Widespread Changes in Dendritic and Axonal Morphology in Mecp2-Mutant Mouse Models of Rett Syndrome: Evidence for Disruption of Neuronal Networks

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ABSTRACT
Rett syndrome (RTT) is a neurodevelopmental disorder caused by mutations in the X-linked gene MECP2. Girls with RTT show dramatic changes in brain function, but relatively few studies have explored the structure of neural circuits. Examining two mouse models of RTT (Mecp2B and Mecp2J), we previously documented changes in brain anatomy. Herein, we use confocal microscopy to study the effects of MeCP2 deficiency on the morphology of dendrites and axons in the fascia dentata (FD), CA1 area of hippocampus, and motor cortex following Lucifer yellow microinjection or carbocyanine dye tracing. At 3 weeks of age, most (33 of 41) morphological parameters were significantly altered in Mecp2B mice; fewer (23 of 39) were abnormal in Mecp2J mice. There were striking changes in the density and size of the dendritic spines and density and orientation of axons. In Mecp2B mice, dendritic spine density was decreased in the FD (–11%), CA1 (14–22%), and motor cortex (–16%). A decreased spine head size (–9%) and an increased spine neck length (–12%) were found in Mecp2B FD. In addition, axons in the motor cortex were disorganized. In Mecp2J mice, spine density was significantly decreased in CA1 (14–26%). In both models, dendritic swelling and elongated spine necks were seen in all areas studied. Marked variation in the type and extent of changes was noted in dendrites of adjacent neurons. Electron microscopy confirmed abnormalities in dendrites and axons and showed abnormal mitochondria. Our findings document widespread abnormalities of dendrites and axons that recapitulate those seen in RTT. J. Comp. Neurol. 514:240–258, 2009.© 2009 Wiley-Liss, Inc.

Indexing terms: Rett syndrome; MeCP2; mouse models; brain; spines; axons; synapses; mitochondria; morphometry

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DENDRITIC AND AXONAL PATHOLOGY IN Mecp2-MUTANT MICE

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MATERIALS AND METHODS

All experiments were conducted in accordance with the National Institutes of Health guidelines for the care and use of animals and with an approved animal protocol from the Stanford University Institutional Animal Care and Use Committee.

Mecp2-mutant mice

Female heterozygotes with an Mecp2<sup>241D</sup> mutation lacking exons 3 and 4 in the Mecp2 gene (Mecp2<sup>B</sup>; Guy et al., 2001) were maintained on a C57BL/6J background, and Mecp2<sup>241D</sup> mice with an exon 3 deletion in Mecp2 (Mecp2<sup>J</sup>; Chen et al., 2001) were maintained on a mixed 129/Sv, C57BL/6J, and BALB/c background. To obtain the litters of WT and Mecp2<sup>J</sup> animals for this study, we mated two Mecp2<sup>241D</sup> female littermates to a WT BALB/c male.
mice with these mutations, in which changes are expected to be more severe; the changes are easier to identify, quantify, and interpret compared with female mutant mice and heterozygous female subjects with RTT.

Lucifer yellow microinjection of neurons

Mice were anesthetized with sodium pentobarbital (200 mg/kg i.p.; Abbott Laboratories, North Chicago, IL), transcardially perfused for 1 minute with 0.9% sodium chloride (10 ml) and then for 10 minutes with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4 (100 ml). The brains were sectioned coronally at 200 μm on a Vibratome and transferred to an injection chamber. Lucifer yellow (LY) microinjections were performed on lightly fixed Vibratome slices, as described (Belichenko et al., 2004). Two slices per mouse were used: 50–60 cells for the FD per mouse (with an n = 3, resulting in a total of 150–180 cells); 20–30 cells for the CA1 region and for layers II–III of the motor cortex per mouse (with n = 3, resulting in a total of 60–90 cells) were analyzed. For each dendrite type a specific domain was examined: 1) for the FD we examined each of the three subdivisions of the molecular layer in their entirety; 2) for CA1 basal and apical oblique, and for motor cortex basal, we examined dendrites immediately after the first bifurcation and then for 20–40 μm; and 3) for the motor cortex apical, we examined dendrites immediately after the appearance of the first apical oblique dendrite.

