Disease Modeling Using Embryonic Stem Cells: MeCP2 Regulates Nuclear Size and RNA Synthesis in Neurons

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Key Words. Rett syndrome • Nuclear size • Transcription • Astrocytes • Brain-derived neurotrophic factor • Synaptophysin

Abstract
Mutations in the gene encoding the methyl-CpG-binding protein MECP2 are the major cause of Rett syndrome, an autism spectrum disorder mainly affecting young females. MeCP2 is an abundant chromatin-associated protein, but how and when its absence begins to alter brain function is still far from clear. Using a stem cell-based system allowing the synchronous differentiation of neuronal progenitors, we found that in the absence of MeCP2, the size of neuronal nuclei fails to increase at normal rates during differentiation. This is accompanied by a marked decrease in the rate of ribonucleotide incorporation, indicating an early role of MeCP2 in regulating total gene transcription, not restricted to selected mRNAs. We also found that the levels of brain-derived neurotrophic factor (BDNF) were decreased in mutant neurons, while those of the presynaptic protein synaptophysin increased at similar rates in wild-type and mutant neurons. By contrast, nuclear size, transcription rates, and BDNF levels remained unchanged in astrocytes lacking MeCP2. Re-expressing MeCP2 in mutant neurons rescued the nuclear size phenotype as well as BDNF levels. These results reveal a new role of MeCP2 in regulating overall RNA synthesis in neurons during the course of their maturation, in line with recent findings indicating a reduced nuclear size in neurons of the developing brain of mice lacking MeCP2.

Introduction
Rett syndrome (RTT) is a progressive neurodevelopmental disorder observed in about one in 10,000 females at birth [1, 2]. Affected girls show a variety of neurological symptoms including microcephaly early in life, autistic features, breathing arrhythmia, and mental retardation [2]. Mutations in the X-linked gene MECP2 encoding the methyl-CpG-binding protein 2 are the major cause of RTT [3]. As in humans, MeCP2 is X-linked in the mouse and the selective deletion of MeCP2 in postmitotic forebrain neurons has been shown to recapitulate important aspects of the disease [4, 5]. Most interestingly, global or neuron-selective re-expression of MeCP2 in animals deprived of MeCP2 for a few weeks after birth markedly improves the symptoms of affected animals and significantly increases their lifespan, suggesting that the lack of MeCP2 even early during brain development does not cause irreparable brain damage [6, 7]. Yet in spite of important progress in modeling RTT in mice, the functional role of MeCP2 in the developing brain is still unclear. MeCP2 is expressed at high levels in adult neurons, comparable to those of histone H1 [8], suggesting a general function in the arrangement of the chromatin. While mRNA-based microarray studies revealed that almost 10% of annotated genes in the mouse genome is, for the most part, underexpressed in the adult brain of MeCP2−/− mice [9, 10], no studies so far analyzed and compared total transcriptional activity of wild-type versus mutant neuronal nuclei. Such measurements necessitate access to uniform populations of neurons. Indeed, nuclear size has long been known to correlate with transcriptional activity and neurons with different identities typically have nuclei of different sizes and therefore rates of transcription that may vary by several folds [11]. In addition, nuclear size rapidly increases during neuronal differentiation such that developmental synchrony is a prerequisite for meaningful comparisons to be made between wild-type and mutant neurons. To circumvent the problems resulting from temporal and cellular heterogeneity, we used here an in vitro system based on the differentiation of mouse embryonic stem cells (ESCs). We previously reported that homogenous populations of neuronal progenitors can be generated from mouse ESCs [12, 13], thus allowing a precise monitoring of the earliest steps of neuronal differentiation. This synchronous system can then be used to measure and quantify the impact of the lack of MeCP2 by comparing mutant with wild-type ESCs. Causality can also be firmly established in this system by restoring MeCP2 expression in mutant neurons.

Authors contributions: M.Y., R.D., and Y.-A.B.: conception and design, collection and assembly of data, data analysis and interpretation, and manuscript writing; I.G. and A.B.: provision of material, data analysis, and manuscript writing; R.A.P.: data analysis and interpretation and manuscript writing. M.Y. and R.D. contributed equally to this article.

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**RESULTS**

**Generation of Neurons from Wild-Type and Mecp2<sup>−/−</sup> ESCs**

To study the role of MeCP2 during the earliest stages of neuronal maturation, we used E14 TG2a wild-type (Mecp2<sup>+/+</sup>) and Mecp2<sup>−/−</sup> ESCs [4]. Since the success of our neuronal differentiation procedure relies on the uniformity and pluripotency of the ESC populations used [12, 13], we first examined and quantified the expression of Oct-4 and Nanog in wild-type and Mecp2<sup>−/−</sup> ESCs. As illustrated in Figure 1A and 1B, the vast majority of wild-type and Mecp2<sup>−/−</sup> ESCs were colabeled with both Oct-4 and Nanog, suggesting that the lack of MeCP2 does not affect pluripotency. Both ESC lines were then used to generate cellular aggregates that were treated with retinoic acid as previously reported [12, 13]. This procedure has been shown to generate essentially pure populations of Pax6-positive progenitor cells [12]. As observed with other ESC lines [13], both wild-type and ESCs lacking MeCP2 generated neuronal cultures of high purity, with more than 90% of the cells expressing neuronal markers after a few days in culture. These neurons were previously characterized as glutamatergic using antibodies to a glutamate vesicular transporter and to form functional contacts [12]. Three days after dissociation of the progenitor aggregates, MeCP2 antibodies revealed a distinct nuclear staining that was absent in Mecp2<sup>−/−</sup> neurons (Fig. 1C).

