mouse (B)

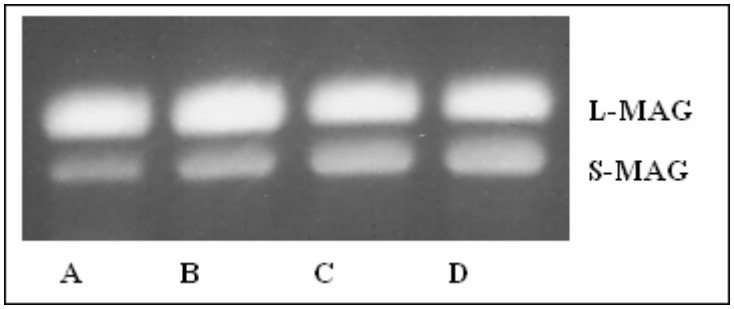

Figure 9. Preliminary RT-PCR results of Myelin-associated glycoprotein gene on the Hindbrain (A and B) and Forebrain (C and D). There are no apparent differences in

isoform ratio expression between the Wild Type mouse (B and D) and the MeCP2 null mouse (A and C)

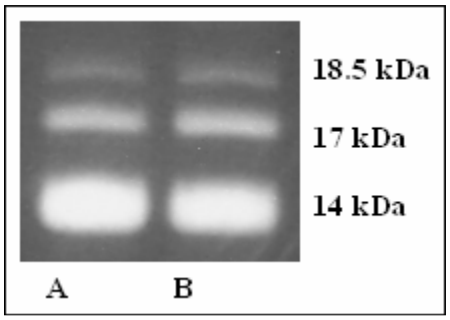

Figure 10. Preliminary RT-PCR results of Myelin Basic Protein on the hindbrain. There are no apparent differences in isoform ratio expression between the MeCP2 null mouse (A) and the control mouse (B). Bands at 14, 17, 18.5 kDa are represented by 354, 422 and 442, and 479 bp respectively.

Table 3. Preliminary Myelin gene isoform expression in the Wild Type mouse compared to the MeCP2 null mouse based on densitometry analysis from forebrain and hindbrain RT-PCR agarose gels (n =1)

PRIMERS		FOREBRAIN		HINDBRAIN	
		Wild Type	**Null**	**Wild Type**	**Null**
	Isoform				
MBP	*18.5 kDa*			0.0758	0.0486
	17 kDa			0.2874	0.21516
	14 kDa			1	1
PLP	*PLP*	1.9159	1.5460	3.2072	2.6229
	DM-20	1	1	1	1
MAG	*S-MAG*	1.9145	2.233	3.5579	4.5110
	L-MAG	1	1	1	1

qRT-PCR

After qRT-PCR was performed, with data collected at the extension and annealing stages, analysis revealed a significant increase in MBP gene expression (2.38 fold, $p < 0.05$) (**Figure. 11**) in the null brain. In addition, MAG expression ratio had a highly significant increase (4.59 fold, $p<0.0001$) within the null brain (**Figure. 11**). However there was a significant reduction in NG2 gene expression (0.33 fold, $p<0.0005$) (**Figure. 12**) as well as a significant reduction found in PLP in the null mouse (0.45 fold, $p<0.001$)

(**Figure. 12**) compared to the WT. Integrity was verified by running 3 random samples by agarose gel electrophoresis.

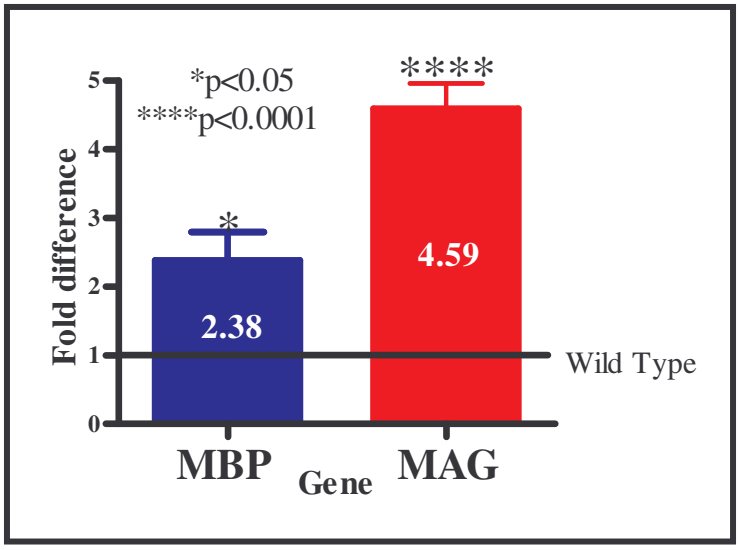

Figure 11: qRT-PCR results showing a significant increase of MBP and MAG gene ratio expression in the middle brain region of the MeCP2null mouse, compared to the wild type mouse (one mouse, 7 repeated experiments).

