MECP2 is progressively expressed in post-migratory neurons and is involved in neuronal maturation rather than cell fate decisions

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Available online 17 September 2004

Rett syndrome is a neurodevelopmental disorder and one of the causes of mental retardation and autistic behavior in girls, as well as in a small group of boys. It was recently discovered that mutation of the methyl-CpG-binding protein 2 (MECP2) gene encoding a transcriptional repressor on the X chromosome causes Rett syndrome. Although it is evident that phenotypes of MECP2 mutant mice that resemble those of Rett syndrome are attributable to lack of the MECP2 gene in the central nervous system (CNS), there is little understanding of the neuropathological abnormalities in the CNS of MECP2-null mice. Here, we investigated the developmental regulation and specific cellular expression of MECP2 during neural development both in vitro and in vivo. MECP2 is expressed in mature neurons, but not in astroglia or oligodendroglia, and is increasingly expressed during development of the mouse neocortex. In addition, in vitro culture studies suggest that MECP2 is expressed in more differentiated neurons rather than in less differentiated neuroblasts. Under in vitro conditions using neural precursor cultures, we find that MECP2 mutant neural precursors differentiate into morphologically mature neurons and glia, and no significant differences in differentiation are detected between cells from wild-type and MECP2 mutant mice, suggesting that MECP2 may play a different role in mice than it does in Xenopus embryos. In agreement with this hypothesis, neocortical projection layers in MECP2 −/− mice are thinner than those in wild-type mice, and pyramidal neurons in layer II/III in MECP2 −/− mice are smaller and less complex than those in wild-type mice. Taken together, our results indicate that MECP2 is involved in the maturation and maintenance of neurons, including dendritic arborization, rather than in cell fate decisions.

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Introduction

Rett syndrome is a neurodevelopmental disorder affecting 1 in every 10,000–15,000 girls, and also a small group of boys, and is a primary cause of mental retardation and autistic behavior in girls (Armstrong, 2002; Glaze, 2002; Hagberg, 2002; Kriaucionis and Bird, 2003; Moog et al., 2003; Shahbazian and Zoghbi, 2002). Affected children have apparently normal development until 6–18 months, after which they undergo a period of rapid regression with loss of purposeful hand use, deceleration of head growth, and onset of repetitive, stereotyped hand movements. They develop gait ataxia and apraxia, autistic features, seizures, and respiratory dysfunction (Armstrong, 2002; Glaze, 2002; Hagberg, 2002; Kriaucionis and Bird, 2003; Moog et al., 2003; Shahbazian and Zoghbi, 2002).

Amir et al. (1999) identified the cause of Rett syndrome as a defect in the methyl-CpG-binding protein 2 (MECP2) gene on the X chromosome, and a variety of mutations on the MECP2 gene have been identified in girls and boys (Christodoulou et al., 2003; Moog et al., 2003). The MECP2 gene encodes a transcriptional repressor, which selectively binds methylated CpG dinucleotides in the mammalian genome and mediates transcriptional repression through interaction with histone deacetylase complex (HDAC) and corepressor SIN3A (Lewis et al., 1992; Nan et al., 1997, 1998).

To date, three independent lines of MECP2-deficient mice have been established, and these mutant mice are all characterized by stiff, uncoordinated gait, hind limb claspig, irregular breathing, seizures, and reduced brain growth, all of which mimic symptoms of Rett syndrome (Chen et al., 2001; Guy et al., 2001; Shahbazian et al., 2002a). In addition, it is evident that these phenotypes are due to loss of MECP2 in the central nervous system (CNS), because specific deletion of the MECP2 gene in the brain mimics the germline loss of the MECP2 gene (Chen et al., 2001; Guy et al., 2001). However, there is little understanding of the neuropathological abnormalities in the CNS of children with Rett syndrome or in MECP2 mutant mice. Apart from somewhat reduced brain size of MECP2-null mice, no specific structural abnormality has yet been detected in mice.

A recent study investigated the molecular mechanisms of REST/NRSF-mediated gene silencing of neuronal-specific genes in non-neuronal cells (Lunyak et al., 2002). This study showed that there are two independent mechanisms for silencing neuron-
specific genes by REST/NRSF: gene silencing via HDACs and via MECP2. In the latter, REST/NRSF binds to the RE element on the promoter region of target genes, such as the sodium channel type II (SCN2A) gene, and recruits the co-repressor Co-REST. The REST–CoREST complex recruits MECP2 and inactivates target gene transcription via methylation of histone H3 by mammalian histone lysine methyltransferase SUV39H1 (Lunyak et al., 2002).

In the brain, in contrast, it is postulated that MECP2 binds to methyl CpG on the promoter region of target genes and then represses them via histone deacetylation by a HDACs–SIN3A complex. This prediction is supported by the finding of hyper-acetylation in MECP2-deficient mouse brains (Shahbazian et al., 2002a), the regulation of xHairy2a, a neuronal repressor in Xenopus embryos (Stancheva et al., 2003), and brain-derived neurotrophic factor (BDNF) in mice by MECP2 (Chen et al., 2003; Martinowich et al., 2003). These recent findings suggest that the role and target genes of MECP2 are tissue and cell-type specific, and strongly argue that it is imperative to investigate MECP2 directly in the CNS to understand the neuropathogenesis of MECP2 mutant mice and Rett syndrome.

The neocortex is the most complex region of the brain and plays critical roles in cognition and motor-sensory integration (Miller, 2000). MRI studies indicate that some Rett patients display cortical abnormalities such as atrophy (Casanova et al., 1991; Gotoh et al., 2001; Naidu et al., 2001). Because of its likely involvement in Rett syndrome and because the formation of the neocortex is relatively well characterized, the neocortex is an ideal model system in which to investigate expression of MECP2 and neuronal maturation.

In the experiments reported here, we have examined the expression patterns of MECP2 in the embryonic and postnatal CNS, and in neural precursor cultures. Our data show that MECP2 is expressed in the neuronal lineage, but not in either the astroglial or the oligodendroglial lineages, and that MECP2 expression is activated after neuronal migration occurs, and increases as neuronal maturation progresses. In addition, to investigate whether MECP2 mutations affect cell fate decisions of neural precursors, we examined the proliferation and differentiation of neural precursors derived from MECP2-null mice. We find that MECP2 mutation in mice does not affect these early aspects of precursor cell behavior, in contrast to findings in Xenopus embryos (Stancheva et al., 2003). Rather, MECP2−/−y mice display reduced thickness of the neocortex, and the cell density of the neocortex in MECP2−/−y mice is increased. Golgi staining reveals that pyramidal neurons in layer II/III in MECP2−/−y mice are smaller and less complex than those in wild-type mice, suggesting that reduced thickness of neocortex in MECP2−/−y mice is due to reduced size and less arborization of projection neurons, rather than to loss of neurons. Taken together, our results indicate that MECP2 is involved in the late stage, post-migratory maturation and maintenance of neurons, including dendritic arborization, rather than in early neural cell fate decisions.