To test whether wild-type and Mecp2<sup>+/+</sup> ESC-derived neurons begin to express synaptic markers at similar rates, we monitored between day 1 and day 21 the levels of synaptophysin, a protein associated with presynaptic vesicles. As previously reported, these levels increase dramatically during the second week of neuronal maturation (Fig. 1D, 1E), reflecting synaptogenesis and functional connectivity during this period [12]. No significant differences were found between wild-type and Mecp2<sup>−/−</sup> neurons (Fig. 1E). In this system, synaptic transmission can be readily detected by electrophysiological recording at 12 days following progenitor plating [12] as was found to also be the case with neurons lacking MeCP2 (data not shown). Western blots were also performed with nuclei isolated both from cultured cortical and ESC-derived neurons (Fig. 1F) and when normalized for histone H3, the levels of MeCP2 were found to be very similar between these two cell types. While no significant differences were observed in the levels of histone H3 between wild-type and mutant neurons, those of histone H1 were markedly elevated in neurons lacking MeCP2 10 days after progenitor plating (Fig. 1H, 1I: 100% ± 7.18%, n = 3 vs. 217.8% ± 18.7%, n = 3, t test p = .021), in line with previous in vivo findings [8].

**Nuclear Size of ESC-Derived Neurons**

As chromosomes typically attach to the nuclear membrane, Hoechst staining (Fig. 2A) of cultured cells can be used to monitor nuclear diameter as verified using antibodies to Lamin B (Supporting Information Fig. S1). We found that 3 days after plating, the size of both wild-type and mutant neuronal nuclei is indistinguishable (Fig. 2B). Note that the levels of MeCP2 are still low at this stage (Fig. 2C). Subsequently, there is a marked increase in MeCP2 levels and nuclear size in wild-type neurons (Fig. 2B–2D), but in the absence of MeCP2, the size of the nuclei failed to increase at the same rate as wild types. Even at 26 days, the latest time point analyzed, the nuclei of Mecp2<sup>−/−</sup> neurons were still significantly smaller than those of wild-type neurons (Fig. 2B). To rule out the possibility that the smaller nuclear size observed in neurons lacking MeCP2 may result from additional differences between the mutant and wild-type ESCs used to generate neurons, we examined two additional mutant lines: Mecp2<sup>loxP/loxP</sup> ESCs carrying a Cre-excisable (“floxed”) version of the gene [4] and Mecp2<sup>loxP/loxP</sup> with a floxed stop cassette inserted before exon 3 preventing the expression of the gene [6]. Western blot analyses showed a roughly 40% reduction of MeCP2 levels in Mecp2<sup>loxP/loxP</sup> neurons and negligible levels in Mecp2<sup>loxP/loxP</sup> neurons (Fig. 2E, 2F). While 3 days after progenitor plating, the nuclei of neurons generated from wild-type and the three mutant ESC lines were essentially of the same size (Fig. 2G), after 8 days both Mecp2<sup>−/−</sup> and Mecp2<sup>loxP/loxP</sup> neurons had smaller nuclei compared with age-matched wild-type neurons. The addition of brain-derived neurotrophic factor (BDNF) (40 ng/ml added every other day starting 2 days after progenitor plating) did not increase nuclear size when measured 10 days following aggregate dissociation, neither with wild-type nor with Mecp2<sup>−/−</sup> neurons (Supporting Information Fig. S1).

**Re-Expression of MeCP2 Rescues the Small Nuclei Phenotype in a Cell-Autonomous Fashion**

We then tested whether the nuclear size phenotype detected in the Mecp2<sup>loxP/loxP</sup> cells could be rescued by the re-expression of MeCP2 following the removal of the stop cassette. To this end, Mecp2<sup>loxP/loxP</sup> cells were infected with lentiviruses containing a dual Synapsin promoter construct driving the neuronal expression of both Cre and green fluorescent protein (GFP) [15] (see Materials and Methods). This approach ensures a very high rate of infection as evidenced by the observation that the majority of neurons expressed GFP (data not shown). Western blot analyses revealed that Cre-mediated excision led to a re-expression of MeCP2 from its endogenous promoter back to normal levels (Fig. 3A) and that these neurons had significantly bigger nuclei compared with cells infected with GFP-encoding lentiviruses used as control (Fig. 3B) (22.01 ± 0.4949 µm<sup>2</sup>, n = 49, vs. Cre viruses: 31.87 ± 0.8158 µm<sup>2</sup>, n = 51). In a complementary approach, we expressed MeCP2 in neurons generated from Mecp2<sup>−/−</sup> ESCs using the same viral delivery system. To also test whether MeCP2 regulates nuclear size in a cell-autonomous fashion, we used low viral titers so that only about half of the neurons would be infected. Neurons were infected with either a control virus (GFP only) or a virus encoding both MeCP2 and GFP (see Materials and Methods) and nuclear size was measured 18 days following infection. While the nuclear size of the Mecp2<sup>−/−</sup> neurons expressing MeCP2 had enlarged (31.16 ± 0.6361 µm<sup>2</sup>, n = 132), the nuclei of neighboring noninfected neurons remained small (22.02 ± 0.4081 µm<sup>2</sup>; n = 89), as small as the nuclei of control neurons from noninfected sister cultures (22.67 ± 0.4320 µm<sup>2</sup>; n = 108) (Fig. 3C). These results indicate that the regulation of the nuclear size by MeCP2 is cell-autonomous.