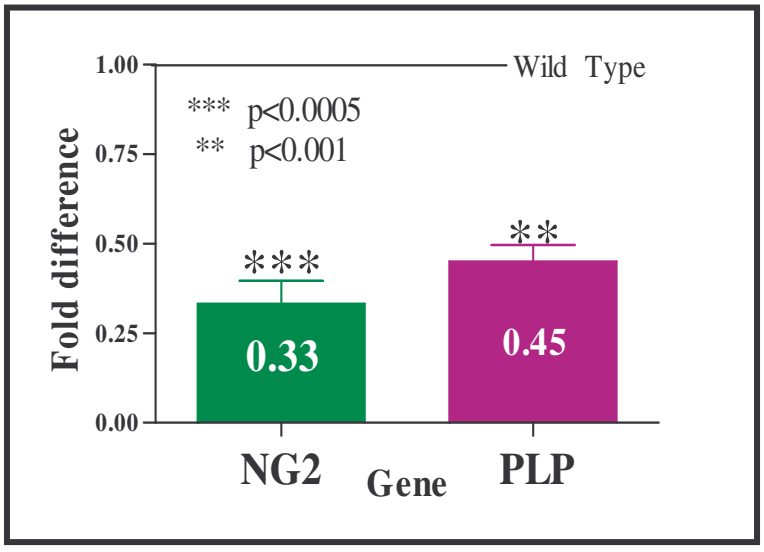

Figure 12: qRT-PCR results showing a significant reduction in NG2 (green) and PLP (purple) ratio expression in the MeCP2 null mouse, compared to the wild type mouse (one mouse, 7 repeated experiments).

Discussion

Most published studies have implied that MeCP2 is expressed only by neuronal cells. However, there is published evidence that glial cells also express MeCP2. For example, in the study by Colantuoni *et al*. (2001) their microarray data show changes in several glial associated genes. In addition, Mnatzakanian *et al.* (2004) show MeCP2 expression in glial cells using RT-PCR analysis. The data in this project provide supporting evidence that OLs express MeCP2, supporting the importance of investigating OLs and myelin formation in the absence of MeCP2.

An increased MBP and MAG expression from qRT-PCR results (**Figure. 11**) might indicate that there is an increased OL maturation in the MeCP2 null mouse, meaning more OLs are at a more mature stage. Alternative splicing of MBP, PLP and MAG occurs during development, with different isoforms being expressed as the OL matures (**Appendix A**). After assessing the pattern of gene isoform expression in the MeCP null mouse relative to wild type controls by RT-PCR, there were no apparent differences in the expression patterns. It is expected that if OL maturation is indeed affected in the MeCP2 null brain, there would be significant differences between the ratio of isoform expression compared to the control mouse. Based on the middle brain, and preliminary forebrain and hindbrain densitometry analyses, there were no apparent differences to suggest dysregulation in maturation. Therefore the increased expression of MBP and MAG seen in the MeCP2 null mouse cannot be due to more mature OLs. An alternative hypothesis is that there is more proliferation and thus more OLs in the MeCP2 null brain, and NG2 gene expression would determine whether in fact this is true. The significant reduction in NG2 gene expression (**Figure. 12**) supports the theory of fewer

cells in the early stages of OL development, reinforcing the original hypothesis that OL maturation is affected. However, the significant reduction in the expression of PLP in the null mouse (**Figure. 12**) disproves that hypothesis. It is expected that PLP would be significantly increased if there were more OLs in the more mature state. This suggests that dysregulation of myelin genes is not due to altered OL maturation or proliferation in the MeCP2 null mouse brain.

MeCP2 depletion and the inability to silence genes can directly and indirectly affect myelin gene expression, which would explain the opposing levels of MBP, PLP, NG2 and MAG. It is possible that MeCP2 is a transcriptional repressor of MBP, so when it is absent MBP expression is never switched off (**Figure. 13b**). This could suggest that MBP may be a direct target of MeCP2. In the case of PLP, it is possible that PLP is regulated by MBP expression, and when MBP is not switched off, PLP is not expressed (**Figure. 13b**). More research would need to be done for further evidence.

MAG was shown to be involved with axon growth inhibitory activity in the CNS, since MAG expression is localized adjacently to the axon in the myelin membrane, and binds to Nogo66 receptor, a neurite growth inhibitor (McKerracher and Winton, 2002). Should MAG or Nogo66 be indirectly or directly affected in MeCP2 depletion, this could result in the dysregulation of MAG expression seen, and would support the findings of smaller, less mature neurons in the RTT brain.

OL development is said to be regulated by a hierarchy of lineage-specific transcription factors (Liu *et al*., 2007). Since MeCP2 likely interacts with many transcription factors, they may target PLP indirectly, causing PLP gene expression to be continuously repressed in a MeCP2 null mouse. Other transcription factors that may be

affected by MeCP2 depletion are Olig2, Sox10 and Nkx2.2, responsible for OL specification and differentiation (Liu *et al.*, 2007; **Appendix B**). Without Sox10 activity in the spinal cord, OL differentiation is considerably inhibited or delayed (Liu *et al.*, 2007). The overexpression of Sox10 protein in heterologous cells lines can induce gene expression from the myelin basic protein (MBP) promoter (Liu *et al.*, 2007). However the regulatory relationship and functional interactions among these transcription factors have not been determined.