Results

MECP2 is expressed throughout the adult mouse brain

We used an immunocytochemical approach to analyze the temporal, spatial, and cellular expression patterns of MECP2 in the mouse CNS from embryonic day 16.5 (E16.5) to adulthood. In 9-week-old young adult mice (just before the typical time of death of MECP2-null mice; Chen et al., 2001; Guy et al., 2001), MECP2 is expressed throughout the brain, including the pyramidal cell layer (CA1, CA2, and CA3) of the hippocampus, the granule cell layer of the dentate gyrus, as well as in the thalamus, striatum, and neocortex (Figs. 1A–C). MECP2 staining is detected in the nucleus and can be categorized into two patterns. The first is a “punctate” nuclear staining pattern (Fig. 1D) and the other is marked by uniform staining of the nucleus (Fig. 1G). The strong punctate expression (arrowheads in Fig. 1D) was coincident with the location of the heterochromatin stained with Hoechst 33258 (arrows in Fig. 1E). Uniform chromatin staining is predominant at embryonic and neonatal stages, but the punctate pattern becomes much more frequent in the adult brain.

Fig. 1. MECP2 is expressed in neuron-rich layers and regions of the adult CNS. (A–C) MECP2 is expressed in the pyramidal cell layer (CA1, CA2, and CA3) of the hippocampus and the granule cell layer of the dentate gyrus (A), as well as in the striatum (B) and thalamus (C) in the adult brain. (D–I) Two MECP2 staining patterns are observed: “punctate” nuclear staining (D) and “uniform” nuclear staining (G). The strong punctate staining (arrowheads in D) corresponds to the heterochromatin region stained with Hoechst 33258 nuclear counter stain (arrows in E). On the other hand, “uniform” MECP2 staining throughout the nucleus does not correspond with the heterochromatin (H). F and I are merged images of D and E, and G and H, respectively. Scale bar 500 μm in A, 200 μm in B and C, 10 μm in D–I. Abbreviations: ctx, cortex; thal, thalamus; DG, dentate gyrus.
At E16.5, MECP2 is very weakly detected in cells in the cortical plate that will become layers V or VI in the adult, with "uniform" nuclear labeling (Figs. 2A and F). Superficial layer Cajal–Retzius cells, one of the earliest born neuron classes, which secrete Reelin (Frotscher, 1998), express MECP2 more intensely than other cells (Figs. 2P–R). On the other hand, MECP2 is hardly detectable at E16.5 in the deeper layers of the ventricular zone and intermediate zone, containing neural precursors and migrating less-differentiated neurons (Figs. 2A and K).

Expression of MECP2 changes dramatically from this early pattern over the next few weeks. At postnatal day 0 (P0), MECP2 is detected in the superficial and the deep layers (Figs. 2B, G, and L), and Cajal–Retzius cells strongly express MECP2 (Figs. 2S–U). By P7, some of MECP2 expression in deep cortex has transitioned to punctate nuclear staining (Figs. 2C and H), in contrast to the continued uniform distribution in superficial cortex (Fig. 2M). By P21, the intensity of MECP2 expression in superficial cortex becomes similar to that in deep cortex (Figs. 2D, I, and N); nuclear staining becomes progressively more punctate as development progresses (Figs. 2E, J, and O). This progressive developmental expression of MECP2 in the neocortex is consistent with that reported previously (Cassel et al., 2004; Cohen et al., 2003; Coy et al., 1999; Mullaney et al., 2004; Shahbazian et al., 2002b) and suggests that MECP may be involved in maturation of the CNS.

**MECP2 is expressed in more differentiated neurons, but not in immature neuroblasts or glia**

The CNS is mainly composed of three cell types: neurons, astroglia, and oligodendroglia. To analyze which cell types express MECP2 in the adult mouse neocortex, immunocytochemistry was employed using cell-type specific markers: NeuN protein, a DNA-binding protein that is mainly detected in the nuclei of mature neurons (Mullen et al., 1992); glial fibrillary acidic protein (GFAP) and S100β that are used as astroglial markers (Debus et al., 1983; Kligman and Hilt, 1988); and 2,3-cyclic nucleotide 3-phosphodiesterase (CNPase), a myelin-associated protein that is an oligodendroglial marker (Sprinkle, 1989) in the CNS. In the adult

![Fig. 2. MECP2 is increasingly expressed with maturity in the developing neocortex, and Cajal–Retzius cells express MECP2 earliest.](image)

(A–E) The expression of MECP2 in the neocortex at progressive developmental stages: A at E16.5; B at P0; C at P7; D at P21; and E at 9 weeks of age. (K–O) MECP2 expression increases in deep layers as development progresses. (F–J) In contrast, expression in superficial cortex is delayed. (P–U) MECP2 is expressed in Cajal–Retzius cells. (P–R) At E16.5, some Reelin-positive Cajal–Retzius cells (arrow in Q) weakly express MECP2 (arrow in P). (S–U) At P0, almost all Reelin-positive Cajal–Retzius cells (arrowhead in S) strongly express MECP2 (arrowheads in T). R and U are merged images of P and Q, and S and T, respectively. Scale bar 200 μm in A–E, 20 μm in F–O, 20 μm in P–U. Abbreviations: CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone.
mouse neocortex, virtually all MECP2-expressing cells are NeuN positive (Figs. 3A–C). On the other hand, MECP2-expressing cells do not colabel with either GFAP (Figs. 3D–F), S100β (data not shown), or CNPase (Figs. 3G–I). MECP2 is expressed in both glutamatergic projection neurons and in GABAergic interneurons, as assessed by both retrograde labeling of projection neurons with FluoroGold and by immunocytochemical analysis of neurotransmitter phenotype (data not shown). These data strongly indicate that MECP2 is expressed in mature neurons, but not in astroglia or oligodendroglia.

