Methyl-CpG binding proteins in the nervous system

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ABSTRACT

Classical methyl-CpG binding proteins contain the conserved DNA binding motif methyl-cytosine binding domain (MBD), which preferentially binds to methylated CpG dinucleotides. These proteins serve as transcriptional repressors, mediating gene silencing via DNA cytosine methylation. Mutations in methyl-CpG binding protein 2 (MeCP2) have been linked to the human mental retardation disorder Rett syndrome, suggesting an important role for methyl-CpG binding proteins in brain development and function. This mini-review summarizes the recent advances in studying the diverse functions of MeCP2 as a prototype for other methyl-CpG binding proteins in the development and function of the vertebrate nervous system.

Keywords: MeCP2, MBD proteins, DNA methylation, neuronal differentiation, chromatin remodeling, gene silencing, histone modification.

INTRODUCTION

In vertebrate animals DNA cytosine methylation is one of the major epigenetic factors, which regulates many cellular events including developmental gene regulation, X chromosome-inactivation, genome defense, and genomic imprinting [1]. The methylation pattern is established during embryogenesis by a family of DNA methyltransferases (Dnmts). Mutant mice lacking either the maintenance enzyme Dnmt1 or both *de novo* methylases Dnmt3a and Dnmt3b exhibit demethylation in the genome and die at the mid-gestation stage, indicating that methylation is essential for embryogenesis [4-6]. Alterations in DNA methylation machinery have been linked to human diseases such as cancer and several mental retardation disorders, including Rett, ICF, Fragile-X, and ATRX syndromes, suggesting an important role for DNA methylation in brain development and function [5]. Consistent with this notion, both Dnmt1 and Dnmt3 proteins are expressed at high levels in embryonic and mature central nervous system (CNS) [6-8]. To directly examine the effect of DNA hypomethylation on the CNS, we have used the cre/loxP system to conditionally delete the *Dnmt1* gene in the entire embryonic CNS [9]. Conditional mutant mice with brains consisting of 95% hypomethylated cells die at birth due to respiratory distress, confirming that DNA methylation is required for the vital CNS function [9]. Furthermore, in mosaic animals containing 30% of Dnmt1$^{-/-}$ cells in the CNS, hypomethylated cells are quickly eliminated from the brain within 3 weeks of postnatal life [9], indicating that DNA methylation is of critical importance for the survival of postnatal CNS neurons.

The effect of DNA methylation on CNS development and function is postulated through its regulatory role in neuronal gene expression [5, 9]. Methylation-mediated gene regulation is proposed to occur through the following two mechanisms. Firstly, CpG methylation within the transcription factor binding domain may directly interfere with the binding of certain transcription activators to the target sequence [10, 11]. More generally, methylation-mediated gene silencing is through the action of a family of methyl-CpG binding proteins such as MeCP2 and MBD1, which preferentially bind to methylated CpG(s) [12-14]. These methyl-CpG binding proteins are transcriptional repressors themselves, which are further coupled to other co-repressor proteins and histone modification enzymes that lead to repressive chromatin-remodeling and gene silencing [15-17]. By employing sequence homology searches to the conserved methyl-binding domain (MBD) of MeCP2 and

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MBD1, additional three members MBD2-4 have been identified (Fig. 1) [18]. MBD4 turns out to be a DNA T:G mismatch repair enzyme and may function to minimize mutation at methyl-CpG sites [19]. Biochemical assays have shown that MBD3 cannot bind methylated DNA directly, so strictly speaking, MBD3 does not qualify as a methyl-CpG binding protein [18]. MeCP2 protein is particularly abundant in the mature CNS [14, 20-22], and transcripts of MBD1-3 are easily detected by Northern blot analysis in the adult brain [18]. In this mini-review, we will focus on the recent advances in studying MeCP2 as well as MBD1-3 function specifically in the nervous system.