A possible MeCP2 target candidate could be Myelin Transcription Factor 1, MyT1 (Romm *et al*., 2005). The expression of MyT1 precedes PLP/DM-20 transcription in OL precursors (Kim and Hudson, 1992). It is predominantly expressed in OL progenitors compared to mature OLs (Campagnoni and Skoff, 2001). MyT1 has binding sites on the PLP/DM-20 gene, suggesting its regulation of PLP transcription (Campagnoni and Skoff, 2001; Kim and Hudson, 1992). MyT1 also contains a binding domain that matches the MeCP2 gene promoter (Liu and Francke, 2006). It is known to interact with the protein, Sin3B, which binds to histone deacetylases in transcriptional repression (Romm *et al.,* 2005). So an interaction between MeCP2 and MyT1 may be involved in regulating PLP expression (**Figure. 13c**). Should MyT1 be a transcriptional repressor of PLP gene, the downregulation of MyT1 by MeCP2 may result in the upregulation of PLP expression. Thus without MeCP2 to regulate MyT1 expression, it is continuously expressed and thus downregulates PLP expression, which may account for what was seen in the MeCP2 null mouse (**Figure. 13d**). MyT1 is also believed to have a role in the initial stages of activation of myelin gene loci (Kim and Hudson, 1992). In situ hybridization studies have also shown MyT1 inhibits differentiation of OL

progenitors into mature OLs (Neilsen *et al.,* 2004). Without regulation by MeCP2, MyT1 may also prevent OL differentiation however this was not indicated in the results of this project. More studies will need to be completed for further evidence.