Both the progressive expression of MECP2 in the developing neocortex and the expression of MECP2 exclusively in mature neurons as opposed to glia indicate that MECP2 expression increases in neurons as neuronal maturation progresses. To test this hypothesis, we examined the developmental expression of MECP2 using a neural precursor–stem cell culture system. Neural precursors are proliferative and multipotent cells with the capacity to differentiate into neurons, astroglia, and oligodendroglia. In the developing brain, neural precursors in the germinal layers differentiate into neuroblasts or glial precursors, and finally into mature neurons and glia (Gage, 2000; Jessell and Sanes, 2000; McKay, 2000; van der Kooy and Weiss, 2000). Even in the adult brain, neural precursors exist in the hippocampal subgranular zone of the dentate gyrus and in the subventricular zone, and provide newborn neurons throughout life in the dentate gyrus and olfactory bulb (Cameron and McKay, 1998; Morshead and van der Kooy, 2001; Taupin and Gage, 2002).

Neural precursors were isolated from the neocortex of E13.5 mouse brains and expanded in growth medium containing the mitogen FGF-2 (Johe et al., 1996). By incubation in differentiation medium containing 1% fetal bovine serum, but not FGF-2, neural precursors differentiate into neuroblasts or glial precursors, and eventually differentiate into neurons, astroglia, and oligodendroglia, in a manner that mimics in vivo neural development. Using this in vitro culture system, we examined MECP2 expression with a series of cell type-specific and stage-specific markers: nestin, an intermediate filament transiently expressed during neural ontogeny and used as a neural precursor–stem cell marker (Lendahl et al., 1990); Doublecortin (DCX), a microtubule-associated protein required for normal neocortical and hippocampal development in humans, and found in migrating and differentiating neuroblasts and immature neurons, but not in mature neurons (Francis et al., 1999; Gleeson et al., 1999; Magavi et al., 2000); Hu, an ELAV-like RNA binding protein that is expressed in postmitotic immature neurons (Marusich et al., 1994); microtubule associated protein 2 (MAP-2), a mature neuronal marker (Tucker, 1990); GFAP, an astroglial marker; and O4, an oligodendroglial marker (Sommer and Schachner, 1982).

When grown under these conditions with FGF-2, most neural precursors proliferate, maintain their undifferentiated status, and express Nestin (Fig. 4A). We could not detect any nestin+/MECP2+ cells under these conditions (Figs. 4A–C and Table 1), in contrast to recent data from dissociated rat embryonic cortical cells (Jung et al., 2003).

Under differentiation conditions, neural precursors gradually differentiate into neuroblasts or glial precursors. Three days after exposure to differentiation medium, some cells express DCX, but only 22.3% of DCX+ cells express MECP2, and those DCX+ cells that do express MECP2 have low levels of expression (Figs. 4D–F and Table 1). Seven days after differentiation, however, 82.5% of Hu-positive cells also express MECP2 (Figs. 4G–I and Table 1), and essentially all MAP-2-positive cells express MECP2 (Figs. 4J–

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Fig. 3. MECP2 is expressed in mature neurons, but not in astroglia or oligodendroglia. (A–D) Essentially all MECP2-expressing cells (B) in the adult neocortex are positive for NeuN, a mature neuron-specific marker (A). (E–H) However, no MECP2-expressing cells (E and H) are positive for GFAP (D), a marker for astroglia, or (I–J) for CNPase (G), a marker for oligodendroglia. C, G, and K show counter-staining of nuclei with DAPI. D, H, and L are merged images of the three markers, DAPI, and MECP2. Scale bar 50 μm in main frames and 20 μm in insets.
Interestingly, in this in vitro system, all MECP2-expressing cells display punctate nuclear staining, rather than uniform staining, suggesting that additional variables affect the expression of MECP2 in vivo, compared to under these in vitro conditions. On the other hand, MECP2 is not expressed in GFAP+ astroglia (Figs. 4M–O) or O4+ oligodendroglia (Figs. 4P–R), consistent with our in vivo data.

Taken together, MECP2 is expressed only in the neuronal lineage, and MECP2 expression levels increase as neuronal differentiation progresses, consistent with previously reported results using the olfactory bulb and epithelium (Cohen et al., 2003). This late developmental regulation and neuronal specificity suggest that MECP2 plays a role in neuronal maturation and maintenance.
MECP2 mutation does not affect proliferation or differentiation of neural precursors in mice

Our data on MECP2 expression suggest that MECP2 is involved in neuronal maturation and maintenance, rather than in neural cell-fate decisions. The phenotypes of MECP2 mutant mice also suggest that MECP2 mutation affects events at postnatal stages rather than embryonic neurogenic events. Interestingly, in light of our in vivo data in mice, a recent in vitro study found that methylation of the GFAP promoter regulates GFAP expression (Takizawa et al., 2001). In addition, a recent study in Xenopus reports that MECP2 is involved in cell fate decisions during primary neurogenesis in Xenopus embryos by recruiting the SMRT complex (Stancheva et al., 2003). Some studies report that MECP2 is expressed even at early stages of embryonic neurogenesis (Jung et al., 2003; Shahbazian et al., 2002b). To test whether MECP2 mutation affects cell fate decisions of neural precursors, we examined proliferation and differentiation of neural precursors using neural precursor culture systems.

To investigate the proliferation of neural precursors, we used neurosphere cultures (Reynolds et al., 1992). In the presence of the mitogens EGF and FGF-2, neural precursors proliferate in a self-renewing manner to generate cell aggregates called “neurospheres”, containing mostly undifferentiated cells (Reynolds et al., 1992). Cells containing neural precursors were dissociated from the cortex of individual E13.5 mice (Fig. 5A) with each of three genotypes: wild-type (wt); heterozygous MECP2 +/−; or hemizygous null MECP2 −/−, which were generated by crossing MECP2 +/− mice with wild-type males. Dissociated cells containing neural precursors were plated at clonal density, one cell per 10 µl, a dilution at which individual cells are sufficiently separated for clonal analysis (Hulspas et al., 1997). These clonal cultures were grown for 9 days to allow neural precursors to generate neurospheres. In this experiment, the number of neurospheres is indicative of the percentage of neural precursors in dissociated cells. We employed this neurosphere generation assay to assess the proliferation of neural precursors of each of the three genotypes: wt, +/−, and −/−.

In this neurosphere generation assay, there is no significant difference in neurosphere formation ability among the three genotypes (Fig. 5B). In addition, there are no apparent differences in neurosphere size among the genotypes. Taken together, these results indicate that MECP2 mutation does not affect the proliferation of neural precursors.