MECP2 AS A PROTOTYPE OF METHYL-CpG BINDING PROTEIN

eCP2 was the first identified methyl-CpG binding protein through biochemical purification [12]. This ~80 kD nuclear protein preferentially binds to methylated CpG site *in vitro* with high affinity [12, 23]. *In vivo*, MeCP2 exists in two alternatively spliced isoforms with the longer isoform of MeCP2α more abundantly expressed in the nervous system [24, 25]. However, this longer form of splice variant does not confer any differences to the original functional assessment of the slightly shorter isoform MeCP2β as a transcriptional repressor. Serial deletion analysis of MeCP2β protein mapped the MBD domain at the N-terminus, specifically the first 78–162 aa of the 486 aa long human MeCP2β isoform (Fig. 1) [23]. DNase I *in vitro* foot printing indicated that MeCP2 binding could protect a 12-nucleotide region surrounding any single methyl-CpG site [23]. Further biochemical and functional studies reveal that MeCP2 contains three functionally critical domains that are thought to mediate its proposed function as a transcriptional repressor. The three domains are: 1) the methyl CpG binding domain (MBD) that confers binding to methylated CpG dinucleotides [23]; 2) a transcriptional repression domain (TRD), which interacts with various co-repressor complexes such as mSin3A-HDAC1, c-Ski, and histone methyltransferases [15-17, 26]; and 3) the carboxyl terminus, which shares some homology to the forkhead transcription factors BF1 and FKH4 [27]. Thus MeCP2 unites DNA methylation, chromatin remodeling, and transcription to provide a prototypical mechanism for gene silencing via MBD containing proteins.

The link between MeCP2 mediated gene silencing and nervous system disorders further highlights the importance of MeCP2 for proper nervous system function. Human genetic studies of Rett syndrome (RTT) cases mapped the disease gene in the Xq28 region and sequencing analysis identified the gene mutations in the X-linked *MECP2* gene [27, 28]. Typically, in female heterozygous

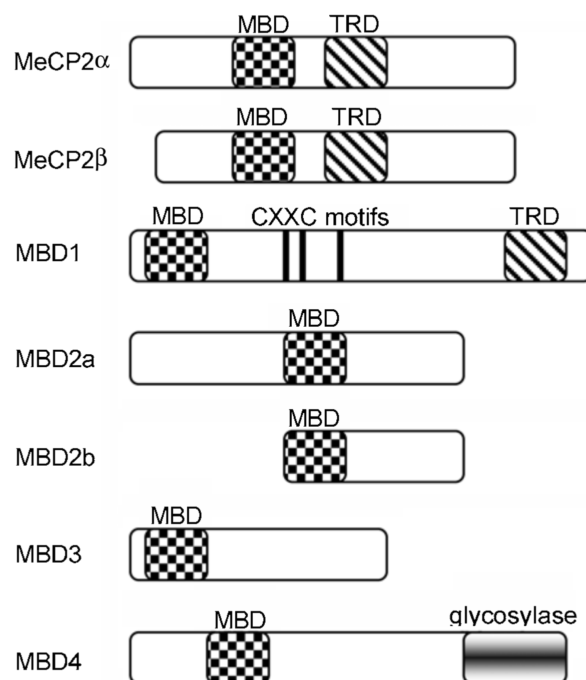

Fig. 1 Schematic drawing of a family of methyl-CpG binding proteins. Members of this family contain the conserved methyl-CpG-binding domain (MBD, checkerboard box). MeCP2 isoforms are represented, with the mMeCP2α long isoform differing from the well characterized MeCP2β isoform in its transcription start site and lack of exon 2 [25,26]. Both MeCP2 transcripts and MBD1 contain transcription repression domains (TRD, diagonal striped box), allowing for interaction with various co-repressor complexes. MBD1 contains cysteine-rich CXXC motifs (black bars) that bind specifically to unmethylated CpG [52]. MBD4 contains a thymine glycosylase domain and has been reported to function as a DNA mismatch-repair enzyme [20]. Note that for the simplicity of the graph, several alternatively spliced isoforms of MBD1 and MBD3 are not depicted here.

patients due to random X-chromosome inactivation, wild-type MeCP2 protein is expressed in 50% of cells, while mutated MeCP2 protein is expressed in the other half. This mixture of functional wild-type and MeCP2 mutant cells leads to RTT etiology. Males that are hemizygous for comparable *MECP2* mutations rarely live beyond 2 years, exhibiting severe phenotypic differences than that of RTT females coupled with congenital encephalopathy. Most missense mutations in *MECP2* gene linked to RTT syndrome are clustered at the MBD domain [27, 28]. Analysis of MeCP2 expression indicated that MeCP2 expression is the highest in neurons [20-22]. Moreover, the initiation of MeCP2 expression is coincident with the synaptogenesis process, suggesting that MeCP2 plays an important role in neuronal maturation [20-22]. In all, research to date into MeCP2 function and RTT syndrome lays the foundation

for future research focusing on the specific role of MBD proteins in CNS development and function.