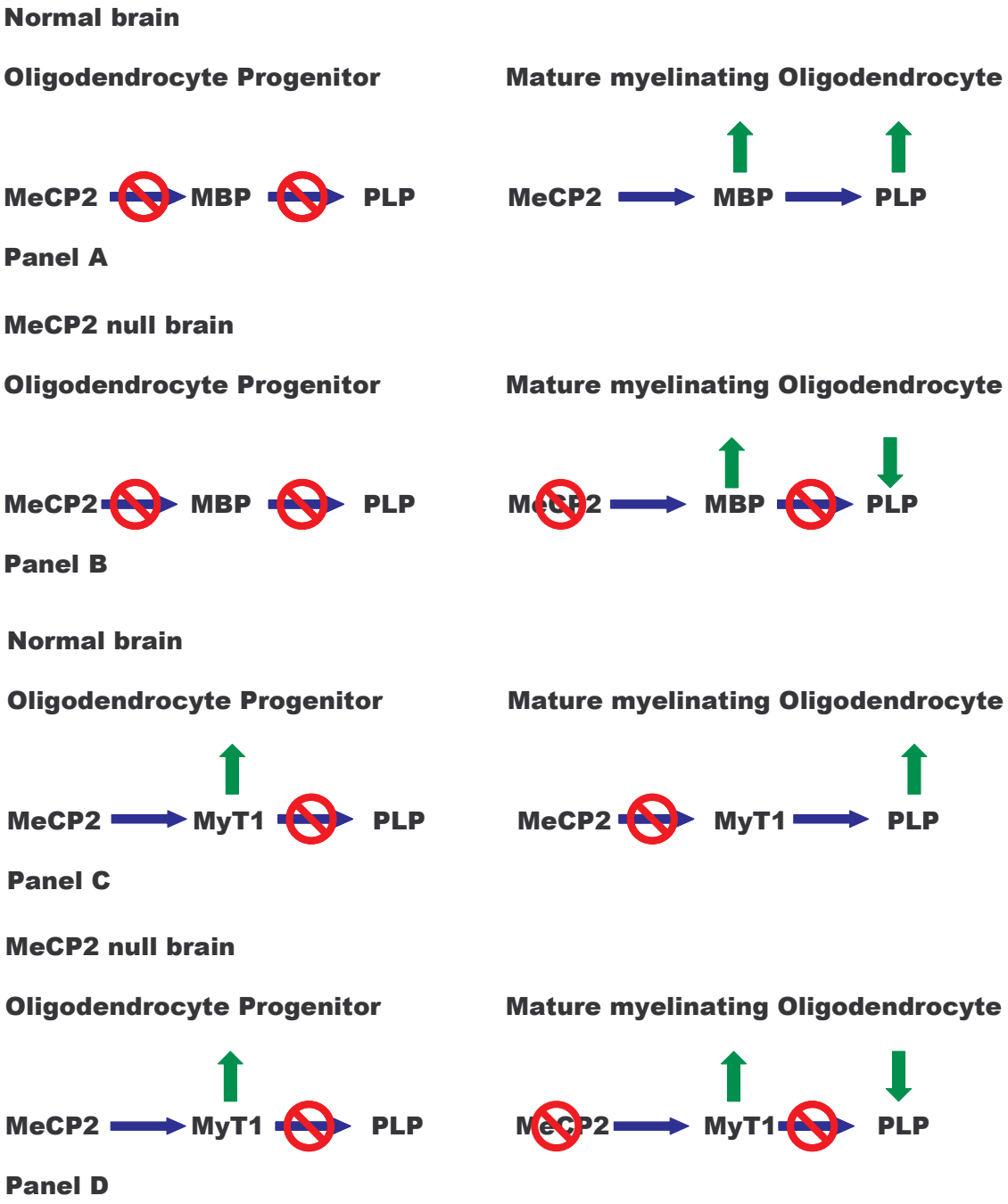

Figure 13. Schematic diagram of possible interactions of MeCP2 and myelin genes. *Panel A*: In a normal wildtype brain, MeCP2 may regulate the expression of MBP and PLP during the oligodendrocyte lineage, expressing the myelin genes at oliogodendrocyte maturity. *Panel B*: In the null nouse, overexpression of MBP may downregulate the expression of PLP. *Panel C*: In a normal wildtype brain, MyT1 regulates the expression of PLP, allowing transcription at oligodendrocyte maturity. *Panel D*: In the null brain, without MeCP2 to repress MyT1, PLP is never switched on.

The results observed in this project may follow a similar regulation seen in Brain derived neurotrophic factor (BDNF), which is known to be a target of MeCP2 (Chang *et al*., 2006). MeCP2 allows BDNF transcription to occur, when calcium ions flow into neurons during activation (Chen *et al.*, 2003). BDNF is necessary for the survival, differentiation and synaptic plasticity of neurons (Liu *et al.*, 2007). It is believed that because BDNF expression depends on neuronal activity, MeCP2 deficiency reduces neuronal activity and thus indirectly affects the decrease in protein level (Chang *et al.*, 2006). Other research has also suggested that mutations in MeCP2 cause other functional disturbances that include reduction of dendritic arborization, which is in part due to the dysregulation of the BDNF gene (Kishi and Macklis, 2004). BDNF deficient mice have similar phenotypes with MeCP2 deficient mice, indicating a possibility that BDNF has a functional role in late RTT defects, such as those seen in the small brain, neuron and reduced synapse (Chang *et al*., 2006). With loss of BDNF in mice models, brain size was found to be reduced, with smaller neurons, and characteristic hind-limb clasping in the mouse, a similar phenotype in RTT (Chang *et al.*, 2006). Further deletion of BDNF in MeCP2 mutant mice resulted in an earlier onset of RTT symptoms (Chang *et al.*, 2006). BDNF overexpression rescued many of the RTT like symptoms and slowed disease progression (Chang *et al.*, 2006). This in itself presents a possible therapeutic strategy that may be used to mediate late RTT pathologies. With overexpression and underexpression of the myelin genes seen in the qRT-PCR results, it would be interesting to determine the affects of rescuing the myelin differences in RTT pathology.

Studies have suggested that MeCP2 can independently of methylated DNA, bind and organize chromatin structures (Abuhatzira *et al.*, 2007). This was seen in the

repression of RE1 silencing transcription factor and (REST) and CoREST, where MeCP2 was able to bind to unmethylated promoters (Abuhatzira *et al.*, 2007). While the mechanism involving histone modifications is still unknown, MeCP2 may bind and inactivate unmethylated promoter regions (Abuhatzira *et al.*, 2007). Is it proposed that MeCP2 depletion upregulates REST and CoREST neuronal transcription factors (Abuhatzira *et al.*, 2007). They are thus overexpressed in the MeCP2 null mouse, which may then downregulate BDNF, and in turn increase production of TRKB, a tyrosine receptor kinase, which is important in activating multiple signaling pathways with roles in neuronal proliferation, differentiation and survival (Abuthatzira *et al.*, 2007). For future studies, it would be important to see whether protein expression correlates with gene expression. The gene expression of BDNF is disconnected with its protein expression in the MeCP2 null mouse (Abuthatzira *et al.*, 2007; Chang *et al*., 2006). Therefore despite MBP and MAG with significant increases in gene expression, the protein level may still be decreased in the MeCP2 null mouse and in turn, NG2 and PLP decreases in gene expression may still have an increased protein expression.

There are some difficulties with qRT- PCR gene expression results, as well as RT-PCR. The isoform ratios show no apparent differences based on densitometry between the WT mouse and null mouse from the RT-PCR experiments, but significant differences were seen in whole gene expression from the qRT-PCR experiments. qRT-PCR does not discriminate between the various isoforms of the myelin gene and looking at the isoform patterns of expression by RT-PCR would reveal possible differences in maturation. In practice, qRT-PCR is expected to give a quantitative ratio of differences between the wild type and null mouse, obtained during the early phases of the reaction.

These results are obtained during the exponential phase, when all reagents are still readily available, reactions are still occurring and where every sample is still producing copies. Agarose gels for detection of RT-PCR amplification are expected to show the final phase/plateau or end-point of the reaction. By this time the reaction has stopped and reagents have been exhausted. Thus it was expected that RT-PCR would show how many possible copies were made based on starting material and total amount of product. However, by the plateau phase, it is possible that reagents will deplete at different rates for each replicate or sample, due to different reaction kinetics. This varies the plateau phase from sample to sample, therefore the initial amounts of starting target material would not be fully represented in the results, and even replicate samples having the same quantity of starting material in the beginning of the reaction would still have differences in the plateau phase.