To further investigate potential effects of MECP2 mutation on the differentiation of neural precursors, neural precursors dissociated from wt, +/−, and −/− E13.5 embryonic brains were cultured as monolayers plated on poly-L-ornithine and fibronectin-coated coverslips (Johe et al., 1996), and differentiated in differ-

Table 1
Percentage of MECP2+ cells with neuronal lineage-specific markers during differentiation

<table>
<thead>
<tr>
<th>Days in vitro</th>
<th>Marker</th>
<th>Percentage (total cells analyzed)</th>
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<tbody>
<tr>
<td>0 DIV</td>
<td>MECP2+/Nestin+ cells</td>
<td>0 ± 0 (%) n = 380</td>
</tr>
<tr>
<td>3 DIV</td>
<td>MECP2+/DCX+ cells</td>
<td>22.3 ± 2.9 (%) n = 551</td>
</tr>
<tr>
<td>7 DIV</td>
<td>MECP2+/Hu+ cells</td>
<td>82.5 ± 2.1 (%) n = 381</td>
</tr>
<tr>
<td>7 DIV</td>
<td>MECP2+/MAP2+ cells</td>
<td>98.9 ± 0.6 (%) n = 321</td>
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Fig. 5. MECP2 mutation does not affect the proliferation of neural precursors. (A) Neural precursors were isolated from neocortex of E13.5 embryos (wild type, MECP2 +/−, and MECP2 −/−) and plated at low cell density. Nine days after expansion, single dissociated neural precursors were cultured to generate “neurospheres”, as an assay for neural precursor activity. (B) Quantification of the number of neurospheres generated from 5000 dissociated cells from the three different genotypes of E13.5 embryos. MECP2 deficiency does not affect the generation or proliferation of neurospheres. Scale bar 50 µm. Values represent the mean ± standard error of the mean (SEM).
entiation medium containing 1% serum but no FGF-2. Ten days after transition to differentiation medium, immunocytochemical analysis was employed to assess expression of the following cell type-specific markers: MAP-2 for neurons, GFAP for astroglia, and O4 for oligodendroglia. Neural precursors from mice with each of the three genotypes (wt, +/−, −/−) differentiate equivalently into mature neurons, astroglia, and oligodendroglia (Figs. 6A–I), without any significant morphological abnormalities in the neurons, astroglia, or oligodendroglia derived from neural precursors from MECP2 −/− mice (higher magnification insets in Figs. 6G–I). In addition, there are no significant quantitative differences in the percentages of neurons, astroglia, or oligodendroglia between differentiated cultures from mice of the three genotypes (Fig. 6J). Taken together these data suggest that MECP2 mutation does not modify early cell fate decisions of mammalian neural precursors, in contrast to recent data in Xenopus embryos (Stancheva et al., 2003).

Our in vivo and in vitro data suggest that MECP2 is involved in neuronal maturation or maintenance, and suggest the hypothesis that its mutation may lead to abnormal neuronal maturation, including dendritic arborization or axonal projection. Recent reports indicate that MECP2 binds selectively to the BDNF promoter III, which is activated in response to neuronal depolarization, and regulates BDNF expression (Chen et al., 2003; Martinowich et al., 2003). In addition, recent work has shown that BDNF–TrkB signaling is required for the maintenance of cortical dendrites using TrkB null and forebrain-specific TrkB and BDNF mutant mice (Gates et al., 2000; Gorski et al., 2003; Xu et al., 2000).

To investigate whether MECP2 −/− mice display abnormal neuronal morphologic maturation, including dendritic arborization,
we first performed Nissl staining of both wild-type and MECP2 −/− mice to analyze the thickness of the neocortex overall and each layer individually (Figs. 7A and B). To determine anatomical position within the neocortex, brains were sectioned coronally at a thickness of 50 μm, and cytochrome oxidase staining was performed on alternate sections to determine the location of the barrel cortex. Cortical thickness was measured every 400 μm from the most rostral position of the barrel cortex (Figs. 7C–G). At each of the four rostrocaudal positions analyzed, the neocortex was reduced in thickness in MECP2 −/− mice compared to littermate controls at 8 weeks of age (a time at which MECP2 −/− mice display phenotypic characteristics), and this reduction is statistically significant (Fig. 7C, P < 0.05, asterisks), while there is no significant difference at 2 weeks of age (a time at which MECP2 −/− mice do not show any phenotype) (data not shown).

We next measured each cortical layer individually in wild-type and MECP2 −/− mice to investigate which layers are most affected in MECP2 −/− mice. There is significant reduction of thickness of pyramidal neuron layers II/III, V, and VI (Figs. 7D, F, and G), but no reduction of granular layer IV (Fig. 7E). There is a significant reduction of thickness at three out of four rostrocaudal positions analyzed in layer II/III (Fig. 7D, P < 0.05, asterisks), the layer most affected in forebrain-specific BDNF mutant mice (Gorski et al., 2003). In addition to layer II/III, there is significant reduction of thickness in the sensorimotor cortex region analyzed in each of layer V and VI (Figs. 7G and F, P < 0.05, asterisks).

There exist at least two possibilities for why MECP2 −/− brains have a thinner neocortex: (1) loss of neurons or other cells (more likely neurons, based on MECP2 expression within neurons); and (2) reduced size or complexity of neurons. To investigate these two possibilities, we quantitatively analyzed cell density in each layer of each genotype (Fig. 7H). The cell densities of layers II/III, IV, V, and VI in MECP2 −/− mice are significantly higher than those in wild-type mice at 8 weeks of age (Fig. 7H, P < 0.05, asterisks), while there is no significant difference at 2 weeks of age (Fig. 7H).

Given that the thickness of neocortex in MECP2 −/− mice is reduced by approximately 10% (Fig. 7C) without loss of cells, the cell density might be predicted to increase by approximately 20%. Consistent with this prediction, cell density in MECP2 −/− mice increases by approximately 15% on average in our analysis and is close to this estimation. Combined with the fact that neuronal death has not been observed in MECP2 mutant mice or in patients with Rett syndrome, our data suggest that the reduction in thickness of the neocortex in MECP2 −/− mice is due to reduced size of neurons, rather than to loss of neurons.