**No Changes in the Size of Gial Cells Nuclei Lacking MeCP2**

Given newly proposed role(s) for MeCP2 in glial cells (see below and Discussion), the nuclear size of these cells was also measured in primary cultures of astrocytes and of
Figure 1. Neuronal differentiation of wild-type and MeCP2−/− embryonic stem cells (ESCs). Cultures of wild-type and MeCP2−/− ESCs were stained with Oct4 and Nanog antibodies and the nuclear dye Hoechst. Arrowheads point to Oct4- and Nanog-negative nuclei of fibroblasts (A). The number of double-positive nuclei for Oct4 and Nanog was quantified and the significance of differences was assessed by Student’s t test (n = 400 for each cell type) (B). Three DIV ESC-derived wild-type or MeCP2+/− neurons were analyzed by immunostaining using MeCP2 and Tuj-1 antibodies (C). Neuronal lysates of wild-type and MeCP2−/− ESC-derived neurons were tested at 1, 3, 8, 14, and 21 DIV for Synaptophysin and β-III-Tubulin (Tuj-1) expression by Western blot (D). The signal intensity of synaptophysin was quantified and normalized to Tuj-1 levels (n = 3) (E). Quantitative Western blot with dilution series using 0.1, 0.2, and 0.4 million nuclei isolated from either cortical neurons or ESC-derived neurons labeled here as ESCD neurons (F, G). Note that the levels of MeCP2 normalized to histone H3 levels are similar when 16 DIV cortical neurons and 8 DIV ESC-derived neurons are compared. However, while the levels of histone H3 remain constant in the absence of MeCP2, those of H1 remain more than double (mean ± SEM, n = 3, **, p < .01, Student’s t test) (H, I). Nuclei of MeCP2−/− were used as negative loading control. Abbreviations: DIV, days in vitro; ESCD, ESC-derived.
Figure 2. The size of nuclei in embryonic stem cell (ESC)-derived neurons correlates with their levels of MeCP2. ESC-derived neurons were stained at DIV 8 with antibodies against the neuron-specific marker β-III Tubulin (Tuj-1) and Hoechst. The nuclei of the Mecp2−/− neurons are smaller than those of wild-types neurons (A). Nuclear area of neurons was measured during the course of neuronal maturation (mean ± SEM, *** p < .0001, Student’s t test) (B). Mecp2, Tuj-1, and actin levels were analyzed by immunoblotting using lysates of wild-type ESC-derived neurons (1, 3, and 8 DIV) and Mecp2−/− ESC-derived neurons (8 DIV) (C). Mecp2 levels were quantified and normalized to actin levels (D). MeCP2 protein levels in neurons generated from four ESC lines with various mutations in the Mecp2 gene (wild-type: Mecp2+/y, Mecp2−/−, Mecp2loxP/y, and Mecp2stop/y) were assessed by Western blot (E). Levels of MeCP2 were normalized to Tuj-1 levels (F). Quantification of nuclear size of wild-type and Mecp2 mutant neurons during the course of neuronal maturation at 3 and 8 DIV (mean ± SEM, *** p < .0001, Student’s t test) (G). Abbreviation: DIV, days in vitro.
In Vivo Measurements of Nuclei Size

The size of neuronal nuclei was then measured in the CA3 area of the hippocampus in 1-week and of 7-month-old mice. As in vitro, there is a significant increase in the size of the neuronal nuclei as they mature in vivo (1 week: 85.08 µm² ± 0.9491 µm², n = 304, 7 months: 150.2 µm² ± 2.519 µm², n = 258) (Fig. 5A, 5B). By contrast, in the same brain area of 1-month-old mice lacking MeCP2, the neuronal nuclei were significantly smaller (Fig. 5C, 5D) than wild-type animals (MeCP2+/−; 138.2 µm² ± 2.146 µm², n = 56, MeCP2−/−; 98.73 µm² ± 1.662 µm², n = 92).

We also measured the nuclear size of astrocytes in hippocampus of 1-month-old mice and found that the size of the nuclei in wild-type and MeCP2−/− astrocytes was similar (Fig. 5E, 5F; MeCP2+/−; 57.71 µm² ± 1.674 µm², n = 93, MeCP2−/−; 61.99 µm² ± 1.601 µm², n = 69, p = .329, Student’s t-test).