Carbocyanine dye tracing

To visualize axons in the brain, we used the carbocyanine dye tracing method as previously described (Belichenko and Dahlstrom, 1995b). Three pairs of WT and Mecp2B littermates at age 21 days were anesthetized with sodium pentobarbital (200 mg/kg i.p.) and transcardially perfused for 1 minute with 0.9% sodium chloride (10 ml) and then for 10 minutes with 4% paraformaldehyde in 0.1 M PBS, pH 7.4 (100 ml). A tiny crystal (100 μm in diameter) of 1.1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI, D-282, lot 4591-17, Mole- cular Probes, Eugene, OR) was placed into the white matter of the motor cortex, and the brains were immersed in 4% paraformaldehyde in 0.1 M PBS, pH 7.4, at room temperature. Seven to 8 months later, the brains were sectioned perpendic- ular to the pial surface on a Vibratome at 30–100-μm-thick sections, and the sections were mounted on a microscope slide, coverslipped with PBS, and immediately imaged with confocal microscope.

Confocal microscopy

Confocal microscopy was performed as described (Belichenko et al., 2004). Slices labeled with LY or DiI were exam- ined and scanned in a Radiance 2000 confocal microscope (Bio-Rad, Hertfordshire, UK) attached to a Nikon Eclipse E800 fluorescence microscope. The laser was an argon/krypton mixed gas laser with exciting wavelengths for LY (488 Å) or for DiI (568 Å). LaserSharp software (Bio-Rad) was used to estab- lish optimal conditions for collecting images. The morphology of LY-microinjected neurons and their spines was studied under the following optimal conditions: the lens was a 60× objective (Nikon; Plan Apo 60×/1.40 oil); laser power was 10%; optical sections were scanned at increments of 0.5 μm, which correlates with the resolution value at z-plane; the zoom factor was 10; scanning was at 500 ips; each optical section was the result of three scans followed by Kalman filtering; pixel size was 0.039 μm; and the size of the image was 512 × 512 pixels. Dil-labeled brain sections were studied in the same manner except that the lens was a 20× objective (Nikon; Plan Apo ×20/0.75) and laser power was 50%, optical sections were scanned at 2-μm increments, and the zoom factor was 3. Each image was saved as a stack of individual optical sections and as a z-projection.

Image analysis

LaserPix software analysis of the z-projection image of individual LY-microinjected dendrites was used to measure the length and width of dendrites, count dendritic spines, and estimate the sizes of heads and the lengths of necks. The number of spines was calculated per 100-μm length of den- drite. To quantify the incidence of dendrites with swelling, the widths of dendrites in three locations (the beginning, the mid- dle portion, and the end of a 5-μm segment of dendrite) were measured and exported to Excel. The average value, standard deviation, and standard deviation/average ratio of widths of dendrite were calculated with Excel. If the standard deviation/ average was >0.2 (i.e., more than 20% variation in widths of dendrite), the dendrite was considered swollen. We also counted the occurrence of dendrites with areas lacking spines (“spineless” spots) and with long spines. Dendrites with no spines along 2.5 μm of their length on one side of dendrite were counted as dendrites with spineless spot(s). A dendrite was considered to have long spines, if the average spine neck was longer than 1.25 μm for the FD and CA1 and longer than 2.4 μm for the motor cortex (i.e., ~2.5 times longer than average length of spine neck for these structures). LaserPix software analysis of the z-projection of 10 optical images (increment was 2 μm) of Dil-labeled axons was used to mea- sure the total number of Dil-labeled axons by densitometry, and z-projection of Dil-labeled axons was used to evaluate the orientation of fibers by measuring the angles between individ- ual fibers and the pial surface in motor cortex.

