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Novel Role of MeCP2 in Developing Oligodendrocytes and Myelination

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Novel Role of MeCP2 in Developing Oligodendrocytes and Myelination

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
at Virginia Commonwealth University

by

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List of Abbreviations

5-AzaC........................................................................5-azadeoxy-citidine
ANOVA........................................................................Analysis of variance
bFGF........................................................................Basic fibroblast growth factor
CDM........................................................................Chemically defined medium
C_m........................................................................Membrane capacitance
CNPase.................................................................2’, 3’-Cyclic nucleotide 3’-phosphodiesterase
CNS........................................................................Central nervous system
CREB-1..............................................................Cyclic AMP response element binding protein-1
CV........................................................................Conduction velocity
d..............................................................................Axonal diameter
DMEM/F12..............................Dulbecco’s modified Eagle’s medium / Ham F12 medium
DNA.................................................................Deoxyribonucleic acid
DTI........................................................................Diffusion tensor imaging
fMRI...............................................................Functional magnetic resonance imaging
GalC.................................................................Galactocerebroside
HDAC..................................................................Histone deacetylase
hnRNP..........................................................Heterogenous nuclear ribonucleoprotein
HRP.................................................................Horseradish peroxidase
IGF-1..............................................................Insulin-related growth factor-1
kDa........................................................................Kilo Dalton
MAG...............................................................Myelin-associated glycoprotein
MBD..................................................................Methyl-binding domain
MBP..............................................................Myelin basic protein
MeCP2.........................................................Methylated CpG-binding protein-2
MOG..........................................................Myelin oligodendrocyte glycoprotein
NLS.............................................................Nuclear localization signal
NT-3............................................................Neurotrophin-3
OPC............................................................Oligodendrocyte progenitor cell
PAGE..........................................................Polyacrylamide gel electrophoresis
PBS.............................................................Phosphate-buffered saline
PDGF..........................................................Platelet-derived growth factor
PDGFr-α......................................................Platelet-derived growth factor receptor-alpha
PLP............................................................Proteolipid protein
PNS............................................................Peripheral nervous system
R_{in}...........................................................Cytosolic resistance
R_{m}...........................................................Membrane resistance
RNA...........................................................Ribonucleic acid
mRNA..........................................................Messenger ribonucleic acid
SDS............................................................Sodium dodecyl sulfate
siRNA..........................................................small interfering RNA
SVZ...........................................................Subventricular zone
T3..............................................................Triiodothyronine
TSA...........................................................Trichostatin A
TRD...........................................................Transcription repressor domain
Abstract

Novel Role of MeCP2 in Developing Oligodendrocytes and Myelination

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Methyl-CpG-binding protein 2 (MeCP2 is) is an epigenetic regulator that binds to methylated DNA. Initially identified as transcriptional repressor, MeCP2 also binds to different proteins functioning as gene activator. Importantly, MecCP2 gene mutations and changes in MeCP2 levels are associated to several forms of mental retardation and autism-related disorders; including Rett, a neurodevelopmental disorder affecting primarily girls. While brain MeCP2 was considered to be exclusively neuronal, this regulator is also present in glia. We found that oligodendrocytes, the myelinating cells of the central nervous system (CNS), express particularly high MeCP2 levels at a developmental stage that precedes their final maturation. Moreover,
downregulation of MeCP2 levels by treatment of immature oligodendrocytes with small interference RNA (siRNA), reduced the expression of 14 kDa myelin basic protein (MBP) and MOG, two markers of mature oligodendrocytes. These observations raise the possibility that oligodendrocytes have a direct participation in Rett syndrome and other autism-related disorders.
Introduction

Since the 19th century, when Golgi and Ramón y Cajal first described them, the cells of the mammalian nervous system have captivated neuroscientists. Over the course of the past century and a half of cellular neuroscience research, the neuronal doctrine has dominated the study of the brain. Essentially, neurons were identified as the functional elements of the nervous system, while the glial cells were merely regarded as support for these highly specialized cells. In recent years this dogma has been called into question; it is now well known that glial cells are far more than just the “glue” that holds the nervous tissue together, supplying energy and neurotrophic factors, regulating axonal growth and modulating synaptic plasticity and neuronal function (Fields 2006).

Among the glial cells are the oligodendrocytes, the cells responsible for the synthesis of the myelin membrane in the CNS. These cells have been implicated in the pathogenesis of a number of illnesses of the CNS, including among others multiple sclerosis, leukodystrophy, schizophrenia and autism. Rett syndrome, first described by Andreas Rett in 1966, is a neurodevelopmental disorder in which girls between 6 and 18 months of age develop profound mental retardation and other neurological maladies. This syndrome results from mutation of the gene encoding MeCP2 (Amir et al. 1999), a protein initially thought to be solely localized in neurons(Shahbazian et al. 2002). As later described in this thesis, altered expression of this protein is also observed in some cases of autism and other related neurodevelopmental disorders.
Studies presented in this thesis confirmed the presence of MeCP2 in developing oligodendrocytes and began to investigate the role of this transcriptional regulator in potentially controlling myelination. Our results indicated that MeCP2 is an important player in the mechanisms that control oligodendrocyte development, a finding that has direct implications for the understanding of Rett syndrome and autism-related disorders also associated with abnormal MeCP2.

The myelin membrane

Myelin structure and function

Myelin is a highly specialized multilamellar membrane structure that wraps around the axons of the nervous system in successive concentric layers, akin to paper spirally rolled around a tube. Generated by the Schwann cells in the PNS and oligodendrocytes in the CNS (Bunge 1968; Baron and Hoekstra 2010); the myelin membrane insulates the axons and facilitates the rapid conduction of action potentials along the fibers. Myelin exists in almost all vertebrates, with the exception of the agnathans (jawless fishes) (Waehneldt 1990), and as described later, recent research on the etiology of different diseases suggests that this membrane also plays a crucial role in CNS development and plasticity. The following sections will discuss in detail the structure, electrophysiology, and molecular composition of the myelin membrane.

Myelin Structure

Myelin is a specialized structure formed by the extension and concentric wrapping of axons by Schwann cell membrane in the PNS or the extended process of an oligodendrocyte in the CNS [Fig. 1]. As myelination occurs, extrusion of the glial cytoplasm and subsequent
compaction of the wrapping membrane results in the formation of a continuous multilamellar sheath or “myelin internode”. These myelin segments are 150-200µm in length (Butt and Ransom 1989), and alternate with “Nodes of Ranvier”, intercalating unmyelinated sections of the axons which vary in length according to their location in the CNS (Bjartmar et al. 1994), and at which sodium selective voltage-gated ion channels cluster. The region directly adjacent to the Node of Ranvier is the paranode, characterized by loops of uncompacted myelin membrane. These paranodal loops are tethered to the axolemma by intermembrane proteins that include Caspr (paranodin), contactin, and neurofascin-155 (NF-155) (Einheber et al. 1997; Menegoz et al. 1997; Charles et al. 2002). On the other hand, the myelinated section of the axon between the paranode and the internode is termed the juxtaparanode, and is characterized by a high concentration of potassium ion channels in the axonal membrane. As described later, this regionalization of ion channels plays a highly important role in the function of the myelinated axon.