RODENT MODEL OF MECP2 DEFICIENCIES AND MUTATIONS

To address the MeCP2 function *in vivo*, transgenic mice bearing null or truncation mutations have been generated [29-31]. These mutant mice exhibit neurological phenotypes mimicking the RTT phenotype [29-31]. MeCP2 null male mice and female mice do not exhibit phenotypes until 6–8 weeks postnatally. After this presymptom period, mutant male mice show a period of rapid regression exhibiting reduced spontaneous movement, clumsy gait, irregular breathing, hindlimb clasping, and tremors, culminating in death before the age of 12 weeks [29, 30]. Detailed histological examination revealed that mutant mice have smaller brains and neuronal sizes than those in wild-type animals [30]. Mutant mice carrying *MeCP2* C-terminal truncation mutation can survive much longer but also develop progressive RTT phenotypes starting 6 weeks of age [31]. In an attempt to identify whether neuronal gene expression was deregulated, DNA microarray analysis was carried out to compare the gene expression profiles between control and mutant brain samples [32]. Surprisingly, only slight differences in the expression levels of a small subset of neural genes were found [32]. However, such a DNA microarray analysis may have sensitivity limitations for detecting differential gene expression in MeCP2 mutant brain samples.

Using conditional gene knockout approach, Chen *et al* [30] further showed that postnatal neuronal deficiency of MeCP2 is the main etiology of RTT phenotype in mice. This raises the possibility whether RTT phenotype can be rescued by postnatal expression of MeCP2. Towards this goal, a *MeCP2* transgene was expressed under the control of endogenous neuronal specific promoter Tau [33]. Neuronal expression of the *MeCP2* transgene indeed rescues the RTT phenotype of *MeCP2* knockout mice, confirming the maintenance role of MeCP2 in the central nervous system [33]. Interestingly, over-expression of MeCP2 in neurons is detrimental and leads to symptoms of severe motor dysfunction and seizures; moreover, approximately 30% of animals overexpressing *MeCP2* die by 1 year of age [33, 34]. These results suggest that a proper dosage of MeCP2 is critical for neuronal function and that levels of MeCP2 in the nervous system are tightly regulated.

A CLASSICAL MODEL OF MeCP2 IN REGULATING NEURONAL GENE EXPRESSION

To identify the MeCP2 target genes, several groups including our lab have taken the candidate gene approach. Using a paradigm of depolarization-induced neuronal gene expression, we and others found that MeCP2 is involved in regulating the expression of a neurotrophin gene brain-derive neurotrophic factor (BDNF) [35, 36]. BDNF promotes neuronal survival and modulates neuronal synaptic plasticity such as long-term potentiation (LTP). It has been reported that the *Bdnf* promoter is activated upon membrane depolarization in cultured embryonic cortical and hippocampal neurons via KCl (50 mM) treatment, which leads to calcium influx, activation of signaling cascades and the subsequent activation of an array of genes involved in neural plasticity [37, 38]. It was found that in resting/non-depolarized neurons the mouse *Bdnf* exon IV promoter is more methylated and more tightly associated with the methyl-CpG binding protein, MeCP2, which triggers inactive chromatin remodeling that involves histone deacetylation and histone H3-K9 methylation [35, 36]. Upon depolarization, a subpopulation of MeCP2 protein, presumably the population bound to the *Bdnf* promoter, becomes phosphorylated, which leads to the dissociation of MeCP2 from the *Bdnf* promoter. The dissociation of MeCP2 is coupled with the release of co-repressor proteins such as mSin3a and HDAC1, leading the active chromatin-remodeling for *BDNF* gene activation [35, 36]. These results provide the first example that MeCP2 is important for activity-dependent neuronal gene regulation.

MeCP2 is also implicated in silencing neuronal gene expression in non-neuronal cells. It has been suggested that MeCP2 in non-neuronal cells will interact with REST/CoRest repressor complex and lead to the silencing of neuronal genes such as the type II sodium channel (*Nav1.2*) gene [39]. Through a series of experiments probing the expression of a cluster of neuronal gene located at the chromosome 3q21-q36 region in rat fibroblasts, the authors show that interruption of either MeCP2 function or REST/CoREST binding to the RE1/NRSE elements derepresses neuronal genes in this locus, even for genes whose promoters do not contain the RE1/NRSE element [39]. Chromatin immunoprecipitation assays reveal REST, CoREST, and MeCP2 association with the promoters of neuronal genes within the rat 3q21-36 locus; moreover, this association was interrupted by 5-azacytadine treatment-induced demethylation. These results support a model that site-specific DNA methylation and MeCP2 binding coordinate with REST/CoREST repressor complex in silencing neuronal genes in non-neuronal cells [39]. These examples, coupled with extremely specific neural dysfunction of both RTT patients and rodent MeCP2 null mutants, strongly implicate MeCP2 in epigenetic regulation of neu-

ronal genes.