Electron microscopy

Ultrastructural analysis of dendritic and axonal architecture was performed as previously described (Belichenko et al., 2004). Mice were deeply anesthetized with sodium pentobarbital (200 mg/kg i.p.) and transcardially perfused with ice-cold 0.9% sodium chloride for 1 minute, followed by ice-cold 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 10 minutes. Brains were immediately re- moved and placed in the same fixative for 1 week at 4°C; then brains were coronally sectioned (at 300 μm) on a Vibratome, postfixed in 1% osmium tetroxide, and stained with saturated uranyl acetate in 50% ethanol. Sections were then dehydrated through ethanol, infiltrated with 2-hydroxypropyl methacrylate and Scipoxy 812 resin (Energy Beam Sciences, Agawam, MA), and polymerized. Thin sections (80 nm) were cut on a Reichert Ultracut E Ultramicrotome (Leica, Vienna, Austria), picked up onto 200-mesh copper grids (Electron Microscopy Sciences, Fort Washington, PA) and poststained in ethanolic uranyl acetate followed by bismuth nitrate (Electron Microscopy Sci- ences). The sections were analyzed with a Zeiss EM10 electron microscope. Ten randomly acquired EM images of the neuropil from the hippocampus and motor cortex from each animal were photographed at a final magnification of 5,000× or 20,000×. The electron micrographs were digitally scanned at 2,400 dots per inch (DuoScan T2500, Agfa, Teterboro, NJ).
and stored as TIFF files. For quantitative analysis of mitochondrial cross-sectional area, a total of 5 electron micrographs (at magnification of 5,000 x) from the hippocampus were studied. Mitochondrial radii were determined automatically by the Laserscan program from mitochondrial cross-sectional area. Mitochondria were identified by the presence of both a distinct double-membrane and identifiable cristae.

Digital images were imported, enhanced for brightness and contrast, assembled and labeled in Adobe Photoshop CS2 color printer (Tektronix by Xerox, Norwalk, CT).

Statistical analyses

The data for density of spines, width of dendrites, area of spine head, length of spine neck, mitochondrial cross-sectional area, density of DiI-labeled axons, and angle of orientation of axons were exported to Excel, and statistical comparisons were performed by using two-way analysis of variance (ANOVA) and for two samples by using two-tailed Student’s t-tests. All results are expressed as mean ± SEM, and P values < 0.05 were considered to be significant.

RESULTS

Changes in spine density and dendritic morphology in MeCP2-mutant mice

To define the morphology of dendrites and dendritic spines, we microinjected LY into the cell bodies of individual neurons in brain slices from MeCP2B, MeCP2J mutants, and their WT littermates. The dendrites of injected neurons were brightly filled, allowing for a detailed quantitative analysis of granular cells in the FD, pyramidal neurons in the CA1 area of hippocampus, and layers II–III pyramidal neurons of motor cortex by confocal microscopy (Figs. 1, 3, 5, 7). We quantitated in z-projections of dendrites of LY-microinjected neurons: 1) the number of spines per 100-μm length of dendrite; 2) the width of individual dendrites; 3) the area of the spine head and the length of the spine neck; 4) the frequency of dendrites with swellings; 5) the frequency of dendrites with spineless spots; and 6) the frequency of dendrites with extremely long spines. Striking changes were noted in the mutants, and the changes differed quantitatively among these three brain region (Tables 1–3, S1). The findings provide evidence for significant changes in the morphology of dendrites and spines in MeCP2 mutant mice.

Comparing spine densities in WT and MeCP2B mice, there was a statistically significant 11% decrease in the FD of MeCP2B mice (Table 1, Fig. 1a–e). Decreases were statistically significant for all three subdivisions of the FD: inner (9%), middle (8%), and outer (17%) layer (Table 1). For the MeCP2J