From our Nissl staining data, we hypothesized that MECP2 mutation might lead to reduced size or complexity of neurons. To investigate the characteristics of individual neurons, we performed Golgi staining to visualize the complete structure of individual, randomly labeled, and systematically selected neurons (Figs. 8A and B). As the Nissl staining data indicated that layer II/III in MECP2 −/− mice is the most affected layer (Fig. 7D), we drew the morphology of pyramidal neurons in layer II/III using a camera lucida device (Figs. 8C and D; n = 36 layer II/III neurons from six mice of each genotype), then analyzed dendritic arborization by Sholl analysis (Sholl, 1953), as well as soma area and dendritic spine density (Figs. 8E–G). At 8 weeks of age, layer II/III pyramidal neurons in MECP2 −/− mice have significantly smaller somas than those in wild-type mice (Fig. 8E). In addition, Sholl analysis, which evaluates the extent of dendritic arborization, reveals that layer II/III pyramidal neurons in MECP2 −/− mice have significantly less complex dendritic arborization than do those in wild-type mice at 8 weeks of age (Fig. 8F, asterisks). These data are consistent with previous findings in hippocampal CA2 neurons in MECP2 −/− mice (Chen et al., 2001) and in patients with Rett syndrome (Armstrong et al., 1995; Bauman et al., 1995a,b), and suggest that MECP2 mutation leads to abnormal neuronal maturation or insufficient maintenance of morphologic complexity associated with neuronal maturity. However, we did not detect any significant reduction in dendritic spine density, as was previously described in a study using autopsy material from patients with Rett syndrome (Belichenko et al., 1994) (Fig. 8G), suggesting that the reduction of dendritic spine density reported by these authors might have resulted from secondary effects due to long-term illness, rather than to primary effects of MECP2 mutation.

Discussion

The expression patterns of MECP2

Taken together, our data indicate that MECP2 functions during postmigratory maturation and stabilization stages of CNS neuronal differentiation rather than at early stages of proliferation control, neuronal specification, or migratory positioning. MECP2 expression coincides with the time during neuronal differentiation when neurons develop dendritic complexity, axonal projections, circuitry, and connectivity.

Recent MRI studies on patients with autism and Rett syndrome report cerebral atrophy (Casanova et al., 1991; Gotoh et al., 2001), and MECP2 may be particularly involved in cerebral development and stabilization. Involvement in cerebral development, and particularly neocortical development, might be expected, given the behavioral and cognitive effects of MECP2 mutation in Rett syndrome.

In this study, we examined the expression patterns of MECP2 in the developing and adult mouse brain, especially focusing on the developing neocortex. Our results are fully consistent with previous studies, including those on human postmortem tissue, rat, and mouse (Coy et al., 1999; Jung et al., 2003; Mullaney et al., 2004; Shahbazian et al., 2002b). MECP2 is not strongly expressed in the murine neocortex during embryonic stages, except in Cajal–Retzius cells, one of the earliest-generated populations of neurons in the developing neocortex, involved in regulation of cortical lamination by secretion of Reelin (Frotscher, 1998). Expression of MECP2 develops progressively from deep cortical layers to superficial layers as development progresses, further suggesting that MECP2 expression increases during post migration neuronal development, following the “inside-out” sequence of cortical laminar development (Super et al., 1998). The strong expression of MECP2 in Cajal–Retzius cells at early embryonic stages compared with neurons in other layers also supports this idea, as Cajal–Retzius cells are the most mature neurons in the embryonic neocortex. Furthermore, the concept that MECP2 is increasingly expressed as development progresses is one common to other regions, such as the cerebellum and the olfactory epithelium (Cohen et al., 2003; Mullaney et al., 2004; Shahbazian et al., 2002b), suggesting that MECP2 is involved in the maturation of neurons throughout the CNS.

Transition from “uniform” nuclear staining with MECP2 to “punctate” nuclear staining of heterochromatin during cortical development, in parallel with neuronal maturation, suggests a relation between MECP2 localization in the nucleus and MECP2
Fig. 7. Neocortical projection layers II/III, V, and VI in MECP2 −/− mice are thinner than those in wild-type mice, and the cell density of the neocortex in MECP2 −/− mice is increased compared to wild-type mice. (A and B) Nissl staining of the neocortex in wild-type (A) and MECP2 −/− mice (B) (8 weeks of age). (C–G) Quantification of thickness of total neocortex (C), layer II/III (D), IV (E), V (F), and VI (G) in both 8-week-old wild-type and MECP2 −/− mice. The thickness of the barrel cortex was measured at four positions, every 400 μm, from the most rostral aspects of barrel cortex. The neocortex of MECP2 −/− mice is significantly thinner than that of wild-type mice (n = 6 for each genotype, *P < 0.05). The most dramatic reduction in thickness is present in layer II/III in MECP2 −/− mice. (H) Quantification of cell density of each layer. Cell density was measured in wild-type and MECP2 −/− at both 2 and 8 weeks of age. At 8 weeks of age, the cell densities of layers II/III, IV, V, and VI in MECP2 −/− are significantly higher than those in wild-type mice (n = 24 grids of each layer of each genotype, *P < 0.05). Scale bar 500 μm in A and B. Values represent the mean ± SEM.
MECP2 is localized to nuclei in all neurons, and this transition to heterochromatin localization occurs with maturation in the vast majority of neurons. It is thought that localization of MECP2 in the highly methylated heterochromatic region represents MECP2 binding to genomic methylated DNA. Mutation of the methyl-CpG binding domain (MBD) of MECP2 or reduced DNA methylation is thus thought to lead to diffusion of MECP2 localization in mouse ES cells (Nan et al., 1997), suggesting that changes in DNA methylation or histone modification may affect the transition of MECP2 staining patterns. In addition, a recent study demonstrated that MECP2 can also bind to unmethylated nucleosomal arrays and assembles secondary chromatin structures independent of DNA methylation in vitro (Georgel et al., 2003), suggesting that MECP2 also plays a role as a general chromatin
condensing factor, as well as a transcriptional repressor to specific target genes. These binary functions of MECP2 may be related to the two different MECP2 localization patterns in mice.

Our results indicate that MECP2 is expressed in mature neurons, but not in less differentiated neural precursors, neuroblasts, astroglia, or oligodendroglia, in vivo or in vitro. While one recent report using rat dissociated cortical cells reported that 25% of Nestin-positive cells are MECP2 positive (Jung et al., 2003), our results are not in concordance at all in mice; nestin+ cells are MECP2−/−. Both species differences between mouse and rat, and the sensitivity of antibodies against MECP2, may very well explain this difference. Consistent with the potential for at least gross species differences, in situ hybridization studies in mouse brain did not detect any MECP2 signal in the neocortex at E12 (Coy et al., 1999), whereas moderate signal was detected in the rat neocortex at E14, which roughly corresponds to E12 in the mouse brain (Jung et al., 2003). Furthermore, recent reports indicate that the MECP2 gene has two open reading frames, generating two isoforms (Kriaucionis and Bird, 2004; Mnatzakanian et al., 2004). The epitope of the antibody we used is in the N-terminus of MECP2A/2β. This antibody cannot detect the newly described isoform of MECP2, MECP2B/2α, which lacks the N-terminus of MECP2A/2β, suggesting that this antibody would fail to localize MECP2B/2α.