Interestingly, several lines of evidence have shown that myelin and oligodendrocytes are both involved in the induction and/or maintenance of the different axonal domains and their characteristic ion channel distribution (Rasband and Trimmer 2001a; DUPREE et al. 2004; Pillai et al. 2009; Thaxton et al. 2011).
**Figure 1:** A) Depiction of an oligodendrocyte extending processes to different axons. Note that each process forms a single myelin internode, adjacent internodes can be formed by different oligodendrocytes [not shown]. Modified from Science Photo Library http://www.sciencephoto.com  B) Diagram of a single myelin segment that has been unwound from an axon. Oligodendrocytes in culture will form similar uncoiled structures, called leaflets. The outer edges of the leaflet contain uncompacted cytoplasm, permitting the transfer of material. Modified from *Basic Neurochemistry*, edited by Siegel, Albers, Brady, and Price, Elsvier, 2006. C) An alternate depiction of a myelinating oligodendrocyte. Nodes of Ranvier are naked axonal regions limited by successive myelin sheaths. Modified from Canadian Institutes of Health Research: Institute of Neurosciences, Mental Health and Addiction
A. Oligodendrocyte soma

B. Outer cytoplasmic tongue

C. Oligodendrocyte

Axons

Node of Ranvier

Myelin sheath

Myelin

Compact myelin

Inner cytoplasmic tongue

Major dense line

Lateral loops
The formation of myelin is a highly regulated process, with different areas of the CNS having different degrees of myelination, as well as different classes of nerve fibers showing distinct patterns of myelination. Importantly, both myelin formation and axonal growth are thought to be tightly coordinated by axo-glial interactions (Waxman and Sims 1984; Baumann and Pham-Dinh 2001). During development, maturing myelinated axons experience a remarkable increase in radial growth. Interestingly, this dramatic growth is restricted to the myelinated areas of the axons and its induction has been attributed to the myelinating glial cells (de Waegh et al. 1992; Colello et al. 1994; Sanchez et al. 1996a). The molecular mechanisms that control this glial function are not fully understood, however certain interactions have been characterized. Larger axons have thicker myelin sheaths, and the thickness of the myelin sheath on an individual axon is believed to be regulated by axo-glial interactions (Waxman and Sims 1984; Baumann and Pham-Dinh 2001). The extracellular integrin β1 has been shown to have a role in initiating the process of myelination (Câmara et al. 2009). It has also been noted that electrical activity along the axon appears to be required for proper myelination (Zalc and Douglas Fields 2000; Fields 2005) and that oligodendrocytes respond to different neurotransmitters in a developmentally regulated manner (Sato-Bigbee et al. 1999a), observations that further emphasize the importance of axo-glial communications.

**Electrophysiology of Myelinated Axons**  
In the nervous system, action potentials are propagated along axons, allowing transmission of electrical signals over exceedingly long distances. The plasma membrane surrounding all animal cells allows the separation of charge between the extracellular and intracellular environment. By wrapping the axons of the nervous
system in successive layers of insulating lipid bilayer, myelin allows for greater conduction speeds. The equation \( CV = 1/ [C_m \cdot \sqrt{R_m \cdot R_{in}}] \) relates conduction velocity (CV) with membrane capacitance \((C_m)\), membrane resistance \((R_m)\) and cytosolic resistance \((R_{in})\). Decreased capacitance of the axolemma due to the presence of the myelin membrane allows a substantial increase in conduction velocity. The myelinated axon magnifies the effect of small changes in ion concentrations, ionic charges are not balanced by opposing charges outside the myelin sheath and current is thus quickly transmitted from node to node, which has the result of increasing conduction velocity. This phenomenon is known as “saltatory” (from the Latin ‘saltare’: to hop) conduction, so called because the membrane depolarization “hops” from one Node of Ranvier to the next, rather than gradually moving down the entire length of the axonal membrane (Tasaki 1939; Huxley and Stämpeli 1949). The end result of this adaptation is signal conduction along an axon at an incredible speed, allowing electrochemical messaging to rapidly proceed through the nervous system. CV is directly related to axon diameter. In unmyelinated axons, this is given by the equation \( CV = \sqrt{d} \) where \( d = \text{axon diameter} \). In myelinated axons, the equation is \( CV = d \).

As described before, the ion channels that permit ion flow across the axolemma exhibit a highly segregated localization along the axon. \( K^+ \)-selective channels are localized to the juxtaparanode (Wang et al. 1993). On the other hand, \( Na^+ \)-selective channels are localized within the nodal region itself (Shrager 1989). This localization, coupled with the insulation provided by myelin, allows for the rapid changes in membrane voltage to be localized to the Nodes of Ranvier. The reduced capacitance of the myelinated axon means that small changes in ion concentrations on either side of the membrane result in large changes in voltage for the entire length of the internode, this increases the efficiency of the system as compared to an
unmyelinated axon. First, the aggregate number of ions that must be moved back across the axolemma is substantially reduced. Multiple discharges are possible with less energy required to transport ions against their concentration gradients, and less time required to repolarize the axon following an action potential. The efficiency of unmyelinated axons is much reduced, as far greater concentrations of Na\(^+\)/K\(^+\) ions must be transported against their concentration gradients to repolarize the axonal membrane after each action potential, necessitating the use of more Na\(^+\)/K\(^+\) active transporter proteins, and the expenditure of far more energy in the form of ATP as compared to myelinated axons (Richie 1973; Wang et al. 2008). It is also important to point out that insulation by myelin, which facilitates rapid conduction with relatively smaller caliber axons, together with the capacity of a single oligodendrocyte to myelinate multiple internodes and axons, allows for maximal efficiency in the restricted space available for the CNS.

**Myelin Composition and Structure**

As indicated above, myelin can be considered as an extension of the Schwann cell or oligodendroglial plasma membrane. However, unlike most biological membranes, myelin contains a very high proportion of lipids (70-80% of dry weight) and a low amount of proteins (20-30%). Cholesterol is present in a far higher molar ratio in myelin than in other tissues of the body (Baumann and Pham-Dinh 2001). Myelin also has a high content of phospholipids and is particularly enriched in glycolipids, predominantly galactocerebrosides (GalC) and their sulfated derivatives, sulfatides (Jahn et al. 2009).

When viewed in cross-section through an electron microscope, layers of myelin can be visualized as alternating thick (major dense) and thin (intraperiod) lines (Sjostrand 1950). The major dense line is generated from the extrusion of cytoplasm and close contact of the
intracellular sides of the rolling membrane. The intraperiod line consists of the apposed extracellular surfaces of adjacent myelin wraps. The periphery of each myelin leaflet is uncondensed, allowing metabolites to move within the sheath.