The resolution from agarose gels can be very poor, making it difficult to differentiate changes, however qRT-PCR is more sensitive and can detect as little as a two-fold changes. Because qRT-PCR collects data as the reaction is proceeding, it may be deemed more accurate for DNA and RNA quantification.

The One-Step kit for RT-PCR will most likely produce a higher yield of amplified product versus the Real-Time kit for qRT-PCR. This is because more enzyme is required to ensure maximum product is amplified, whereas in qRT-PCR, it is more sensitive to amplifying a low starting material, there is not as much enzyme and may not produce as high a yield. Theoretically, products run on gels of both kits, with the same primers should result in the same fluorescence of bands, however because of the exhaustion of materials, and differences in plateau phases, it will be difficult to identify the true

reflection of starting material. The problem is that qRT-PCR reveals the quantitative amount of products expressed for the whole gene, so unless specific primers for each isoform of the gene are used, then it will be hard to say if the bands on the gel reflect the actual amount based on starting material.

It would therefore be optimal to decrease the amount of amplification cycles to 25, where most likely the enzyme and reagents are not yet exhausted and can show more accurately which band is being amplified more, based on starting material. Given more time in the project, this would have been verified. In order to continue, it is best to use primers specific to each isoform, in order to analyze with certainty whether expression ratios are altered.

In light of many diseases found to be deficient in myelination (Schizophrenia, Multiple sclerosis, Age-related cognitive decline) it is important to investigate myelin in the RTT brain. In addition to the common clinical presentations found in RTT, autism and Angelman Syndrome (AS), they also all appear to have overlapping gene dysregulation (Reviewed by Jedele, 2007). 15q11-13, a chromosomal region containing ubiquitin-protein ligase E3A gene (UBE3A) is subject to gene silencing via imprinting (Reviewed by Jedele, 2007). Over 80% of AS patients have a loss of function of UBE3A, and the expression of one protein product, E6-associated protein, and its subunit GABA-A are reduced with MECP2 deficiency (Reviewed by Jedele, 2007). This is seen in RTT, AS and autistic brains (Reviewed by Jedele, 2007). This data supports the need for further investigation of MeCP2 regulation on myelin in the pathology of RTT.

Conclusions

1. OLs do express MeCP2 based on western blotting and immunocytochemistry
2. There are no apparent difference in alternative splicing of myelin isoforms as shown by RT-PCR
3. MBP, MAG, PLP and NG2 are significantly differentially expressed in the MeCP2 null brain as shown by qRT-PCR
4. **Results indicate that OL proliferation and maturation are not the causes of myelin protein dysregulation in the MeCP2 null brain**

Further studies would include completing the investigation of myelin gene expression in the forebrain and hindbrain, as well as looking at different developmental ages of mice, to see at what time point significant myelin changes occur. These results suggest that it is important to compare changes in gene expression, with protein expression. There may be dysregulation in the expression of myelin genes and the actual translation of myelin proteins. In situ hybridization of MeCP2 would reveal where in the brain effects are occurring, and looking at the myelin sheath ultrastructure would reveal if the changes in myelin gene expression results in any physical defects of the myelin wrapping/sheaths.

Dysfunctional myelin is known to play a significant role in the pathology several brain disorders including Schizophrenia, bipolar mood disorders, and age-related cognitive decline. With RTT often misdiagnosed and misclassified as an autistic disease, identifying it as a dysmyelinating disease would thus provide better therapeutic strategies. These data provide positive insight and progress into identifying the underlying pathology of the disease, as well as potential regulatory mechanisms of MeCP2.

REFERENCES

Abuhatzira, L., Makedonski, K., Kaufamn, Y., Razin, A. and R. Shemer. 2007. MeCP2 deficiency in the brain decreases BDNF levels by REST/CoREST-mediated repression and increases TRKB production. Epigenetics 2 (4): 1-9.

Amir, R., Van den Vyver, I., Wan, M., Tran, C., Francke, U., and H. Zoghbi. 1999. Rett syndrome is caused by mutations in X-linked *MECP2*, encoding methyl-CpG-binding protein 2. Nature Genetics 23: 185-188

Armstrong, R.C. 1998. Isolation and characterization of immature oligodendrocyte lineage cells. A Companion to Methods in Enzymology 16: 282-292.

Bienvenu, T., Carrie, A., de Roux, N., *et al*. 2000. MECP2 mutations account for most cases of typical forms of Rett syndrome. Human Molecular Genetics 9 (9): 1377-1384.

Bourdon, V., Phillipe, C., Labrune, O., Amsallem, D., Arnould, C. and P. Jonveaux. 2001. A detailed analysis of the MECP2 gene: prevalence of recurrent mutations and gross DNA rearrangements in Rett syndrome patients. Human Genetics 108: 43 – 50

Bozzali, M. and L. Wrabetz. 2004. Axonal signals and oligodendrocyte differentiation. Neurochemical Research 29(5): 979-988.