Reduced Rate of Transcription in MeCP2-Deficient Neurons

To test whether the reduced size of nuclei in neurons lacking MeCP2 may affect global transcription, we compared radioactive ribonucleotide incorporation in nuclei isolated at different time points from neurons generated from wild-type and mutant ESCs. This procedure reflects the biosynthesis of mostly ribosomal and transfer RNA as especially ribosomal RNA is far more abundant than mRNA. After incubation of purified nuclei with unlabeled ATP, CTP, GTP, and radiolabeled [32P]-UTP, total RNA was extracted and the amount of radioactivity incorporated was measured [18]. Three days after progenitor plating, MeCP2−/− neuronal nuclei were found to be transcriptionally as active as wild-type nuclei (Fig. 6A). However, neurons lacking MeCP2 had significantly reduced rate of RNA synthesis after 8 days when compared with wild-type neurons (Fig. 6A). MeCP2−/+ neurons were also significantly less transcriptionally active compared with aged-match wild-type nuclei (8 days after plating wild-type and mutant progenitors, data not shown). The levels of transcriptional activity were found to be indistinguishable (Fig. 6B).

MeCP2 and BDNF Levels in Neurons and Astrocytes

As mice lacking MeCP2 or BDNF in the CNS show striking phenotypic similarities including hind limb clamping, excessive weight in female mutants, reduced mobility, and a behavior suggestive of anxiety [4, 19], we examined the levels of BDNF in neurons derived from MeCP2−/− and MeCP2+/− ESCs and found them to be reduced in mutant neurons (Fig. 7A–7C). By contrast, the (very low) levels of BDNF in wild-type neurons were indistinguishable between wild-type and MeCP2−/− cells (Fig. 7F, 7G). As MeCP2 re-expression in MeCP2−/− neurons rescued the small nucleus phenotype, we investigated that this manipulation would also be accompanied by a restoration of BDNF levels. We found that MeCP2+/− neurons infected with Cre-encoding lentiviruses had significantly higher levels of BDNF compared to neurons infected with GFP-encoding lentiviruses (Fig. 7D, 7E).
DISCUSSION

Our study uncovers several novel aspects related to the functional role of MeCP2 in neurons. First, MeCP2 regulates the size of neuronal nuclei and their global transcriptional activity. All previous studies on the role of MeCP2 in transcription relate to mRNA levels and neither to total RNA nor to comparative rates of biosynthesis. The large decrease observed in nuclei isolated from mutant neurons was observed in the absence of any gross retardation of neuronal differentiation. Second, the regulation of nuclear size is observed very early,
even before detection of spontaneous electrical activity, during a phase of rapid growth of postmitotic neurons. MeCP2 regulates nuclear size in a cell-autonomous fashion while its absence does not affect the rate of neuronal differentiation as measured by synaptophysin expression. Third, the requirement for MeCP2 with regard to nuclear growth and transcriptional activity is specific for neurons and is not observed in astrocytes, in spite of similar levels of MeCP2 in both cultured cell types. Fourth, the restoration of BDNF levels in neurons previously lacking MeCP2 indicates that this regulation takes place directly in neurons and does not require other cell types or hormones.

Figure 5. In vivo measurements of nuclear size of neurons and astrocytes in the CA3 area of the hippocampus. Hoechst staining of nuclei in the CA3 area of the hippocampus of 1 week and 7 months old wild-type mice (A). Quantification of nuclear size in CA3 area showed a significant increase in nuclear size with age (mean ± SEM, ***, p < .0001, Student’s t test) (B). Hoechst staining of the nuclei in CA3 area of the hippocampus in 1-month-old wild-type (Mecp2+/−) and Mecp2+/− mice (C). The nuclear size in CA3 is significantly reduced in neurons lacking Mecp2−/− compared with wild-type neurons (mean ± SEM, ***, p < .0001, Student’s t test) (D). Hippocampi of a 1-month-old mouse were stained with antibodies against the astrocyte-specific protein GFAP and nuclei with Hoechst (E). Quantification of the nuclear size of astrocytes in hippocampi of 1-month-old mice revealed that wild-type and Mecp2−/− astrocytes have similar nuclear sizes (mean ± SEM; Student’s t test) (F). Abbreviation: GFAP, glial fibrillary acidic protein.
An Early Role for MeCP2 in Neurons

Several previous studies indicate that MeCP2-deficient neurons are smaller in some brain areas (for review, see [2]) and smaller nuclei have been noted in the adult CA1/CA2 and cortical layer V [5, 7]. A number of previous reports have also addressed the role of MeCP2 in neuronal differentiation using cultured cells, including recent reports based on differentiated mouse and human induced pluripotent (iPS) cells.

Figure 6. Transcriptional rates are reduced in nuclei isolated from MeCP2−/− embryonic stem cell-derived neurons but not from MeCP2−/− astrocytes. Equal numbers of nuclei at all ages were incubated with unlabeled ATP, CTP, GTP, and 1 μCi of [32P]-UTP for 0 and 30 minutes. The reaction was stopped by the addition of TRIzol and total RNA extracted. The amount of radioactivity incorporated in total RNA was measured and standardized to the values obtained from wild-type nuclei (mean ± SEM, n = 3, *, p < .05, **, p < .01 Student’s t test) (A). Similar experiments as in (A) were also performed with nuclei isolated from wild-type and MeCP2−/− astrocytes (B). Abbreviation: DIV, days in vitro.