Myelin basic protein (MBP) is essential for the formation of compact myelin (Privat et al. 1979); it localizes to the intracellular surface of the myelin membrane and contributes to the stability of the myelin sheath. MBP is used as a marker to label myelinating oligodendrocytes, and comprises up to 30% of the protein content of myelin. The protein binds to negatively charged lipids present on the inner leaflet of the plasma membrane and this is thought to contribute to myelin compaction. There are several MBP isoforms resulting from alternative splicing of a single gene called Golli-MBP. This is one of the most complex mammalian genes, containing a minimum of three transcription start sites and ten exons (Pribyl et al. 1993). Interestingly, some of the proteins encoded by the Golli-MBP gene are not localized to the myelin membrane or the oligodendrocytes but to neurons and cells of the immune system. DNA transcribed from start site 3 usually encodes MBP proteins. While the significance of the different MBPs is not fully understood, it is known that these isoforms are differentially expressed during development and also exhibit differential intracellular localization. Isoforms containing exon II of the MBP gene do localize to the nucleus and the cytoskeleton, while exon II-lacking isoforms are primarily found in the myelin membrane (Baumann and Pham-Dinh 2001). During active myelination, MBP synthesis is rapidly upregulated and MBP mRNAs destined to generate isoforms localized in myelin are transported along the oligodendrocyte processes to the distant sites of translation to facilitate local membrane production (Ainger et al. 1993; Boggs 2006; Min 2009; Laursen et al. 2011). Multiple animal models exist in which the MBP encoding regions have been subject to insertion, deletion and
substitution mutations, showing that the MBP gene is necessary for myelination, but not necessary for oligodendrocyte development (Privat et al. 1979).

Another major component of myelin is the transmembrane proteolipid protein (PLP), which constitutes about 50% of the total myelin protein. The extracellular domains of PLP serve as linkages that tightly bind together the apposed extracellular surfaces of the layers of myelin (Klugmann et al. 1997).

Myelin-associated glycoprotein (MAG) is a type I transmembrane glycosylated protein present in myelinating cells and periaxonal layer of the myelin membrane. This heavily glycosylated protein is believed to have a role in glial-axonal interactions. MAG has been shown to be important for the maintenance of myelinated axons; its absence results in a reduction of axonal caliber, neurofilament spacing, and neurofilament phosphorylation (Yin et al. 1998b). MAG also functions as an inhibitor of neurite outgrowth in white matter, and acts as a ligand for the Nogo receptor on neurons, inhibiting axonal regeneration (Filbin 1996).

Another glycoprotein, myelin/oligodendrocyte-associated glycoprotein (MOG) is like MAG a member of the immunoglobulin superfamily. Both MOG and MAG are expressed in the oligodendrocytes themselves, but unlike the periaxonal location of MAG, MOG is present on the external surface of the myelin sheath (Pham-Dinh et al. 1993). MOG’s exact function is still unclear, though evidence has lead to three likely roles, that of a surface adhesion/receptor molecule, a regulator of oligodendrocyte microtubule stability via MBP degradation, or as an activator of complement in the CNS (Johns and Bernard 1999).

Myelin in Disease
Myelin is critical for the normal function of the nervous system. Moreover the process of myelination is highly interconnected with plasticity and remodeling of the developing brain. The original perception of myelin as an inert insulating structure only responsible for saltatory conduction has radically changed to that of a complex membrane which, as mentioned above, is also implicated in controlling axonal extension and radial growth (Yin et al. 1998a), axolemmal integrity (Bruce et al. 2010), and the establishment of nodal and paranodal structures and ion channel localization at the nodes of Ranvier (Rasband and Trimmer 2001b). Results from different laboratories also indicate that the oligodendroglial cells themselves could regulate axonal function by maintaining bidirectional glial-neuronal interactions (Fields 2008). Moreover, in agreement with a large number of animal studies, there is evidence correlating increased myelin formation with experience and enhanced function of the infant brain (Als et al. 2004; Pujol et al. 2006). It is known that human cognitive development and memory as well as learning experience beyond early infancy are positively correlated with white matter deposition in functionally related areas of the brain (Bengtsson et al. 2005; Deutsch et al. 2005; Niogi and McCandliss 2006; Whitaker et al. 2008; Rimrodt et al. 2010; Takeuchi et al. 2010; Uhlhaas et al. 2010). Furthermore, the importance of myelin in the later phases of brain maturation is also underscored by the growing number of neurological diseases being associated to white matter pathology during adolescence, including among others schizophrenia (Chambers and Perrone-Bizzozero 2004; Kubicki et al. 2005; Kerns et al. 2010; Whitford et al. 2010), bipolar disorder (Chambers and Perrone-Bizzozero 2004; Brambilla et al. 2009; Mahon et al. 2010), and autism (O'Hearn et al. 2008).

Disease states that are associated with demyelination include multiple sclerosis, leukodystrophy, transverse myelitis, and optic neuritis. The classic example of a demyelinating
disease is multiple sclerosis (MS), in which lesions are present in the white matter of the CNS. Disruption of the myelin sheath on the axons of the CNS cause the symptoms of MS, including muscle weakness, clonus, and ataxia. At the cellular level, demyelination can result in conduction failure as demyelinated axons in the CNS fail to conduct action potentials; additionally, *in silico* modeling suggests that demyelination may be responsible for spontaneous discharges of the axon (Coggan et al. 2010). In response to the loss of myelin, ion channels begin to diffuse from their previously well defined areas and propagate throughout the membrane of the axon, allowing action potentials to traverse the demyelinated axonal membrane, though at a far reduced conduction velocity (Bostock and Sears 1978).

Potential therapies for these diseases are aimed at remyelination by the oligodendrocytes and therefore, understanding the biology of these cells is of significant importance.

**Oligodendrocytes**

Virchow first identified the glial cells as a novel category of nervous system cells in the mid-19th century. He described these cells as “nervenkitt” (neural putty), or glia. Rio Hortega coined the term ‘oligodendroglia’ in the early 20th century to describe a population of neuroglial cells with a few processes. As exploration of the CNS progressed, scientists theorized that these glial cells were a support system for the highly specialized neurons. Thus, for more than a century, the neuron was considered to be the functional unit of the nervous system. However, in recent years this dogma has been challenged by findings from numerous laboratories indicating that glial cells, including oligodendrocytes, astrocytes and microglia, play multiple and crucial functions in the CNS.
As described before, Oligodendrocytes are responsible for the synthesis of myelin in the CNS. Additionally, oligodendrocytes have been shown to have a role in axonal growth and maintenance, and inhibition of axonal regeneration, through surface-surface receptor interactions (Sanchez et al. 1996b). Moreover, a recent report indicated an important function of oligodendrocytes in the establishment of neuronal networks during early development of the brain (Doretto 2011).