MECP2 mutation does not affect cell fate decisions in mice

To date, three lines of MECP2 mutant mice have been generated, and each displays behavioral phenotypes that mimic the symptoms of Rett syndrome patients (Chen et al., 2001; Guy et al., 2001; Shahbazian et al., 2002a). It appears that these phenotypes are truly due to loss of MECP2 in the CNS, because specific deletion of MECP2 in the brain mimics the germline loss of MECP2 (Chen et al., 2001; Guy et al., 2001). However, there is little neuropathological understanding of the abnormalities in the CNS of MECP2-null mice, except for the reduced brain size. Recently, Stancheva et al. showed in Xenopus that MECP2 binds to the promoter region of the xHairy2a gene. This gene encodes a neuronal repressor that is a target gene of Notch signaling involved in a variety of cell fate decisions. This repressor recruits the SMRT corepressor complex, then the MECP2-SMRT complex silences the gene (Stancheva et al., 2003). One question that arises from this Xenopus work is whether the phenotypes of MECP2-null mice are actually due to abnormal cell fate decisions demonstrated in Xenopus embryos. MECP2-null mice begin to display abnormal phenotype at approximately 3 weeks of age (Chen et al., 2001; Guy et al., 2001), whereas the phenotype observed with MECP2 antisense morpholino in Xenopus embryos was apparent during embryonic stages (Stancheva et al., 2003). If abnormal cell fate decisions in the mouse nervous system caused the observed phenotype, the phenotypic abnormalities would appear during embryonic development when neurogenesis occurs. However, this does not appear to be the case; no abnormalities, including any of brain size, are detected until 3 weeks of age. To investigate the possibility that subtle, abnormal cell fate decisions during mouse embryonic development lead to the phenotypes observed at the later stages, we examined the proliferation and differentiation ability of neural precursors derived from wild-type, MECP2 +/−, and MECP2 −/− mice. However, we could not detect any significant differences among the three genotypes in proliferation or differentiation, arguing that MECP2 mutation does not affect these events of early neural development in mice. In addition, a recent report, which showed that transgene expression of MECP2 under the control of a neuron-specific promoter rescues the phenotype in MECP2 mutant mice, also supports our conclusion (Luikenhuis et al., 2004). One possible explanation for this difference between mice and Xenopus is that there is more redundancy for MECP2 function in mice than in Xenopus. In mice, four additional methyl CpG binding proteins have been identified: MBD1, 2, 4, and Kaiso (Kriaucionis and Bird, 2003). In particular, mouse MBD1 is known to be expressed in immature cells expressing Nestin, as well as in mature neurons in the adult hippocampus (Zhao et al., 2003). It is possible that mouse MBD1 partially or fully compensates for loss of MECP2 function during embryonic neurogenesis. Another possibility is a difference in the role played by DNA methylation between mice and Xenopus, as the spectra of DNA methylation levels and patterns are very broad among species (Bird, 2002).

Potential roles of MECP2 in Rett syndrome

Our data demonstrate that MECP2 is expressed in the neuronal lineage in vivo and in vitro, that the expression of MECP2 increases as neuronal maturation progresses, and that MECP2 mutation does not affect the proliferation and differentiation of neural precursors in mice. These data indicate that MECP2 is involved in the maturation and maintenance of neurons, rather than in early cell fate decisions or neuronal migration (Fig. 9). In what steps might MECP2 play a role? Neural development does not mean only the birth of neurons and neuronal migration to appropriate positions during embryonic stages. In order for a neuron to become fully mature and participate in neuronal circuitry, it must develop axonal projections to targets, extend its dendrites, form synapses, and be able to release neurotransmitter (Fig. 9). It is notable that both the substantial increase of MECP2 expression in the neocortex and the transition of MECP2 staining from “uniform” to “punctate” occur over the time when neurons in the neocortex become more mature. We speculate that MECP2 may play a role in some of these events.

Dendritic arborization is an important maturation process during the formation of neuronal circuitry. The previous findings using human postmortem tissue indicated less complex dendritic arborization and smaller neuronal size in patients with Rett syndrome (Armstrong et al., 1995; Bauman et al., 1995a,b). Our study using MECP2 mutant mice strengthens this evidence that MECP2 mutation causes these phenotypes, and is not simply due to nutritional, respiratory, or other secondary medical issues. Although highly speculative, there may be a connection between recent data on BDNF as one target gene for MECP2-modified regulation (Chen et al., 2003; Martinowich et al., 2003) and a reduction in dendritic complexity in TrkB and BDNF conditional mutant mice and TrkB−/− neurons (Gates et al., 2000; Gorski et al., 2003; Xu et al., 2000). Recent reports indicate that MECP2 binds selectively to the BDNF promoter III, which is activated in response to neuronal depolarization, and functions as a negative regulator of BDNF expression (Chen et al., 2003; Martinowich et al., 2003). Other recent reports indicate that the neocortex is reduced in thickness due to dendritic retraction in TrkB and BDNF conditional mutant mice (Gorski et al., 2003; Xu et al., 2000). Interestingly, BDNF conditional mutant mice (Gorski et al., 2003) and TrkB−/− neurons (Gates et al., 2000) begin to display less complex dendritic arborization at 3 weeks of age, the same age at
that MECP2 neural circuitry. In agreement with this speculation, our data show abnormal neuronal maturation and synapse formation in related phenotype observed in BDNF conditional null mice due to expression in MECP2 mutant mice might partially mimic the dysregulated BDNF which MECP2-null mice begin to show reduced brain size. Whereas one interpretation of recent reports is that BDNF might be modestly basally upregulated in MECP2 mutant mice (Chen et al., 2003; Martinowich et al., 2003), this dysregulated BDNF expression in MECP2 mutant mice might partially mimic the phenotype observed in BDNF conditional null mice due to abnormal neuronal maturation and synapse formation in related neural circuitry. In agreement with this speculation, our data show that MECP2 /−/mice have a thinner neocortex than wild-type mice, and layer II/III pyramidal neurons in MECP2 /−/mice have smaller somas and less complex dendrites than those in wild-type mice, suggesting that dysregulation of BDNF–TrkB signaling may cause some of phenotypes of MECP2 mutant mice. Although MECP2 mutant mice share some phenotypes with BDNF conditional mutant mice, the phenotypes in MECP2 mutant mice are substantially more severe and more widespread than those in BDNF conditional mutant mice. MECP2 /−/mice die between 6 and 12 weeks of age (Chen et al., 2001; Guy et al., 2001), whereas BDNF conditional mutant mice survive until 14–18 months of age (Gorski et al., 2003). These findings suggest that MECP2 mutation causes other functional disturbance, along with less dendritic arborization in part due to dysregulation of BDNF gene.