Campagnoni, A. and R. Skoff. 2001. The Pathobiology of Myelin mutants reveal novel biological functions of the MBP and PLP genes. Brain Pathology 11: 74-91

Chahrour, M. and H. Zoghbi. 2007. The Story of Rett Syndrome: From Clinic to Neurobiology. Neuron 56: 422-437

Chang, Q., Khare, G., Dani, V, *et al.* 2006. The disease progression of MeCP2 mutant mice is affected by the level of BDNF expression. Neuron 49(3): 341-8.

Chen, W.G., Chang, Q., Lin, Y., Meissner, A., *et al.* 2003. Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2. Science 302: 885 - 889

Colantuoni, C., Jeon, O.H., Hyder, K., Chenchik, A., Khimani, A.H., Narayanan, V., Hoffman, E.P., Kaufmann, W.E., Naidu, S. and J. Pevsner. 2001. Gene Expression Profiling in Postmortem Rett Syndrome Brain: Differential Gene Expression and Patient Classification. Neurobiology of Disease 8: 847–865.

Duncan, I., Hammang, J. and B. Trapp. 1987. Abnormal compact myelin in the myelin-deficient rat: Absence of proteolipid protein correlates with a defect in the intraperiod line. Proceedings of the National Academy of Sciences 84: 6287-6291.

Dyer, C.A. 2002. The structure and function of myelin: from inert membrane perfusion pump. Neurochemical Research 27(11): 1279-92.

Frost, E.E., P.C. Buttery, *et al.* 1999. Integrins mediate a neuronal survival signal for oligodendrocytes. Current Biology 9(21): 1251-4.

Fujita, N., Sato, S., Kurihara, T., Kuwano, R., Sakimura, K., Inuzuka, T., Takahashi, Y. and T. Miyatake. 1989. cDNA cloning of mouse myelin-associated glycoprotein: a novel alternative splicing pattern. Biochemical and Biophysical Research Communications 165 (3): 1162-1169.

Hinman, J. and Abraham, C. 2007. What's behind the decline? The role of white matter in brain aging. Neurochemical Research 32: 2023-2031.

Hogart, A., Nagarajan, R., Patzel, K., Yasui, D. and J. Lasalle. 2007. 15q11-13 $GABA_A$ receptor genes are normally biallelically expressed in brain yet are subject to epigenetic dysregulation in autism-spectrum disorders. Human Molecular Genetics 16(6): 691–703.

Horike, S., Cai, S., Miyano, M., Cheng, J-F. and T. Kohwi-Shingematsu. 2005. Loss of silent-chromatin looping and impaired imprinting of DLX5 in Rett syndrome. Nature Genetics 37(1): 31-40.

Jedele, K. 2007. The Overlapping Spectrum of Rett and Angelman Syndromes: A Clinical Review. Seminars in Pediatric Neurology 14:1 08-117.

Jian, L., Nagarajan, L., De Klerk, N., *et al.* 2006. Predictors of seizure onset in Rett syndrome. Journal of Pediatrics 149: 542-547.

Karp, G. Cell and Molecular Biology: Concepts and Experiments. (2005). 4th Ed. John, Wiley & Sons, Hoboken, New Jersey. p. 466

Khong, P.L., Lam, C.W., Ooi, C.G., Ko, C.H. and V.C. Wong. 2002. Magnetic resonance spectroscopy and analysis of MECP2 in Rett syndrome. Pediatric Neurology, 26(3): 205-9.

Kim, J. and L. Hudson. 1992. Novel Member of the Zinc Finger Superfamily: A C2-HC Finger That Recognizes a Glia-Specific Gene. Molecular and Cellular Biology 12 (12): 5632-5639.

Kimura, M., Sato, M., Akatsuka, A., *et al*. 1998. Overexpression of a minor component of myelin basic protein isoform (17.2 kDa) can restore myelinogenesis in transgenic *shiverer* mice. Brain Research 785: 245-252.

Kishi, N. and J. D. Macklis. 2004. MECP2 is progressively expressed in post-migratory neurons and is involved in neuronal maturation rather than cell fate decisions. Molecular and Cellular Neuroscience 27: 306-321.

Lekman, A.Y., Hagberg, B., and L. Svennerholm. 1991. Membrane cerebral lipids in Rett syndrome. Pediatric Neurology 7(3): 186-90.

Li, C., Trapp, B., Ludwin, S., Peterson, A., and J. Roder. 1998. Myelin Associated Glycoprotein Modulates Glia-Axon Contact In Vivo. Journal of Neuroscience Research 51: 210–217.

Liu, J. and U. Francke. 2006. Identification of cis-regulatory elements for MECP3 expression. Human Molecular Genetics 1 (11): 1769 - 1782.

Liu, Z., Hu, X., Cai, J., Liu, B., Peng, X., Wegner, M. and M. Qiu. 2007. Induction of oligodendrocyte differentiation by Olig2 and Sox10: Evidence for reciprocal interactions and dosage-dependent mechanisms. Developmental Biology 302: 683–693.

McKerracher, L. and M. Winton. 2002. Nogo on the Go Minireview. Neuron, Vol. 36, 345–348.