Several new studies have also discovered that glial cells in the white matter of rat brain and spinal cords are interconnected by gap junctions comprised of apposed connexin membrane proteins. These junctions, which permit the flow of water, ions, and proteins between cells, join oligodendrocytes and astrocytes in the so called “paraglial syncitium”. There is documented evidence for the existence of both astrocyte-astrocyte as well as astrocyte-oligodendrocyte connections (Rash et al. 1997). Oligodendrocyte-oligodendrocyte connections have recently been characterized and while their functional significance is not known at this time, some may play a role in modulating local ion concentrations around Nodes of Ranvier (Yamazaki et al. 2007). Importantly, many of the myelin-associated diseases described above, including multiple sclerosis, schizophrenia, leukodystrophies, and leukencephalopathies also affect the oligodendroglia (Baumann and Pham-Dinh 2001). The following sections will describe the development of oligodendrocytes as understanding of these cells' biology may be crucial to the design of therapies for myelin-related diseases.

**Oligodendrocyte development**

Oligodendrocytes have multiple processes extending from a central soma, *in vivo* each of these cell processes has the potential of wrapping around an axon, forming a single
internodal segment of myelin sheath. However, a single oligodendrocyte may extend processes
to and myelinate up to 10-50 different axons. In addition to those that myelinate axons, there are
smaller satellite oligodendrocytes present in the white matter as well. Although the precise
function of these cells remains unknown (Nishiyama 1999), several lines of evidence suggests
that these cells function in remyelination in disorders like multiple sclerosis (Reynolds et al.
2002).

It is important to stress that while each Schwann cell generates a single myelin
internode in a single axon, a single oligodendrocyte can myelinate multiple neurons and form
several myelin segments (Peters 1964). Therefore death of a single oligodendrocyte can generate
areas of demyelination on multiple axons. Additionally, oligodendrocytes exhibit several unique
characteristics that make them susceptible to injury. Evidence of this is seen in demyelinating
mouse models, mice treated with the copper-chelating agent cuprizone and immuno-mediated
models of demyelination (Matsushima and Morell 2001).

Oligodendrocyte lineage is traced to the neuroectodermal cells of the developing
mammalian embryo, sharing an initial developmental pathway with neurons and astrocytes.
Oligodendrocyte precursor cells (OPCs) are mitotically active and characterized by a bipolar
morphology and migratory behavior. OPCs migrate from origin sites within the dorsal spinal
cord and the ventricular and subventricular zone (SVZ) of the mammalian brain, migrating to
their final destinations guided by various extracellular molecules (Small et al. 1987; Ffrench-
Constant 1988). Prior to their maturation into cells capable of myelination, OPCs must undergo
several steps of development (Figure 2). Immunostaining techniques are highly effective means
of both identifying oligodendrocytes and determining their maturational stage. A number of
distinct phenotypic stages have been identified based on the expression of various specific antigenic markers.

Early proliferative OPCs are bipolar cells that express the NG2 chondroitin sulfate proteoglycan and the platelet-derived growth factor alpha receptor (PDGFr-α) (Levine et al. 1993; Nishiyama et al. 1996; Chang et al. 2000) These immature cells can also be labeled with the A2B5 antibody that recognizes several gangliosides including GT3 and its O-acetylated derivative (Dubois et al. 1990; Farrer and Quarles 1999). Those antigens are down-regulated as the cells differentiate into committed oligodendrocytes which are multipolar and can be labeled with both the O4 and O1 antibodies, which react with sulfatides and GalC, respectively (Sommer and Schachner 1981). In the next stage, cells are additionally labeled with antibodies against 2’, 3’-cyclic nucleotide 3’-Phosphodiesterase (CNPase), a myelin-associated enzyme known to be expressed at high levels almost exclusively in both Schwann cells and oligodendrocytes (Baumann and Pham-Dinh 2001). Over-expression of this protein causes a variety of myelin defects in the developing brain (Gravel et al. 1996). The final stage of differentiation is characterized by the expression of other myelin proteins, including PLP, the MBPs, MAG and MOG (Kumar et al. 1988).

Like astrocytes, oligodendrocytes mature mostly in the postnatal period of mammalian animals. Importantly, some populations of OPCs do not continue down the path of differentiation, and remain as OPCs in the SVZ. It is hypothesized that these cells may serve to repair demyelinated axons. Remyelination in cuprizone treated mice has been shown to occur in waves, in which NG2+ cells migrate to sites of demyelination and metamorphose into mature oligodendrocytes (Mason et al. 2000).
At the final stage of maturation, the cells begin to rapidly synthesize the components of the myelin membrane. *In vitro* the multiple branching processes formed in the premature phase begin to form characteristic leaflets, broad sheets of myelin membrane. *In vivo* these same cells will begin to sheathe the axons of neurons with their leaflets, as will oligodendrocytes co-cultured with neurons.

MBP, as previously mentioned, is generated from the Golli-MBP gene in a number of different splicing isoforms and appears to have a crucial role in myelin compaction. The positively-charged lysine and arginine residues of MBP tightly bind to negatively-charged phosphatidylserine, a lipid which is present on the intracellular plasma membrane leaflet of oligodendrocytes causing apposed inner-membrane leaflets to be drawn together and facilitating the compaction of myelin (Min 2009). It is believed that Translation of MBP mRNA must therefore occur near the site of myelin synthesis, avoiding the chances of MBP-induced compaction of other portions of the cell and compromised survival. Research into this problem revealed that oligodendrocytes transport MBP mRNA to relevant regions of the cell via RNA binding proteins such as hnRNP-1 and Kif1b (Lyons et al. 2009; Laursen et al. 2011). MBP mRNA transport from the nucleus to specific sites of translation is one example of oligodendrocyte specialization; another is the active nuclear transport of Exon II-containing MBP isoforms. Exon II contains coding of a nuclear localization sequence (NLS), and translated isoforms containing the NLS may have a role in the regulation of gene expression during development (Pedraza 1997).

Different molecules and growth factors were shown to stimulate oligodendrocyte maturation. Platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1) and neurotrophin-3 (NT-3) stimulate OPC proliferation but
in the presence of triiodothyronine (T3), these cells differentiate into MBP expressing mature oligodendrocytes (Barres et al. 1994).

Details of the molecular mechanisms underlying oligodendrocyte differentiation are still poorly understood. In this regard, previous studies show that epigenetic mechanisms working through histone modification can modulate oligodendrocyte maturation (Copray et al. 2009). However, to our knowledge no studies have yet investigated the role of mechanisms related to DNA methylation. This project began to investigate the potential role of MeCP2, a transcriptional regulator known to bind to methylated DNA. As described below, this protein was originally thought to be exclusively expressed in cells of the neuronal lineage.
Figure 2: Markers in cells of oligodendrocyte lineage. Beginning with precursor and moving clockwise, markers appear and disappear over the course of neural development. CNP (or CNPase), MBP and MOG appear at different time points in maturation. Modified from (Baumann and Pham-Dinh 2001).
MeCP2, a multifunctional gene regulator

It is now well recognized that gene transcription and subsequent gene expression from DNA is in part regulated by epigenetic mechanisms, in which the DNA nucleotide sequence is unaltered, but the architecture of the molecule is modified in such a way so as to alter transcription. Chromatin is the form of DNA and associated proteins in a non-dividing cell, and can exist as euchromatin, or the more condensed heterochromatin. Euchromatin is the less condensed form of nuclear material, in which the DNA can be accessed by the transcriptional machinery of the cell; in contrast to heterochromatin, where the DNA molecule exists in varying states of compaction and cannot be transcribed. Therefore, transcription can be regulated by changing the state of DNA between these two types of chromatin.