Axonal projection is another neuronal maturation process that might be affected by MECP2 mutation. For example, callosal projection neurons (CPN) which are excitatory pyramidal neurons in layers II/III and V of the neocortex and project via the corpus callosum to targets in homologous regions of contralateral cortex (Conti and Manzoni, 1994), begin to extend their axons to the midline via the corpus callosum at approximately E18–E19 in rodents (Floeter and Jones, 1985). At P2–3, the axons begin to innervate contralateral cortex, and target innervation is complete by P6–7 (Spreatco et al., 1995). This similar time course of CPN development and the progressive expression of MECP2 suggest that MECP2 may be involved in the late development of these and related projection neurons. In fact, MRI studies detect a smaller corpus callosum in some autism patients and Rett syndrome patients, suggesting abnormalities of formation or maintenance of these interhemispheric projection neurons (Egaas et al., 1995; Gotoh et al., 2001). Furthermore, a recent study using a proteomic differential display strategy detected differences in expression levels of genes that are categorized as functioning in cytoskeleton arrangement, suggesting that MECP2 deficiency might lead to abnormal axonal projections (Matarazzo and Ronnett, 2004). Because layer II/III and V callosal projection neurons are responsible for interhemispheric association pathways and are thought to be centrally involved in high level associative cognitive function, our data suggest that the observed abnormal projection neuronal complexity may underlie some aspects of the cognitive phenotype in Rett syndrome.

Another critical event in neuronal maturation is the development of electrophysiologic activity. Of note, the sodium channel type II (SCN2A) gene is a target gene for MECP2 in non-neural cells (Lunyak et al., 2002). SCN2A-deficient mice die perinatally due to severe hypoxia, presumably resulting from abnormal respiratory regulation in the brainstem (Planells-Cases et al., 2000). In addition, mutations of the SCN1A and SCN1B genes encoding subunits of the neuronal voltage-gated sodium channels have been identified as genes responsible for human generalized epilepsy with febrile seizures plus type 2 (GEF+2) and 1 (GEF+1), respectively (Escayg et al., 2000; Wallace et al., 1998). Seizures and respiratory problems are major phenotypes of both MECP2 mutant mice and patients with Rett syndrome; thus, these findings suggest that MECP2 mutation may in some way alter ion channel or neurotransmitter expression.

Taken together, our data indicate that MECP2 functions in the maturation and maintenance of neurons in mice. Late-stage developmental events of circuit connectivity, dendritic development, and regulation of electrophysiologic function are critical late developmental events whose subtle perturbation could lead to subtle-yet-critical functional phenotypes. Further investigation of MECP2 function in these processes may contribute importantly to understanding the neuropathogenesis of Rett syndrome.

Experimental methods

Animals

All animal experimental protocols were approved by the institutional animal care and use committee, and adhere to NIH
guidelines. Wild-type C57/B16 mice were purchased from Charles River Laboratories (Wilmington, MA). Female MECP2 heterozygous mice were generously provided by the laboratories of Drs. Rudolf Jaenisch and Adrian Bird (Chen et al., 2001; Guy et al., 2001), and MECP2 hemizygous mice were obtained by crossing heterozygotes with wild-type male C57/B16. For timed gestations, midnight before the morning upon which the vaginal plug was observed was defined as day 0. The genotypes of mice were identified by PCR on tail genomic DNA (forward primer Nsi-5′-CAC CAC AGA AGT ACT ATG ATC-3′; 2lox-3′-5′-GTA GGT AAG AGC TCT TGT TGA-3′; Nsi-3′-5′-ATG CTG ACA AGC TTT CTT CAT-3′; 2 min at 95°C, 30 cycles of 45 s at 95°C, 45 s at 55°C, 1 min at 72°C, followed by 5 min at 2°C. The PCR generates only a 280-bp product for wild-type genomic DNA, and 280- and 300-bp products for heterozygous mice, and only a 300-bp product for hemizygous mice.

**Histology**

Animals were perfused for immunocytochemistry following deep anesthesia with Avertin; and brains were fixed by cardiac perfusion with 0.1 M phosphate-buffered saline (PBS) with 10 U/ml heparin, pH 7.4, followed by 4% paraformaldehyde (PFA) in PBS buffer (4% PFA/1% glutaraldehyde was used for immunocytochemistry for glutamate). Brains were either cryoprotected overnight in 30% sucrose in PBS and sectioned coronally at 30 µm on a freezing microtome (Leica, Nussloch, Germany), or postfixed in 4% PFA (4% PFA/1% glutaraldehyde for glutamate staining) overnight and sectioned at 40 µm with a vibrating microtome (Leica).

**FluoroGold injection**

Three-day-old mouse pups were anesthetized by hypothermia, and FluoroGold (Fluorochrome, Inc., Englewood, NJ; FG; 2% in 75% ddH2O/25% DMSO) was injected into the left neocortex of the left neocortex with a digitally controlled oocyte injector (Nanoinjector II; Drummond Scientific, Broomall, PA) using pulled glass micropipettes with tip diameter of 30–60 µm. FluoroGold was microinjected at depths ranging from 300 µm deep to the pial surface, at 10 sites spaced evenly throughout the parietal cortex, depositing 50 nl at each site, for a total of 500 nl in a hemisphere. The pups were returned to their mothers. FluoroGold was retrogradely transported across the corpus callosum to the contralateral hemisphere for 2 weeks, and the FG-injected mice were perfused at 2 weeks of age for combined FG analysis and immunocytochemistry.

**Isolation and expansion of embryonic CNS neural precursors and neurosphere formation assay**

Timed-gestation female mice were deeply anesthetized with Avertin to obtain E13.5 mouse embryos with each of the three genotypes: wt, +/-, –/y. Embryos were individually dissected in Hank’s buffered saline solution (HBSS), neocortex was collected, tissue was dissociated by gentle mechanical trituration in HBSS, and cells were collected by centrifugation.

Neurosphere formation assays were performed as described (Reynolds and Weiss, 1996) with slight modifications. Dissociated cells were resuspended in DMEM/F12-based serum-free growth medium containing insulin (25 µg/ml), apo-transferrin (100 µg/ml), progesterone (20 nM), sodium selenate (30 nM), putrescine (60 µM) (all from Sigma-Aldrich, St. Louis, MO), EGF (20 ng/ml) (Peprotech, Rocky Hill, NJ), and FGF-2 (20 ng/ml) (Peprotech), and plated in culture dishes at 10 cells/µl, lower than the cell density at which virtually all neurospheres are clonal (Hulspas et al., 1997). During a 9-day expansion, some cells proliferate and form large cell masses called neurospheres (Reynolds and Weiss, 1996). After 9 days, the number of neurospheres greater than 50 µm in diameter was counted from four wells of cells derived from each embryo, from at least three independent embryos from each of the three genotypes.