<table>
<thead>
<tr>
<th>Structure</th>
<th>Genotype</th>
<th>Spine density</th>
<th>Width of dendrites (μm)</th>
<th>Dendrites with swellings (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fascia dentata, total</td>
<td>WT for MeCP2B</td>
<td>170.5 ± 1.3</td>
<td>1.04 ± 0.05</td>
<td>31.1</td>
</tr>
<tr>
<td></td>
<td>MeCP2B</td>
<td>152.5 ± 1.0</td>
<td>0.96 ± 0.06</td>
<td>65.4</td>
</tr>
<tr>
<td></td>
<td>WT for MeCP2J</td>
<td>201.6 ± 0.7</td>
<td>1.03 ± 0.08</td>
<td>23.5</td>
</tr>
<tr>
<td></td>
<td>MeCP2J</td>
<td>201.4 ± 1.0</td>
<td>0.97 ± 0.07</td>
<td>32.3</td>
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<td>Inner ML</td>
<td>WT for MeCP2B</td>
<td>168.5 ± 1.5</td>
<td>1.23 ± 0.03</td>
<td>16.7</td>
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<tr>
<td></td>
<td>MeCP2B</td>
<td>153.2 ± 1.3</td>
<td>1.11 ± 0.03</td>
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</tr>
<tr>
<td></td>
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<td>192.4 ± 4.3</td>
<td>1.30 ± 0.04</td>
<td>10.1</td>
</tr>
<tr>
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<td>MeCP2J</td>
<td>189.7 ± 3.9</td>
<td>1.19 ± 0.04</td>
<td>23.5</td>
</tr>
<tr>
<td>Middle ML</td>
<td>WT for MeCP2B</td>
<td>170.3 ± 0.5</td>
<td>1.02 ± 0.03</td>
<td>23.3</td>
</tr>
<tr>
<td></td>
<td>MeCP2B</td>
<td>157.4 ± 4.6</td>
<td>0.98 ± 0.02</td>
<td>73.5</td>
</tr>
<tr>
<td></td>
<td>WT for MeCP2J</td>
<td>206.2 ± 4.0</td>
<td>1.02 ± 0.03</td>
<td>30.6</td>
</tr>
<tr>
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<td>MeCP2J</td>
<td>208.0 ± 4.6</td>
<td>0.96 ± 0.06</td>
<td>22.8</td>
</tr>
<tr>
<td>Outer ML</td>
<td>WT for MeCP2B</td>
<td>173.9 ± 5.2</td>
<td>0.86 ± 0.02</td>
<td>56.7</td>
</tr>
<tr>
<td></td>
<td>MeCP2B</td>
<td>145.0 ± 4.3</td>
<td>0.77 ± 0.02</td>
<td>81.7</td>
</tr>
<tr>
<td></td>
<td>WT for MeCP2J</td>
<td>206.7 ± 4.4</td>
<td>0.77 ± 0.02</td>
<td>32.2</td>
</tr>
<tr>
<td></td>
<td>MeCP2J</td>
<td>205.0 ± 3.6</td>
<td>0.75 ± 0.02</td>
<td>53.7</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>CA1 basal</td>
<td>137.9 ± 3.4</td>
<td>0.72 ± 0.02</td>
<td>53.7</td>
</tr>
<tr>
<td></td>
<td>MeCP2B</td>
<td>107.9 ± 3.2</td>
<td>0.72 ± 0.02</td>
<td>89.7</td>
</tr>
<tr>
<td></td>
<td>WT for MeCP2J</td>
<td>161.7 ± 3.4</td>
<td>0.81 ± 0.02</td>
<td>56.3</td>
</tr>
<tr>
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<td>MeCP2J</td>
<td>120.0 ± 3.7</td>
<td>0.70 ± 0.01</td>
<td>84.2</td>
</tr>
<tr>
<td></td>
<td>CA1 apical oblique</td>
<td>143.3 ± 4.2</td>
<td>0.68 ± 0.01</td>
<td>54.0</td>
</tr>
<tr>
<td></td>
<td>MeCP2B</td>
<td>122.6 ± 4.2</td>
<td>0.67 ± 0.01</td>
<td>93.3</td>
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<tr>
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<td>WT for MeCP2J</td>
<td>177.3 ± 4.0</td>
<td>0.79 ± 0.02</td>
<td>36.4</td>
</tr>
<tr>
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<td>MeCP2J</td>
<td>152.8 ± 4.4</td>
<td>0.72 ± 0.02</td>
<td>86.9</td>
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<tr>
<td>Motor cortex</td>
<td>Layers II–III basal</td>
<td>163.1 ± 3.8</td>
<td>0.98 ± 0.02</td>
<td>25.7</td>
</tr>
<tr>
<td></td>
<td>WT for MeCP2B</td>
<td>136.8 ± 3.6</td>
<td>0.89 ± 0.02</td>
<td>75.4</td>
</tr>
<tr>
<td></td>
<td>MeCP2J</td>
<td>170.6 ± 4.6</td>
<td>0.96 ± 0.02</td>
<td>29.4</td>
</tr>
<tr>
<td></td>
<td>WT for MeCP2J</td>
<td>170.5 ± 4.9</td>
<td>0.86 ± 0.02</td>
<td>62.2</td>
</tr>
<tr>
<td></td>
<td>MeCP2J</td>
<td>165.0 ± 5.0</td>
<td>1.93 ± 0.07</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td>MeCP2B</td>
<td>128.8 ± 4.5</td>
<td>1.58 ± 0.05</td>
<td>22.8</td>
</tr>
<tr>
<td></td>
<td>WT for MeCP2J</td>
<td>153.6 ± 4.9</td>
<td>1.87 ± 0.05</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td>MeCP2J</td>
<td>152.5 ± 6.5</td>
<td>1.84 ± 0.06</td>
<td>17.0</td>
</tr>
<tr>
<td>Layers II–III apical oblique</td>
<td>WT for MeCP2B</td>
<td>156.8 ± 4.7</td>
<td>0.95 ± 0.03</td>
<td>42.2</td>
</tr>
<tr>
<td></td>
<td>MeCP2B</td>
<td>142.6 ± 4.6</td>
<td>0.89 ± 0.03</td>
<td>80.6</td>
</tr>
<tr>
<td></td>
<td>WT for MeCP2J</td>
<td>167.1 ± 5.0</td>
<td>0.99 ± 0.02</td>
<td>37.5</td>
</tr>
<tr>
<td></td>
<td>MeCP2J</td>
<td>159.2 ± 6.0</td>
<td>0.86 ± 0.03</td>
<td>50.0</td>
</tr>
</tbody>
</table>