Condensation of DNA is in part affected by methylation of the phosphate-deoxyribose backbone of the molecule. CpG sites are dinucleotide sections of the DNA in which a cytosine is adjacent to a guanine in the sequence 5’-CpG-3’, also referred to as CpG islands. These sites are often methylated on the cytosine residue in a reversible reaction carried out by DNA methyltransferase enzymes. Methyl-CpG binding domain (MBD) proteins interact with methylated-CpG islands and can modulate gene expression. The MBD domain is located near the N-terminal region of these proteins. The structure of this domain has been elucidated via x-ray crystallography and NMR spectroscopy, and highly conserved residues of the N-terminal region were visualized as interacting in the major groove of the methylated CpG island (Ohki et al. 2001).
Methylated-CpG-binding-protein-2 (MeCP2) is a nuclear protein that can act as a transcription repressor by binding to methylated DNA. This protein contains an 84-aa MBD and a 104-residue TRD. Human MeCP2 exists as two isoforms, the more common 486 aa protein or the 498 aa form. Post-translational processing has been shown to include one or more serine/threonine phosphorylation sites as well as SUMO-1 modification, giving a molecular weight of 75 kDa in mouse and rat brain, and 100 kDa in human cerebral cortex (Jarrar et al. 2003; Miyake and Nagai 2007). Large sections of the protein lack any discernable secondary structures, such as beta sheets and alpha helixes (Hansen et al. 2010). Lately, several observations have challenged the role of MeCP2 as a methyl-dependent repressor of transcription. Upon binding to methylated DNA, MeCP2 recruits co-repressor proteins. However recent evidence has shown that MeCP2 binding to methylated DNA is only marginally greater than its binding to unmethylated DNA; human MeCP2 binds methylated DNA at only ~3 fold the affinity with which this protein binds unmethylated DNA. Additionally, MeCP2 has been implicated not in inhibition but in the promotion of activity of some genes (Chahrour et al. 2008). These findings have led to categorize MeCP2 as a gene modulator, and its effect on transcription being determined by other factors influencing the strength of the gene’s expression (Chahrour et al. 2008). Furthermore, as later described in greater detail, recent studies have also shown that MeCP2 has a purported role in methyl-independent chromatin remodeling (Georgel et al. 2003), as well as a function in the regulation of RNA splicing (Young et al. 2005).

The MeCP2 gene is located at position Xq28, and mutations of this gene in the region encoding the MBD domain of the MeCP2 protein were shown to cause Rett’s syndrome (Amir et al. 1999), a cause of severe mental retardation in females. In addition, Rett syndrome is characterized by autistic behavior, seizures, autonomic nervous system disorders,
stunted growth, gastrointestinal disorders, ambulatory and motor problems, and poor or nonexistent verbal skills. The clinical severity of the illness has been linked to the activation of the affected X chromosome. When the faulty gene is activated in a larger proportion of cells, the degree of mental retardation is more severe than that observed in patients with activation in a smaller proportion of cells (Archer et al. 2007). MeCP2 mutation was once considered to be lethal in males, however this is now known to be incorrect, in fact a range of neurodevelopmental disorders are also encountered in males possessing a mutation of this gene (Villard 2007).

MeCP2 has been described as a protein highly expressed in neurons, but not in glial cells. However, analysis of a MeCP2 knockout mouse have shown altered levels of myo-inositol, a putative glial marker (Saywell et al. 2006). Additionally, more recent studies of MeCP2 in astrocytes have shown that the protein is not only expressed in theses cells, but that their growth is inhibited when transfected with siRNA for MeCP2 (Nagai et al. 2005). Clinical evidence has also pointed to the alteration of myelin in MeCP2-related illness. White matter abnormalities in Rett syndrome patients have been documented for over twenty years (Oldfors 1990). More recent DTI studies in living patients have shown white matter abnormalities in the corpus callosum, external capsule, and other white matter tracts (Mahmood et al. 2010). These studies taken together indicate a potential role of MeCP2 in oligodendrocytes.

Only one study so far has investigated myelin protein gene expression in a MeCP2 knockout mouse. However, this recent report used an animal model in which the MeCP2 gene was eliminated by a nestin-Cre knockout mechanism. Because nestin is expressed in all neural progenitors, these animals lack MeCP2 expression in neurons and glial cells and thus the findings cannot be ascribed specifically to a direct role of MeCP2 in oligodendroglia. A
reflection of the complexity of this system is the fact that changes in mRNA expression for myelin-related genes were region-specific. Analysis of the mutant animals showed that in the case of MBP, higher levels of mRNA were detected in the corpus callosum and forebrain while levels in the cerebellum were unaffected. Similarly, the results showed MAG mRNA levels in the corpus callosum of MeCP2\textsuperscript{null} mice were three times higher than in the wild-type controls, though they were unaffected in forebrain and cerebellum. PLP mRNA in the forebrain was significantly increased as well(Vora 2010). This study did not indicate the relative levels of protein in this animal model, nor were actual levels of myelin protein measured. Thus, the function of MeCP2 in oligodendrocytes and myelination has yet to be determined.

Previous studies from this laboratory (Sato-Bigbee and Coelho 2009), that MeCP2 may be involved in oligodendrocyte differentiation. In the present thesis, we have examined the role of MeCP2 in cultures of purified oligodendrocytes. Our research not only indicates that MeCP2 is expressed in oligodendrocytes, but also that this protein plays a role in the maturation of these cells. This novel finding, coupled with the role of MeCP2 mutation in Rett syndrome, may have implications for the understanding and future research into the pathology of this disease and other CNS disorders with altered MeCP2 expression.
Materials and Methods

Materials

Percoll, bovine pancreas DNase and papain for cell isolation as well as all cell culture medium components were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco’s modified Eagle’s medium/ Ham’s F-12 (DMEM/F-12) (1:1) medium was obtained from Invitrogen (Grand Island, NY). Reduced growth factor Matrigel was from Becton Dickinson (Franklin Lakes, NJ). All gel electrophoresis reagents and supplies were purchased from Bio-Rad Laboratories (Hercules, CA). The mouse anti-β-actin and rat anti-MBP (82-87 region) monoclonal antibodies were from Sigma-Aldrich and Millipore Corporation (Temecula, CA), respectively. The anti-MeCP2 polyclonal antibody (465-478 region) was purchased from Millipore Corporation (Temecula, CA). Super Signal West Dura reagent was obtained from Pierce (Rockford, IL). The secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Jackson ImmunoResearch Laboratories, Inc (West Grove, PA).

Animals

Sprague-Dawley rats were provided by Harlan Laboratories (Frederick, MD). Animal use and oligodendrocyte isolation were conducted in accordance with the guidelines from the National Institutes of Health and approved by the Virginia Commonwealth University Animal Care and Use Committee.