Mikoshiba, K., Okano, H., Tamura, T. and K. Ikena. 1991. Structure and Function of Myelin Protein Genes. Annual Review Neuroscience 14: 201-17

Mnatzakanian, G.N., Lohi, H., Munteanu, I., Alfred, S.E., Yamada, T., MacLeod, P., Jones, J., Scherer, S., Schanen, N., Friez, M., Vincent, J.B. and B.A. Minassian. 2004. A previously unidentified MECP2 open reading frame defines a new protein isoform relevant to Rett syndrome. Nature Genetics 36(4): 339-341.

Naidu, S., Kaufmann, W.E., Abrams, M.T. *et a*l. 2001. Neuroimaging studies in Rett syndrome. Brain & Development 23: S62–S71.

Nielsen, J., Berndt, J., Hudson, L. and R. Armstrong Myelin transcription factor 1 (Myt1) modulates the proliferation and differentiation of oligodendrocyte lineage cells. Molecular and Cellular Neuroscience 25: 111 –123.

Nishiyama, A., Lin, X., Giese, N., Heldin, C. and W. Stallcup. 1996. Interaction between NG2 proteoglycan and PDGF α-receptor on O2A progenitor cells is required for optimal response to PDGF. Journal of Neuroscience Research 43: 315-330.

Nomura, Y. 2005. Early behaviour characteristics and sleep disturbance in Rett syndrome. Brain & Development 27: S35–S42.

Power, J., M. Mayer-Proschel, J. Smith, and M. Noble. 2002. Oligodendrocyte precursor cells from different brain regions express divergent properties consistent with the differing time courses of myelination in these regions. Development Biology. 245(2): 362-75.

Preuss, P. 2004 Dec. Solving the mechanism of Rett Syndrome: How the first identified epigenetic disease turns on the genes that produce its symptoms. Research News Berkeley Lab. <http://www.lbl.gov/Science-Articles/Archive/LSD-Rett-syndrome.html>. Accessed 2007 25 Sept.

Romm, E., Nielsen, J., Kim, J. and L. Hudson. 2005. Myt1 family recruits histone deacetylase to regulate neural transcription. Journal of Neurochemistry 93(6): 1444–1453.

Shahbazian, M., J. Young, L. Yuva-Paylor, C. Spencer, B. Antalffy, J. Noebels, D. Armstrong, R. Paylor, and H. Zoghbi. 2002a. Mice with truncated MeCP2 recapitulate many Rett syndrome features and display hyperacetylation of histone H3. Neuron 35(2): 243-54.

Shahbazian, M.D., B. Antalffy, D.L. Armstrong, and H.Y. Zoghbi. 2002b. Insight into Rett syndrome: MeCP2 levels display tissue- and cell-specific differences and correlate with neuronal maturation. Human Molecular Genetics 11(2): 115-124.

Southwood, C. and A. Gow. 2001. Molecular Pathways of Oligodendrocyte Apoptosis Revealed by Mutations in the Proteolipid Protein Gene. Microscopy and Research Technique 52:700–708.

Vacca , M., Filippini, F., Budillon, A. *et al.* 2001. Mutation analysis of the MECP2 gene in British and Italian Rett syndrome females. Journal of Molecular Medicine 78:648–655.

Webb, T., Clarke, A., Hanfeld, F., Pereira, J., Rosenbloom, L., and C. Woods. 1998. Linkage analysis in Rett syndrome families suggest that there may be a critical region at Xq28. Journal of Medical Genetics 35 (12): 997-1003.

Willard H. and B. Hendrich. 1999. Breaking the silence in Rett syndrome. Nature Genetics 23: 127- 128

Windebank AJ, Wood P, Bunge RP, Dyck PJ. 1985. Myelination determines the caliber of dorsal root ganglion neurons in culture. Journal of Neuroscience 5(6):1563-9.

Ye, P., Bagnell, R. and J. D'Ercole. 2003. Mouse $NG2^+$ oligodendrocyte precursors express mRNA for Proteolipid Protein but not its DM-20 variant: A study of Laser Microdissection-Captured $NG2^+$ cells. The Journal of Neuroscience 23 (11): 4401 – 4405.

Young, J. and H. Zoghbi. 2004. X-Chromosome Inactivation Patterns Are Unbalanced and Affect the Phenotypic Outcome in a Mouse Model of Rett Syndrome. American Journal of Human Genetics 74: 511–520

Young, J., Hong, E., Castle, J. et al. 2005. Regulation of RNA splicing by the methylation-dependent transcriptional repressor methyl-CpG binding protein 2. Proceedings of the National Academy of Sciences 102 (49): 17551–17558.