NEW HYPOTHESES FOR MeCP2-MEDIATED GENE SUPPRESSION

While genome-wide microarray analysis of gene deregulation in MeCP2 deficient human and mouse cells did not yield the most promising results, several new approaches have been used to map the target genes of MeCP2. Horike *et al* [40] use chromatin-immunoprecipitation-combinded loop assays to identify target genes that are flanked by MeCP2. Among the first 33 MeCP2-binding sequences they analyzed, they found the expression of maternally-imprinted *Dlx5* gene is increased by two-fold in MeCP2-deficient mouse cells and human lymphoblastoid cells, suggesting maternal imprinting of *Dlx5* is tightly regulated by MeCP2 [40]. Dlx5 is involved in the regulation of enzymes that synthesize gamma-aminobutyric acid (GABA), a neurotransmitter for inhibitory GABAergic neurons in the CNS. These results suggest that MeCP2 can regulate gene expression and maternal imprinting through formation of a silent-chromatin loop [40].

MeCP2 has been purified in a number of repressor complexes including ATPase-dependent SWI/SNF chromatin-remodeling complex [41]. Most recently, it has been reported that Brahma (Brm), a catalytic component of SWI/SNF complex, is associated with MeCP2 *in vivo* [42]. SWI/SNF is an ATPase-dependent remodeling complex known to disrupt nucleosome structure, thus altering access to DNA and regulating gene transcription. Chromatin immunoprecipitation experiments reveal that Brm and MeCP2 assemble on methylated genes in cancer cells as well as on the *FMR1* gene in cells from fragile-X syndrome patients, which involves hypermethylation-mediated silencing of *FMR1* due to a triplet CGG repeat expansion [42]. These findings provide a new model for MeCP2-linked SWI/SNF in silencing neuronal gene expression [42].

In addition to the above models of MeCP2-mediated gene regulation, evidence also exists that MeCP2 could serve as a sequence-specific DNA binding protein to repress gene expression independent of DNA methylation. The N-terminus of mammalian MeCP2 is highly homologous to the chicken ARBP, a nuclear protein exhibiting a high affinity interaction with the AT-rich nuclear matrix attachment region and scaffolding in the nucleus [43, 44]. This raises the possibility that MeCP2 may be important for the domain organization of chromatin. Furthermore, human MeCP2 protein can induce compaction of unmethylated nucleosome array *in vitro*; and the region (s) of MeCP2 containing the activity of chromatin compaction resides outside the MBD domain [45]. That MeCP2 can serve as a structural chromatin protein in gene regulation has also recently been reviewed by Bowen *et al* [46]. Considering that MeCP2 is particularly abundant in mature neuronal cells, future studies shall examine whether MeCP2 also regulates neuronal gene expression through its methylation-independent effect on nucleosome-remodeling *in vivo*.

POTENTIAL ROLE FOR MeCP2 IN NEUROGENESIS AND NEURONAL MATURATION

Several expression studies of MeCP2 in rodent and primate brains suggests that MeCP2 is highly abundant in the mature nervous system and relatively low in neural precursor cells, astrocytes, and oligodendrocytes. Using amphibian *Xenopus laevis* embryos as a model system, Stancheva *et al* [47] has shown that MeCP2 is a partner of the SMRT co-repressor complex regulating the expression of the neuronal repressor xHairy2a in differentiating neuroectoderm. Further, a truncated form of MeCP2 (R168X) found in RTT patients cannot interact with SMRT complex properly leading to stable xHairy2a repression and abnormal patterning of primary neurons during neuronal differentiation [47]. However, in the mammalian system, MeCP2 is found to play a less significant role in neurogenesis. Using neural stem cell culture system, Kishi and Macklis [48] did not find any differentiation defect in MeCP2-deficient neural precursor cells. Nevertheless, they found that neocortical projection layers in *MeCP2*$^{-/y}$ mice are thinner than those in wild-type mice; furthermore, pyramidal neurons in layers II/III in *MECP2*$^{-/y}$ mice are smaller and less complex that those in wild-type mice [48]. These results are consistent with the role of MeCP2 in the maturation and maintenance of CNS neurons such as dendritic arborization. In the olfactory system, MeCP2 expression is detected during neurogenesis and prior to synaptogenesis [49]. MeCP2 deficiency causes a transient delay in the terminal differentiation of olfactory neurons, leading to abnormal axonal projections and disorganization of glomerular structure [50]. Thus these initial studies provide insight into MeCP2-mediated gene regulation during neurogenesis and neuronal maturation, a process to be better understood as more gene targets for MeCP2 are discovered.