Isolation of pre-oligodendrocytes and preparation of cell cultures
Pre-oligodendrocytes were directly isolated from 9-10 day-old rat brains as described previously (Colello and Sato-Bigbee 2001) with minor modifications. For this, the animals were sacrificed by decapitation and the brains rapidly dissected out, transferred to ice, and the meninges and main blood vessels removed by rolling the tissue on sterile filter paper. After mincing into 1-2 mm pieces, the tissue was incubated for 25 min. at 37°C in the presence of 1 unit/ml papain and 0.01 mg/ml DNAse. Following extensive washes and filtration through a 75µm pore size nylon mesh, the resulting cell suspension was centrifuged for 15 min at 30,000xg in an isotonic self-generated Percoll gradient. The band enriched in oligodendrocytes was then subjected to differential adhesion on tissue culture-treated Petri dishes to eliminate microglial cells and residual astrocytes. The floating oligodendrocytes were then plated in 25-well plates (Falcon) previously coated with 25 µL/well reduced growth factor-Matrigel™ extracellular matrix. Prior to use, the cells were maintained overnight in chemically defined medium (CDM) (DMEM/F-12 supplemented with 1 mg/mL fatty acid-free bovine serum albumin, 50 µg/mL transferrin, 5 µg/mL insulin, 30 nM sodium selenite, 0.11 mg/mL sodium pyruvate, 10 nM biotin, 20 nM progesterone, 100 µM putrescine, and 30 nM triiodothyronine). Astroglial contamination, as assessed by glial fibrillary acid protein staining, was less than 5%.

**Analysis of MeCP2 expression at different stages of oligodendrocyte development**

To compare MeCP2 levels at different steps of oligodendrocyte maturation, cells were directly isolated from the rat brain as indicated above at 3, 5, 10, 18, and 25 days postnatal. Equivalent numbers of cells were solubilized in 60 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% sodium dodecyl sulfate (SDS), and 5% 2-mercaptoethanol and maintained at -80°C until further analysis by western blotting.
Inhibition of MeCP2 expression

MeCP2 levels were downregulated in cultures of pre-oligodendrocytes one day after isolation from 9-10 rat brains as indicated above. For this, 80% confluent cell cultures were treated with MeCP2 specific siRNA duplexes (100 nM final concentration), designed using the “HiPerformance siRNA Design Algorithm” program from Qiagen (Bethesda, MD). Double-stranded scrambled non-functional /non-targeting siRNA was used as control. Transfection of the RNAs was carried out for 3 hours by using the GeneJammer transfection reagent (Statagene, CA) following the manufacture's recommendations for cultured adherent cells (Saini et al. 2005a). At the end of the transfection procedure, the medium was replaced by fresh CDM. Cell cultures under each condition were then examined for MeCp2 and myelin protein expression at the times indicated later in the text.

Analysis of proteins by Western Blotting

Oligodendrocyte cultures were lysed in 50-75 µL 60 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% SDS, and 5% 2-mercaptoethanol. After boiling for 5 minutes, proteins in 10 µL samples were then separated by SDS-polyacrylamide gel electrophoresis (PAGE) in 15% acrylamide and electro-transferred to nitrocellulose. The membranes were then subjected to immunoblot analysis as previously reported (Saini et al. 2005a), with minor modifications. Nonspecific antibody binding was blocked by incubation in 10 mM Na₂HPO₄, 2.7 mM KCl and 137 mM NaCl, pH 7.4, (PBS) containing 3% nonfat-dry milk and 0.05% Tween-20 (blocking solution), for 1 hr at room temperature. Blots were then incubated overnight with anti-MeCp2 (dil. 1:1,000), anti-MBP (dil. 1:100), anti-CNPase (dil.1:1,000) or anti-MOG
(dil.1:1,000) antibodies. β-Actin levels detected with anti-β-actin antibody (dil. 1:5,000) were used as loading controls. After three 10 minute washes with PBS, blots were incubated for 30 min in blocking solution, followed by incubation with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 2 hrs. All antibodies were diluted in blocking buffer. At the end of the incubations blots were-washed twice for 5 minutes with PBS containing 0.05% Tween-20 and four times for 10 minutes with PBS alone .The immunoreactive bands were then detected by chemiluminescence with Super Signal West Dura reagent. The relative amount of immunoreactive protein in each band was determined by scanning densitometric analysis of the X-ray films and the use of the NIH Image J program. The relative density values for each specific protein in question were divided by the corresponding β-actin levels to correct for total protein concentration and sample loading differences.

**Statistical Analysis**

Statistical analysis was carried out by Student's T test using the GraphPad Prism program. Differences between results were considered statistically significant when p values were less than 0.05.
Results

Analysis of MeCP2 expression at different stages of oligodendrocyte development

As described in the Introduction section, preliminary results from the laboratory detected the presence of MeCP2 in cultured oligodendrocytes and suggested a role in their maturation. Thus, we have now continued those studies and determined the expression of this protein in oligodendrocytes \textit{in vivo} and at different stages of brain development. Our initial studies using direct immunocytochemistry of brain tissue slices failed to clearly assess the extent of protein expression in the oligodendrocytes due to high staining in other cells and elevated background levels. Therefore, we decided to solve this problem by investigating the developmental expression of MeCP2 in oligodendrocytes that were directed isolated from rat brain at different postnatal ages. Previous studies from the laboratory (Sato-Bigbee et al. 1999a) determined that cells isolated from 3- to 5-day-old rat brain are immature OPCs, while cells from 10-day-old animals belong to the pre-oligodendrocyte stage. Cells obtained from 25-day-old rat brains are for the most part already mature myelinating oligodendrocytes. As shown in Figure 3, while MeCP2 is expressed at all developmental stages, the protein levels are the highest in the cells isolated from the 10-day-old pups, a pattern of expression that pointed to a functional role of MeCP2 as pre-oligodendrocytes are primed to mature into myelinating cells.
Figure 3: MeCP2 levels at different stages of oligodendrocyte development.

Oligodendrocyte lineage cells at different developmental stages were directly isolated from the rat brain at postnatal days 3 and 5 (OPCs), 10 (pre-oligodendrocytes), 18 (actively myelinating oligodendrocytes at the peak of rat brain myelination), and 26 (oligodendrocytes at the end of the active period of rat brain myelination). The relative OD values of MeCP2 for each of the samples were normalized by the corresponding OD values for actin.
Analysis of MeCP2 function in pre-oligodendrocytes

Based on the developmental expression of MeCP2 in the oligodendrocytes described above, we next decided to investigate the function of this protein in pre-oligodendrocytes obtained from 9-10 day-old rat brain. For this, the freshly isolated cells were plated at about 80% confluence and on the following day, transfected with double-stranded siRNA specific to MeCP2 mRNA. Controls cultures were treated in a similar manner with a scrambled siRNA. Western blot analysis of the cells 24 hours after transfection showed that this treatment significantly reduces MeCP2 levels to about 50% of the control values (Figure 4).