Monolayer culture assays were performed as described in Johe et al. (1996) with slight modifications. Dissociated cells were resuspended in DMEM/F12-based serum-free growth medium with FGF-2 (20 ng/ml) and plated on plastic tissue culture plates precoated with poly-L-ornithine (10 µg/ml) and fibronectin (1 µg/ml) (Sigma-Aldrich) at 80,000 cells/cm². Media were changed every 2 days. After 4 days, cells were removed from the plates by briefly incubating them in HBSS, and cells were then plated on glass coverslips precoated with poly-L-ornithine and fibronectin at 40,000 cells/cm². After 2 days in growth medium, cells were treated with differentiation medium (growth medium with 1% fetal bovine serum (Invitrogen, Carlsbad, CA), but without FGF-2).

**Immunocytochemistry**

Cryostat or vibrating microtome sections prepared as described above were collected into PBS, incubated in blocking solution (PBS, 5% BSA, 3% goat serum, and 0.3% TritonX-100) for 30 min at room temperature, then incubated in primary antibody overnight at 4°C. Primary antibodies were used at the following concentrations: rabbit polyclonal anti-MECP2 antibody (1:200, Affinity Bioreagents, Golden, CO); mouse monoclonal anti-NeuN antibody (1:500, Chemicon, Temecula, CA); mouse monoclonal anti-GFAP antibody (1:200, Sigma-Aldrich); mouse monoclonal anti-CNPass antibody (1:200, Promega, Madison, WI); and mouse monoclonal anti-Reelin antibody (1:100, Chemicon). Sections were washed three times in PBS for 10 min and incubated in Alexa 546 goat anti-rabbit IgG antibody (1:750, Molecular Probes, Eugene, OR) and Alexa 488 goat anti-mouse IgG antibody (1:250, Molecular Probes) for 2 h at room temperature. Sections were washed three times in PBS, rinsed in distilled deionized water (DDW), and mounted on glass slides.

Cells cultured on coverslips were washed in PBS, fixed in 4% paraformaldehyde in PBS for 10 min, and washed with PBS three times. Cells were preincubated with PBS containing 10% goat serum for 15 min, then incubated with primary antibodies overnight at 4°C. Primary antibodies were used at the following concentrations: rabbit polyclonal anti-MECP2 antibody (1:200 Affinity Bioreagents); mouse monoclonal anti-Nestin antibody (1:100, Chemicon); guinea pig polyclonal anti-DCX antibody (1:500, Chemicon); mouse monoclonal anti-Hu antibody (1:200, courtesy of S. A. Goldman and M. Marusich); mouse monoclonal anti-MAP-2 antibody (1:500, Chemicon); rabbit polyclonal and mouse monoclonal anti-GFAP antibody (1:200 Sigma-Aldrich); mouse monoclonal anti-O4 antibody (1:200, courtesy of P. Follet). Cells were washed three times with PBS for 5 min and incubated in appropriate fluorescent secondary antibodies: Alexa 546 goat anti-rabbit IgG (1:750); Alexa 546 goat anti-guinea pig IgG (1:750); Alexa488 goat anti-mouse IgG (1:250); Alexa 488 goat anti-rabbit IgG (1:250); Alexa 488 goat anti-mouse IgM (1:250) (all from Molecular Probes) for 2 h at room temperature. Cells were washed three times in PBS, rinsed in DDW, and mounted on glass slides.
Determination of cortical layer thickness

Wild-type and MECP2 −/− brains at 2 weeks (n = 5 for each genotype) and 8 weeks (n = 6 for each genotype) of age were sectioned coronally at a thickness of 50 μm. Half of the sections were used for Nissl staining and the rest were used for cytochrome oxidase (CO) staining. To determine the location of the barrel cortex, CO staining was performed on alternate sections. Sections were incubated in 0.3% Triton X-100 at 37°C for 1 h, then incubated at 37°C in PBS containing 0.05% diaminobenzidine, 0.02% cytochrome c, and 0.01% H2O2 until the desired contrast was obtained. Nissl staining was performed with cresyl violet (0.2% cresyl violet, 0.5% acetic acid, 0.01 M sodium acetate, and 0.02 M sodium hydroxide) to determine layer boundaries. The thickness of layers II/III to VI were measured at the middle of the barrel cortex, every 400 μm, from the most rostral position of the barrel cortex that was determined by CO staining. Cells were counted in a 143 × 109 μm grid of each layer in four independent locations in each of six mice of each genotype. Statistical analysis was performed using an unpaired two-tailed t test.

Golgi staining

Golgi staining was carried out using a rapid Golgi method (Morest and Morest, 1966). Six brains of 8-week wild-type and MECPC2 −/− mice were incubated in a solution containing 2% potassium dichromate and 0.2% osmium tetroxide for 7 days in the dark. Then, the brains were rinsed with 0.5% silver nitrate until precipitates disappeared and were incubated in 1% silver nitrate in 2% potassium dichromate and 0.2% osmium tetroxide for 7 days in the dark. The brains were dehydrated and embedded in low-viscosity nitrocellulose. The brains were cut at a thickness of 100 μm and cleared in α-terpineol. The closest layer II/III pyramidal neurons to the center crosshair in six independent microscopic fields for each brain were selected for Sholl analysis (Sholl, 1953), then reconstructed and drawn using a camera lucida device. Concentric circles in 20-μm radius increments were drawn around the center of the reconstructed soma (see Figs. 8C and D), and the number of dendrites crossing each circle was counted. Dendritic spines were counted along 20-μm lengths of primary and secondary dendrites. Soma sizes were measured under a microscope using OpenLab software.

Acknowledgments

We thank Farren Briggs, Kyle MacQuarrie, and Alex Eswar for excellent technical assistance. We thank Pieter Dikkes of the Mental Retardation Research Center at Children’s Hospital for superb histological expertise and assistance. We thank Drs. Jason Emsley and Bartley Mitchell for helpful comments and critical reading of the manuscript. We thank Drs. Adrian Bird, Jacky Guy, and Rudolf Jaensich for generously sharing their MECP2 mutant mice. This work was partially supported by fellowships from the Rett Syndrome Research Foundation and Sumitomo Life Social Welfare Services Foundation to N.K., by infrastructure supported by NINDS grants NS41590 and NS45523 to JDM, and by MRRC HD18655 at Children’s Hospital.

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