Figure 7. Reduced levels of BDNF in MeCP2-deficient neurons are rescued by MeCP2 re-expression. A Western blot analysis of 16 days in vitro (DIV) wild-type (MeCP2+/−), MeCP2−/−, and MeCP2−/− neuronal lysates (A) revealed a significant reduction of BDNF levels (B). BDNF mRNA levels in neurons lacking MeCP2 at DIV 16 were analyzed by qPCR in MeCP2−/− neurons and compared with BDNF mRNA levels in wild-type neurons at DIV 16 (mean ± SEM, n = 3, **, p < .01, Student’s t test) (C). Infection of MeCP2−/− neurons with Cre-encoding lentivirus leading to re-expression of MeCP2 from its endogenous promoter caused an increase in BDNF protein levels compared with neurons infected with GFP-encoding lentivirus (D). Quantification revealed a significant increase in BDNF protein levels (mean ± SEM, n = 3, *, p < .05, Student’s t test) (E). BDNF levels assessed by Western blots of lysates of cultured wild-type and MeCP2−/− astrocytes (F). Lysates prepared from brains of mice carrying a Mapt::Cre-mediated deletion of Bdnf (cBDNF [18]) were used as negative control (F). Very low levels of BDNF protein were detected in astrocytes and no significant differences between wild-type and mutant cells. BDNF mRNA levels (G) were determined in cultured wild-type and MeCP2−/− astrocytes and no significant differences were observed (mean ± SEM, n = 3. Student’s t test). Abbreviations: BDNF, brain-derived neurotrophic factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; WT, wild type.
MeCP2 Regulates Neuronal Nuclear Size and Transcription

[20–24]. However, these previous studies typically reported on maturation defects in cells lacking MeCP2 based on β-III-Tubulin staining [23] or else subtle changes in neurons excitability [24]. Our results show that while Mecp2−/− is required in postmitotic nuclei to sustain their growth during maturation, it is not needed for the dramatic increase of the presynaptic marker synaptophysin during the course of neuronal maturation. We also note that causality can be more readily established with mouse XY ESCs, compared with human iPSCs, especially with XX cells [23, 25, 26]: re-expression of MeCP2 in postmitotic mutant neurons restored nuclear growth in a cell-autonomous fashion (Fig. 3C) as well as BDNF levels (see below). These findings fit well with the reversibility of neurological symptoms observed in mouse model of RTT [6], even when MeCP2 levels are restored only in neurons [7]. Our results also show that cell types other than neurons are not involved: neither do mutant astrocytes alter the size of wild-type neuronal nuclei nor do wild-type astrocytes rescue the small nuclear size of mutant neurons (Supporting Information Fig. S3). This is interesting in view of recent results indicating that cultured astrocytes lacking MeCP2 contribute to decreased neuronal growth [27–29], suggesting secretion of hitherto unknown toxic factors. A role for MeCP2-deficient astrocytes was also demonstrated in vivo following the selective expression of MeCP2 in astrocytes of mutant animals, a manipulation significantly improving the locomotor activity and respiratory abnormalities as well as greatly extending the lifespan of Mecp2−/− mice [30]. In view of increasing evidence for cell types others than neurons playing a role in models of RTT, it is interesting to note that our findings indicate that the nuclear phenotype and the decreased rate of transcription caused by the absence of MeCP2 is limited to neurons and is not observed in astrocytes, in spite of comparable levels of MeCP2. Likewise, BDNF levels (see below) are decreased in mutant neurons but not in astrocytes.

MeCP2 and Nuclear Size

In general, little is known about the regulation of the size and the shape of nuclei and of postmitotic neurons in particular [31–34]. Ours results with astrocytes indicate that MeCP2 is not the sole regulator of nuclear size, since similarly high levels in astrocytes and neurons do not seem to be equally needed to regulate nuclear size and transcriptional activity. Another chromatin binding protein associated with nuclear size regulation is the linker histone H1 [35] and its loss in Tetrahymena results in an increase in nuclear size. Interestingly, MeCP2 is known to compete with H1 in binding to the linker DNA [36]. Recently, quantification of MeCP2 levels revealed that it is nearly as abundant as H1 in adult neuronal nuclei. Also, MeCP2 deficiency was shown to double the levels of H1 in neuronal but not in glial nuclei [8]. In line with this previous report, we found that the H1 levels are more than double in neuronal nuclei lacking MeCP2. These elevated H1 levels may enhance chromatin compaction and contribute to nuclear shrinkage [8].