We then tested whether cells with reduced MeCP2 levels exhibit any changes in the expression of different myelin-oligodendrocyte specific proteins that are expressed at different stages of cell maturation. Pre-oligodendrocytes do not express MBPs but the synthesis of these proteins is upregulated as they differentiate into more mature cells. As shown in Figure 5, analysis of the different MBPs three days after transfection, indicated effects that were splicing variant-specific. No major changes were observed for the levels of the 17.0 kDa, 18.5 kDa, and 21.5 kDa MBP isoforms while cell cultures treated with MeCP2 siRNA exhibited significantly reduced levels of the 14.0 kDa MBP.

We also chose to investigate MeCP2 involvement in the expression of CNPase, a protein that is already present in pre-oligodendrocytes and MOG, a glycoprotein that is expressed at the late stages of oligodendrocyte maturation. As shown in Figure 6, downregulation of
MeCp2 levels did not have any significant effects on the expression of CNPase. Interestingly, cell cultures treated with MeCP2 siRNA exhibited significantly reduced levels of MOG. Because both the 14 kDa MBP and MOG are expressed in the mature oligodendrocytes, these results suggest that MeCP2 may play an important role in the last stages of oligodendrocyte differentiation.
Figure 4: Inhibition of MeCP2 expression by siRNA treatment. Oligodendrocyte cultures prepared from cell directly isolated from 9-10-day-old rat brain were treated with MeCP2 siRNA as indicated under “Methods”. Control cultures were treated in a similar manner with scrambled siRNA. Western blot was used to detect MeCP2 expression 3 days after transfection. The western blot shows a representative experiment. The bar graph shows the mean ± SEM from 3 separate experiments. * p<0.003
Figure 5: Analysis of MeCP2 function in pre-oligodendrocytes. Pre-myelinating oligodendrocytes from 9-10 day old rat brains were treated with MeCP2 siRNA as indicated in “Methods”. Controls were treated with scrambled siRNA. Western blots were used to detect MBP protein levels three days post transfection. A representative western blot is shown. The columns indicate the mean ± SEM from 9 experiments. ** p<0.005
Figure 6: Analysis of MeCP2 function in pre-oligodendrocytes. Pre-myelinating oligodendrocytes from 9-10 day old rat brains were treated with MeCP2 siRNA as indicated in “Methods”. Controls were treated with scrambled siRNA. Western blots were used to detect CNPase and MOG protein levels three days post transfection. A representative western blot is shown. The columns indicate the mean ± SEM from 6 experiments. * p<0.05
Discussion

Previous research into MeCP2 function in Rett syndrome and autism-related disorders has been focused primarily on neurons, however, autopsies have shown the presence of white matter abnormalities in the brains of Rett syndrome patients, and recent fMRI studies in Rett syndrome patients reinforce these studies (Oldfors 1990; Mahmood et al. 2010). Furthermore, as described in the introduction a recent report indicated that MeCP2 null mice exhibit region-specific alterations in the levels of mRNA for different myelin proteins (Vora 2010). While myelin-related problems in both patients and the animal model could reflect neuronal alterations affecting both axon outgrowth and axo-glial interactions, the finding of MeCP2 presence in oligodendrocytes and our present observations raise the possibility that this protein may also play a crucial direct role in these cells controlling myelin formation.

Our findings indicate that MeCP2 is present in oligodendrocytes during the period of active rat brain myelination. Moreover, downregulation of MeCP2 levels in cultured oligodendrocytes has differential effects on the expression of myelin specific proteins that are suggestive of a role of MeCP2 in oligodendrocyte maturation. The proteins that we have examined in this study place the importance of MeCP2 function in the last stages of oligodendrocyte development. MOG as well as the 14 kDa MBP isoform are unique in that they are expressed later in myelin formation and we have found that their levels are significantly reduced in the cells in which MeCP2 expression is downregulated. Understanding of the mechanisms underlying these effects is complicated by the fact that several recent publications uncovered multiple roles of MeCP2 that expand well beyond its originally proposed function as a transcriptional repressor (Newell-Price et al. 2000; Georgel et al. 2003; Nagai et al. 2005; Wade
Based on these multiple functions, possible reasons for the effects we observed in the oligodendrocytes may include: (1) a role for MeCP2 in the silencing of genes which could in turn downregulate MOG and 14KDA MBP expression; (2), a role for MeCP2 as a transcriptional activator perhaps working in conjunction with other transcription factors important for oligodendrocyte development; (3), a potential function in the splicing of the Golli-MBP gene, and/or a role in the expression of transport proteins responsible for the movement of MBP mRNA to the oligodendrocyte processes; and (4), a MeCP2 function in large-scale chromatin remodeling independent of methylated DNA binding.

It has been previously shown that in neurons, MeCP2 seems to function as a developmental brake, slowing down some processes important for cell maturation (Carter and Segal 2001). One possibility in the oligodendrocytes is the function of MeCP2 in gene silencing via methylation-dependent recruitment of histone deacetylases (HDACs). Gene silencing through the binding of methylated CpG sites may be the most easily modeled way in which MeCP2 downregulation affects the expression of myelin proteins. As previously discussed, MeCP2 was first shown to be involved in the silencing of genes by a mechanism in which a protein complex including Sin3A, HDAC, and MeCP2 attaches to methyl-CpG groups utilizing the methyl binding ability of MeCP2. This localization allows HDAC in complex with MeCP2 to remove the acetyl groups from nearby histone proteins, thus promoting compaction of chromatin and by extension silencing local gene expression (Wade 2005). Based on our present results, it is possible to speculate that a DNA-methylation dependent gene silencing mechanism mediated by MeCP2 may be indirectly responsible for controlling the observed results on the level of MOG and 14 kDa MBP protein levels in the oligodendrocyte. This could be the case if
MeCP2 binds to and silences genes encoding products which somehow maintain the oligodendrocytes in a less mature stage of development. While such possibility has not been investigated before, there is indeed evidence for the existence of negative regulators of oligodendrocyte maturation. Among these proteins, Hes-5 is a transcription factor expressed in neural cells which inhibits oligodendrocyte differentiation and is controlled by a promoter region with multiple CG rich regions (Takebayashi et al. 1995). MeCP2 has not been associated with Hes-5 expression, but this has not been investigated, and could be a possible mechanism responsible for our observed results.

Another possibility to explain our present findings is a function of MeCp2 as a transcriptional activator. Evidence has emerged in the last half-decade which questions the exclusive role of MeCP2 as a gene repressor as MeCP2-promoter binding has been shown to increase expression of certain genes. Genomic analysis of hypothalami from six-week old wild-type, MeCP2null and MeCP2 over-expressing mice demonstrated that 85% of the genes showing altered activity exhibited increased expression in the MeCP2 over-expressing mutants. On the contrary, these genes showed decreased expression in the MeCP2null mice (Chahrour et al. 2008). Thus, the classic role for MeCP2 as a silencer of gene expression is still correct in some cases, but the protein is increasingly being characterized as a gene "modulator" that can work both as inhibitor or activator.