APPENDIX A

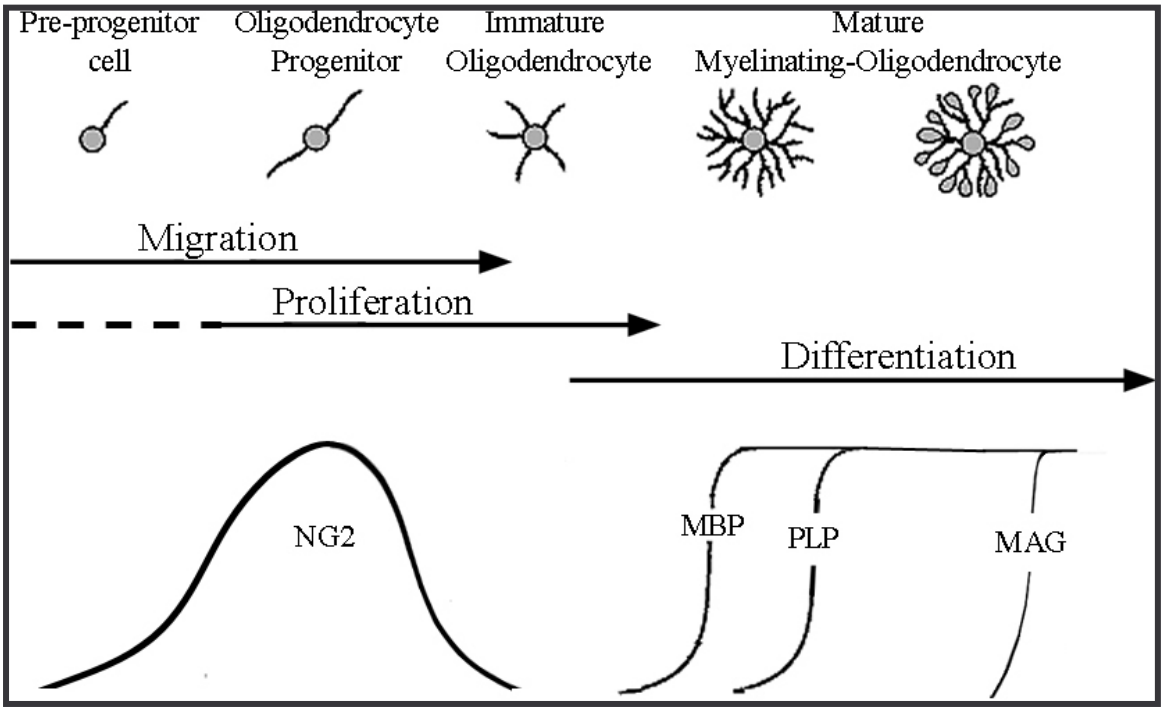

Figure 1. Oligodendrocyte lineage and Markers for differentiation

APPENDIX B

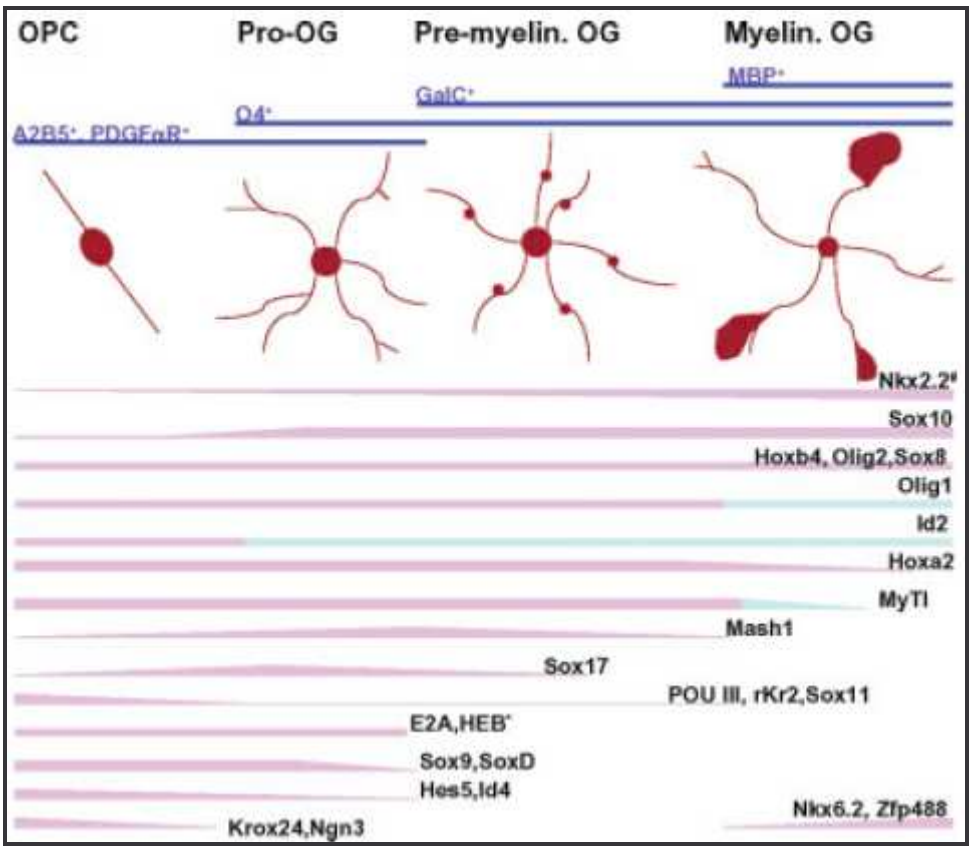

Figure 1. Expression of transcription factors during oligodendrocyte (OG) development[1]

[1] Obtained from Nicolay, D., Doucette, J. and A. Nazarali. 2007. Transcriptional Control of Oligodendrogenesis. Glia 55:1287–1299