Neurons have long been known to be transcriptionally highly active compared with other brain cells or even liver cells [11]. Global rates of transcription are well known to correlate with nuclear size, such that, for example, the comparatively small nuclei of cerebellar granule cells transcribe at much lower rates than those isolated from the cortex [11, 18]. In line with this, we observed that nuclei isolated from wild-type neurons significantly increase their transcriptional activity during the course of neuronal differentiation, which is accompanied by a rapid growth of the nuclei. However, during the same period of neuronal maturation, neither nuclear growth nor increased transcriptional activity is observed in the absence of MeCP2. These run-off assays [18] allow changes in the rates of global transcription to be measured, an approach that differs from steady-state transcription analyses typically performed in microarrays. Our observations fit well with recent findings indicating that the size of neuronal nuclei lacking MeCP2 is decreased early during cortical development in vivo [37]. Indeed, structural RNAs such as ribosomal RNAs contribute to the bulk of ribonucleotide incorporation in our run-off assays. Previous analyses with RNA extracted from brain tissues of Mecp2−/− mice revealed that expression of approximately 2,400 genes is downregulated [9, 10]. However, these results should not be confused with our findings as all previous publications dealt with specific mRNAs and excluded structural RNAs and global transcription.

MeCP2 and BDNF

Early experiments with cultured neurons suggested that MeCP2 may repress the expression of BDNF, the first to establish a link between MeCP2 and BDNF [33, 34]. However, in animals lacking MeCP2, BDNF levels are decreased in several brain areas [35], including nuclei of the brain stem involved in respiratory control [36]. In addition, it has been shown by crossing mouse lines engineered to either increase or decrease BDNF levels on a Mecp2 null background that the progeny of the former showed improved symptoms resulting from the lack of MeCP2, while the progeny of the latter was worse off and died earlier [38]. In ESC-derived neurons lacking MeCP2, BDNF expression levels, both mRNA and proteins, were clearly decreased, in line with in vivo observation. Essentially, normal BDNF levels could be restored by re-expressing MeCP2 in mutant neurons. BDNF levels are much lower in cultured astrocytes compared with neurons and we did not detect any difference in BDNF levels among wild-type and Mecp2−/− astrocytes, neither at the mRNA levels nor at protein levels. We believe that the extraordinary low levels of BDNF expression in astrocytes may explain the apparent discrepancy with a previous report [28]. Note that even in neurons where BDNF levels are far higher, accurate measurements by Western blot remain challenging [39].

Conclusions

The use of a synchronous in vitro differentiation system allowed the demonstration that MeCP2 is required for the growth of neuronal nuclei soon after progenitors become postmitotic. Restoration of MeCP2 levels rescues these phenotypes as well as BDNF levels, in line with the previously reported behavioral rescue of mutant mice engineered to re-express MeCP2 in adulthood.

Materials and Methods

Cells Culture

ESCs were cultured and differentiated into neurons as described previously [12, 13]. Briefly, this procedure selects by frequent splitting for the most rapidly dividing ESCs, ensuring their homo- geneity and pluripotency (Fig. 1A, 1B). Following aggregation and 4 days of exposure to retinoic acid to trigger neural commitment, aggregates of Pax6-positive progenitor cells are dissociated and plated on a polylysine/laminin substrate. The time points indicated in the text and days in vitro in Figures refer to the days following the plating of these dissociated progenitors counted as Day 0. Note that the serum-free, chemically defined medium used
for progenitor and neuron differentiation contains 4 μg/ml insulin [13].

Glial cultures were prepared from P2 cerebral cortex. Briefly, cortex was dissected in phosphate buffer saline (PBS) and dissociated with 0.05% trypsin for 15 minutes at 37°C followed by mechanical dissociation with a 10-ml pipette. After centrifugation (5 minutes at 1,500 rpm), cells were plated on 10-cm cell culture dish in Dulbecco’s modified Eagle’s medium (DMEM) complemented with 20% fetal calf serum (FCS), 1% nonessential amino acids (Sigma, St. Louis, MO, www.sigma-aldrich.com), 2 mM glutamine, and Pen/Strep (Invitrogen, Grand Island, NY, www.invitrogen.com). The genotype of the pups was determined by standard genotyping methods using specific primers for Mecp2.

Wild-type or mutant astrocytes were seeded on 12-well plates and maintained until confluence in DMEM supplemented with 20% FCS, 2 mM glutamine, nonessential amino acids, and Pen/Strep. The medium was changed to neuronal growth medium (complete medium [13]) 2 days before adding the neurons. Wild-type, and mutant neuronal progenitors were plated on N2 medium onto polyornithine/laminin-coated coverslips that were then added after 2 days to the astrocyte-containing wells. Cells were kept in complete medium until the end of the experiment.

Western Blots

After washing twice with ice-cold PBS, cells were lysed for 30 minutes on ice by adding RIPA buffer (Tris-HCl 25 mM pH 7.5, NaCl 150 mM, Triton X-100 1%, sodium deoxycholate 1%, and SDS 0.1%) containing protease inhibitors (Complete; Roche, Basel, Switzerland, http://www.roche-applied-science.com/index.jsp) and phosphatase inhibitor cocktail (PhosSTOP; Roche.) Supernatants were collected after 30 minutes centrifugation at 12,000g, and protein concentration was determined by bicinchoninic acid (BCA) protein assay (BCA protein assay; Pierce, Waltham, Massachusetts, www.thermofisher.com). Equal amounts of total protein (20–25 μg) were loaded and separated by electrophoresis using NuPAGE Novex Bis-Tris 4%–12% gels (Invitrogen) and transferred to nitrocellulose membranes (GE Biosciences, Little Chalfont, United Kingdom, www.gehealthcare.com). Antibodies and concentrations were as follows: rabbit polyclonal anti-BDNF (N-20; Santa Cruz, Santa Cruz, CA, www.scbt.com) 1:500, rabbit polyclonal anti-MeCP2 (Millipore, Billerica, MA, www.millipore.com) 1:2,000, mouse monoclonal anti-β-III-Tubulin (TU-1; clone Covance, Princeton, NJ, www.covance.com) 1:10,000, rabbit polyclonal anti-Histone H3 (ab7766; Abcam, Cambridge, United Kingdom, www.abcam.com) 1:3,000, rabbit polyclonal anti-Histone H1 (Santa Cruz; sc-10806) 1:100, and mouse monoclonal anti-Actin ( Sigma; A2228) 1:10,000. Densitometric quantification of bands was done using ImageJ software (NIH).