OTHER METHYL-CpG BINDING PROTEINS

Expression of MBD1 is ubiquitous in somatic cells, but notably absent in embryonic stem cells [18]. Initially studies showed that MBD1 binds to densely methylated CpG sites and actively represses gene transcription through a C-terminal TRD domain (Fig. 1) [51]. More recently, MBD1 is found to be recruited to both methylated and non-methylated CpGs for gene repression through distinct domains [52]. In dividing cells, MBD1 is coupled with

histone-methyltranferase complex at the replication foci and may be important for the maintenance of histone modification pattern at the region of methylated DNA [53]. *MBD1*$^{-/-}$ mice are healthy and fertile but exhibit decreased neurogenesis, impaired spatial learning, and increased genome instability of adult neural stem cells [54]. However, it is still unclear whether MBD1 is involved in regulating subsets of neuronal gene expression as defined for MeCP2 and how MDB1 plays a role in maintaining genome stability of adult neural stem cells.

MBD2 exists as two differentially expressed isoforms (MBD2a/2b), of which MBD2a differs by a 152 aa N-terminal extension (Fig. 1) [18]. In several independent biochemical purifications of the MeCP1 complex, MBD2 is proven to be a component of MeCP1 complex and is coupled with the Mi2/NuRD complex to mediate the gene silencing effect of NuRD/MeCP1 complex directed to methylated genes [55-58]. MBD2b was suggested to be a putative DNA demethylase [59], but this notion was not supported by follow-up biochemical and genetic analyses [56, 57, 64]. Additionally, MBD2a, but not MDB2b, associates with demethylated cAMP-responsive elements (CRE) to promote transcription via binding to the RNA helicase bridge of the CBP/PolII transcription initiation complex [60]. However, when the CpG site in the CRE element is methylated, both MBD2a and MBD2b act as repressors through the recruitment of histone deacetylases and the NuRD complex [60]. Interestingly, a series of biochemical purification experiments demonstrated that MBD3 protein is in fact a key component of Mi/NuRD complex [55, 58].

Both MBD2 and MBD3 mRNA are expressed in the adult brain [18]. In the developing brain, MBD3 expression can be detected in high levels in neuroepithelial cells whereas MBD2 is barely detected [61]. In adult CNS, MBD3 is strongly expressed in principle neurons of hippocampus and cortex but weakly expressed in outer cortical layer cells [61]. Both MBD2 and MBD3 can be transiently induced by kindling or transient ischemia in rodent hippocampus, suggesting that these proteins may be involved in alteration of gene expression upon lesions [62, 63]. *MBD2*$^{-/-}$ mice appear normal but do exhibit a defect in maternal nursing behavior, whose exact cause is still unclear [64]. On the other hand, the *MBD3*$^{-/-}$ mutation is embryonic lethal, consistent with the possibility that MBD3 is a necessary component for NuRD complex and involved in multiple gene regulatory pathways for embryogenesis [64]. Future study using a strategy for brain-specific conditional knockout of MBD3 will shed light on the specific role of MBD3 in the nervous system.

It is worth noting that there are other candidates of methyl-CpG binding protein that do not contain the conventional MBD domain. A distinctly different methylated DNA binding domain was found in the transcriptional repressor Kaiso (ZBTB33), which binds methyl-CpGs through a zinc-finger (ZF) motif [65]. Kaiso recognizes methylated DNA regions containing at least two methyl-CpGs. The knock-down of *Kaiso* leads to premature zygotic gene activation and subsequent developmental arrest and apoptosis, mimicking the phenotype of hypomethylated embryos deficient in xDnmt1 [66]. In Hela cells, Kaiso is coupled with the N-CoR corepressor to mediate DNA methylation-dependent gene silencing [67]. However, the exact role of Kaiso in the nervous system has yet to be determined.

CONCLUSIONS

The requirement for DNA methylation in the embryonic and postnatal CNS implies that methyl-CpG binding proteins such as MeCP2 and MBD1-3 are also important for the development and function of the nervous system. A major role of MBD proteins may be to mediate gene silencing in the nervous system. Indeed, example of reversible regulation of neuronal genes such as BDNF is shown to be mediated via the MeCP2 repressor complex. It is likely that different methyl-CpG proteins are involved in different repressor complexes to exert specific regulatory control of neuronal gene expression. The coupling of MBDs with nucleosome remodeling complex and histone modification enzymes also connects the gene silencing role of DNA methylation with chromatin-remodeling mechanisms. Future study will likely provide more mechanistic insight into the action of MBD proteins in neuronal differentiation and maturation in the CNS.

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