The mechanisms underlying MeCP2 function as a positive regulator of gene expression are still being determined, but some models have been substantiated. It has been shown that MeCP2 associates with CREB1, an activator of gene expression. The MBD of MeCP2 has been shown as necessary for this particular mechanism of gene enhancement. A genome-wide study has shown upregulation of a multitude of genes, including the activation of
CREB1 which in turn increases expression of the micro-RNA miR134. This micro-RNA downregulates the expression of MeCP2; and so a negative-feedback mechanism may regulate expression of these molecules (Chahrour et al. 2008). Interestingly, previous results from this laboratory have implicated CREB as a mediator of a variety of signals that regulate oligodendrocyte development (Sato-Bigbee and DeVries 1996; Sato-Bigbee et al. 1999b; Afshari et al. 2001; Saini et al. 2004; Saini et al. 2005b). Moreover, CREB was shown to play a crucial role regulating MBP gene expression (Sato-Bigbee and DeVries 1996; Afshari et al. 2001) although this gene lacks CREB binding sites. Thus, it is tempting to hypothesize that both MeCP2 and CREB could be part of a common mechanism that could indirectly stimulate MBP, and perhaps also MOG, gene activities.

Particularly intriguing is the fact that MeCP2 downregulation was accompanied by a decrease in the 14 kDa MBP levels without majorly affecting the expression of the other MBP splicing isoforms. As discussed previously, the 14 kDa MBP is characteristic of mature oligodendrocytes and thus, its reduced expression in the cells with decreased MeCP2 levels may be a reflection of a general effect of MeCP2 in cell development. However, different reports investigating novel roles of this multifaceted modulator also raise the possibility of direct MeCP2 effects on gene splicing. MeCP2 was shown to associate with the RNA splicing factor YB-1 in a specific and RNA dependent manner, and modification to remove the DNA methyl-binding properties of the MBD in MeCP2 did not prevent this association (Young et al. 2005). YB-1 is one of the most highly conserved DNA binding proteins (Kohno et al. 2003) and one of its functions includes controlling the alternative splicing of mRNA for the cell adhesion molecule CD44. Experiments in MeCP2 over-expressing HeLa cells demonstrated that this function does not involve changes in YB-1 levels, ruling out indirect effects on gene splicing by
transcriptional regulation of the YB-1 gene (Young et al. 2005). Another factor that was shown to associate with MeCP2 in the rat brain is the hnRNP TDP-43. TDP-43 is involved in splicing of mRNA during synapse formation, neuron development and RNA processing, including pre-mRNA splicing, mRNA transport and stability (Buratti 2008; Sephton et al. 2011). To our knowledge, none of these splicing factors have been studied in oligodendrocytes, however it would be interesting to examine a potential role of MeCP2 and these proteins in these cells. We cannot discard the possibility that MeCP2 may associate with other RNA binding proteins known to be involved in myelin synthesis, such as hnRNP A1, a protein known to participate in the intracellular transport of MBP mRNA in oligodendrocytes (Shan et al. 2000). As TDP-43 is a related molecule, investigation of a potential association between hnRNP A1 and MeCP2 may be useful in elucidating a mechanism of MeCP2-mediated RNA processing.

Finally, another potential mechanism of MeCP2 function is large-scale chromatin remodeling independent of methylated DNA binding. Large-scale modifications of chromatin, such as looping, may be the primary method by which MeCP2 could affect MOG and 14KDA MBP protein levels during development. It has been demonstrated that the shape of nucleoli and heterochromatic chromocenters is significantly altered in neurons lacking MeCP2 (Singleton et al. 2011). In vitro experiments have shown that MeCP2 compacts nucleosomes into secondary chromatin structures, and does so importantly in the absence of salts (as well as in the presence of physiological salt concentrations). The remodeling is distinct from the methylated CpG binding/deacetylation role as the binding sites for MeCP2 in these examples are often unmethylated (Yasui et al. 2007) and missense and nonsense mutants in which the MBD of MeCP2 has been affected do not cause loss of chromatin condensation induced by this protein (Georgel et al. 2003). Two high-mobility group domains have been implicated in this property of
MeCP2, which is also unaffected by the presence of methyltransferase inhibitors. These chromatin modifications cannot be classified as uniformly downregulatory or upregulatory, but they further substantiate the theory that MeCP2 is in fact a modulator of gene expression. Thus, it would be important to determine if downregulation of MeCP2 expression in our experiments is accompanied by changes in chromatin remodeling in the oligodendrocytes and the extent to which such changes could regulate myelin protein expression.

An important consideration is that the functions described above for MeCP2 are not mutually exclusive, and in developing oligodendrocytes this protein may be performing a combination of several of the regulatory mechanisms described above (Figure x). Maturing oligodendrocytes and myelination are subjected to a myriad of regulatory mechanisms and at this moment it is difficult to ascertain if any of them could at the same time determine a prefer MeCP2 mode of action. As research continues on the increasingly complex role that MeCP2 plays in epigenetics, gene splicing and other gene expression mechanisms, it seems likely that any cell type which expresses this protein will exhibit substantial developmental abnormalities when afflicted with a mutation in the MeCP2 gene.

Regardless of the mechanism, the findings presented in this thesis provide for the first time direct support for an important role of MeCP2 in the control of oligodendrocyte development and myelination. Future experiments should investigate if the role of MeCP2 found in the cultured cells is also observed in vivo and the extent to which this protein function in oligodendrocytes could contribute to the developmental alterations and symptoms observed in Rett syndrome and autism-related disorders with abnormal MeCP2 expression. From a clinical point of view, future studies should also consider how adverse expression or function of MeCP2 in cells of the nervous system other than oligodendrocytes may indirectly affect CNS development.
myelination. Altogether, these studies could also contribute to the design of strategies that either by pharmacological intervention or genetic manipulation may stimulate remyelination in demyelinating diseases like multiple sclerosis.
Figure 7: Hypothetical mechanisms by which MeCP2 increases myelin protein expression.

A) Methylated DNA dependant gene repression. The MBD of MeCP2 localizes the protein to a region of the DNA molecule which regulates expression of Factor X, a downregulator of MOG transcription. Factor X would be an oligodendrocyte development inhibitor such as Id2 or Id4. This mechanism allows MeCP2 to indirectly enhance myelin protein expression.

B) Methylated DNA-dependant myelin protein enhancement. The MBD of MeCP2 localizes it to a DNA region where the protein directly upregulates transcription.

C) RNA splicing. MeCP2 associates with a splicing factor similar to TDP-43, facilitating myelin protein pre-mRNA processing.

D) Large scale chromatin remodeling. MeCP2 causes major structural changes in the architecture of chromatin, in this diagram placing the MOG gene in a conformation where it can be accessed by transcription mechanisms. Importantly MeCP2 may instead change chromatin in such a way as to downregulate transcription of myelin protein downregulators, such as Id2 or Id4.
References


Vitae

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Awards

Charles C. Clayton Award - for outstanding performance in the Department of Biochemistry and Molecular Biology Master’s Program