Quantitative Real-Time PCR

Total RNA (0.5 μg) was extracted using RNeasy-Plus Mini Kit (Qiagen, Venlo, Netherlands, www.qiagen.com) and reverse-transcribed using SuperScript-III Reverse Transcriptase and random primers according to manufacturer instructions (Invitrogen). The primer and probe sequences used are as follows: for Bdnf forward 5’-GGG AGC TGA GCG TGT GTG A-3’, reverse 5’-CTG CCC GCC AGA CAT GTC-3’, TaqMan probe 5’-CGA GTG GTT CAC AGC GCC AGA-3’; and for Gapdh, forward 5’-TGT GTC CCG CTT GGA TCA CCA CCT TCT GTA-3’, reverse 5’-CCT CCT CTA GG TAG TGT C-3’, and TaqMan probe 5’-CCG CCT CTA ACC TCC CCA GTA TG-3’. The levels of the Mecp2-1 and Mecp2-2 transcripts were assessed by PCR reaction-contained SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, www.appliedbiosystems.com), cDNA (5 ng), and the following primers (900 nM each): for Mecp2-1 forward 5’-GGA GAC GAT GAG ACT GGA AAA GTG-3’, reverse 5’-TGG AGA ATG GTC GGA TCA G-3’, for Mecp2-2 forward 5’-GGA TCA GGT TAG GGC-3’, reverse 5’-GAA GTG TGT AGC TGC TCA TG-3’, and for Gapdh 5’-TTG CCA ACA ACC ACC CCT TC-3’, reverse 5’-GCC TGG ACT GTG CCG TTA AA-3’. The levels of Bdnf, Mecp2-E1, and Mecp2-E2 were normalized to the levels of Gapdh transcripts according to the ΔC method, and statistical significance was determined by the Student’s t test. For the MeCP2 splice variants, specificity of the amplification reactions was assessed by melting curves to ensure single product amplifications.

Immunostaining and Nuclear Size Measurement

For immunocytochemistry, cells were grown on glass coverslips, washed twice with warm PBS, and fixed for 15 minutes with 4% paraformaldehyde (PFA) at 37°C. Unspecific antibody binding was prevented with blocking solution (10% horse serum, 0.1% Tween 20 in PBS) for 1 hour at room temperature. The primary antibodies were added in the blocking solution and kept overnight at 4°C. Primary antibodies and dilutions used were as follows: rabbit polyclonal anti-MeCP2 (Millipore; 07-013) 1:200, mouse monoclonal anti-GFAP (Sigma; G3893) 1:500, rat monoclonal anti-CD11b (Abcam; ab66332) 1:300, goat polyclonal anti-lamin B (SantaCruz; sc-6217) 1:100, and mouse monoclonal anti-β-III-Tubulin (Covance; MMS-435P) 1:1,000. Secondary antibodies coupled to the fluorophore were all used at a dilution of 1:500 (Invitrogen).

For immunohistochemistry, animals were sedated by intraperitoneal injection of Ketalar (100 mg/kg; Parke, New York, NY, www.pfizer.com/Rompun (0.2%; Bayer Health Care, Berlin-Wedding, Germany, www.bayer-scheringpharma.de) and perfused transcardially with ice-cold PBS (50–60 ml) followed by 4% PFA in 1× PBS (50–60 ml). The brains were removed and kept in fixative overnight at 4°C. Serial coronal sections (15–20 μm thickness) were obtained with a cryostat (Leica, Solms, Germany, www.leica-microsysystems.com). Immunostaining of brain sections was as with cultured cells.

The freely available software ImageJ (NIH) was used for nuclear size measurement. All the pictures were taken with a ×40 objective and in the software setting, one pixel was set equal to 0.115 μm. With a line drawn around the Hoechst nuclear staining, the software then calculates the surface of the nuclei automatically. Student’s t test was used for statistical analysis.

Nuclei Purification and In Vitro Transcription Assay

Cells were washed twice with PBS, collected in 1 ml of PBS containing protease inhibitors using a cell scraper, and pelleted at 800g for 5 minutes. To lyse the cell membrane, the cellular pellets were resuspended in 10× volume of 250-STM buffer (250 mM sucrose, 50 mM Tris-HCl pH 8.0, 5 mM MgCl2, and protease inhibitors) with 0.1% NP40 and kept on ice for 10 minutes, then centrifuged at 800g for 10 minutes. Nuclear pellets were resuspended in 1 ml of 2 M-STM buffer (2 M sucrose, 50 mM Tris-HCl pH 8.0, 5 mM MgCl2, and protease inhibitors) and centrifuged at 45,000g for 30 minutes to separate nuclei from contaminating cytoplasmic organelles. The pellets, verified to contain highly purified nuclei by phase contrast microscopy, were then resuspended in 600 μl 2× elongation buffer (100 mM Tris-HCl pH 8.0, 5 mM MgCl2, 5 mM MnCl2, β-mercaptoethanol 10 mM, and protease inhibitors). Nuclei were counted using a cell counter (NucleoCounter, Chemometec, Allerod, Denmark, www.chemometec.com). The number of nuclei was adjusted, with 2× elongation buffer, to have desired numbers in 100 μl, typically 250,000 nuclei. In vitro transcription was initiated by adding 100 μl of nuclei suspension to 100 μl of the reaction mixture (ATP, CTP, and GTP ribonucleotides each at a final concentration of 0.6 mM, 1 μCi of [32P]-UTP, and 40 units of RNase Inhibitor from Roche) in water. The reaction was performed at 37°C for 0 and 30 minutes and terminated by adding 80 μl TRIzol (Invitrogen) to each tube. The RNA purification was performed according to the manufacturer’s instructions (TRIzol, Invitrogen). The radioactivity incorporated into total RNA was measured using triti- cary liquid scintillation counter (Packard, Ramsey, MN, www.gmi-inc.com). Statistical differences were determined by the Student’s t test.

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**MeCP2 Mutant Mice**

*MeCP2* mutant mice [4] were obtained from the Jackson Laboratory (stock number 003890; B6.129P2-Cre/+; Bar Harbor, ME, www.jax.org). Male mutants were used in our studies with their wild-type littermates as control. All experiments involving wild-type and mutant animals were performed following the rules of the University of Basel.

**Plasmids and Lentivirus Generation**

In order to express Cre and MeCP2 specifically in neurons, we used the dual synapsin promoter lentiviral vector pLL-Syn-DsRed-Syn-EGFP [15]. Due to the dual synapsin promoter system, only neurons express the proteins of interest plus the reporter EGFP.

The pLL-Syn-CRE-Syn-EGFP and pLL-Syn-Mecp2myc-Syn-EGFP vectors were generated by cloning the respective PCR products into the pLL-Syn-DsRed-Syn-EGFP vector using the BamHI and NotI sites. The Cre sequence was PCR amplified from the pMC-CRE vector using the following primers: Cre forward 5'-GG AGATCT ATG CCC AAG AAG AAG AGG AAG-3' (bold letters: BglII restriction site), Cre reverse 5'-CG CAG GGG ATG TTA GGG CTC AGG-3' (bold letters: NotI restriction site and italic letters encode for the c-myc tag). To amplify the wild-type MeCP2, total RNA from wild-type ESC-derived neurons was extracted and reverse-transcribed into cDNA, which was subsequently used as the template in the PCR reaction. The following primer sets were used to amplify MeCP2: MeCP2 forward Primer 5'-CG CAG GGG ATG TTA GGG CTC AGG-3' (bold letters: BglII restriction site and italic letters c-myc tag), MeCP2 reverse primer 5'-AA GCGGCCGC TCA GCT AAC TCT CTC GGT CAC GG-3' (bold letters: NotI restriction site). Similar to CRE, the PCR product was digested with BglII and NotI and ligated into the BglII/NotI double-digested pLL-Syn-DsRed-Syn-EGFP vector.

Lentiviral particles were produced by cotransfecting the pLL-Syn-Cre-Syn-EGFP or pLL-Syn-Mecp2-Syn-EGFP vectors with the pMD2.G and psPax2 vectors (kindly provided by D. Trono, Ecole Polytechnique Fédérale de Lausanne, Switzerland) into 60% confluent HEK-293T cells. Three days after transfection, the lentiviral particles were concentrated from the medium using the Lenti-X concentrator system according to the manufacturer instructions (Clontech, Mountain View, CA, www.clontech.com), resuspended in complete medium, and kept at –80°C until used. Viruses were added 2 days after plating the progenitor cells on the polyornithine/laminin substrate.

**ACKNOWLEDGMENTS**

We would like to thank the Rett Syndrome Research Foundation for supporting the initial phase of this work, subsequently supported by a “Smergia” program of the Swiss National Foundation, CRSI3_130441, and by the International Rett Syndrome Foundation.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicate no potential conflicts of interest.

**REFERENCES**


6 Kriaucionis S, Bird A. The major form of MeCP2 has a novel N-terminal domain that is essential for CREB binding in neurons. Mol Cell 2001;7:595–609.


16 Kriaucionis S, Bird A. The major form of MeCP2 has a novel N-terminal domain that is essential for CREB binding in neurons. Mol Cell 2001;7:595–609.


19 Kriaucionis S, Bird A. The major form of MeCP2 has a novel N-terminal domain that is essential for CREB binding in neurons. Mol Cell 2001;7:595–609.


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