1The number of mice used was as follows: WT for MeCP2B/MeCP2J/WT for MeCP2J/MeCP2J = 3/3/3/3.
2Per 100-μm length of dendrite.
3P < 0.05, significantly different between WT mice.
4P < 0.05, significantly different from WT mice.
5P < 0.05, significantly different between mutant mice.
mice, there was no significant difference in the mean value of spine density in the FD and their subdivisions (Table 1). Analysis of confocal images of dendritic spine density and their frequency distribution in the FD for both Mecp2B and Mecp2J mice showed significant diversity between individual dendrites. Whereas some dendrites had relatively few spines, others were excessively studded with spines (Figs. 1c–e, h–j, 2a,b). Because spines receive afferent axonal contacts, these findings point to differences in the extent of innervation of individual dendrites. Although overall, the frequency distribution of spine density in the FD of Mecp2B mice shifted to lower values, the data show that the distribution of values is not symmetrical, and raise the possibility of a biphasic distribution (Fig. 2a). The same may be true for Mecp2J mice (Fig. 2b).

Relative to controls, a significant reduction was also recorded for spine density in basal and apical oblique dendrites in CA1 for both mutant mice (Table 1, Fig. 3). In Mecp2B, spine density was reduced by 22% and 15%, respectively, and in Mecp2J by 26% and 14%, respectively. For both mutant mice, the frequency distribution of spine density was shifted to lower values (Fig. 4a,b). Surprisingly, the distribution of spine density in CA1 appeared to be different from that in the FD, where more significant diversity between individual dendrites was revealed. This suggests that these two regions of the hippocampus responded differently to Mecp2 mutation.

In layers II–III pyramidal neurons of the motor cortex of Mecp2B mice, we detected significantly fewer spines on the basal, apical, and apical oblique dendrites (17%, 22%, and 9% fewer, respectively; Table 1, Fig. 5a–e). No significant changes in dendritic spine density were observed in the motor cortex of Mecp2J mice, but the spread of values appeared to be greater (Table 1, Figs. 5h–j, 6b).

Spine densities in the FD and CA1 were significantly greater in WT for Mecp2J than in WT for Mecp2B mice (P < 0.01; Table 1). There was also significantly greater spine density in Mecp2J mice in all areas investigated than in Mecp2B (P < 0.01; Table 1).

Another aspect of dendritic morphology that differed in Mecp2-mutant mice was the width of dendrites. There was an overall pattern of decreased width in both mutants, and in some cases this reached statistical significance (Table 1). In Mecp2B vs. WT mice, the average width of dendrites was significantly smaller for the inner (by 10%) and outer molecular layer (by 10%) of the FD, and in layers II–III of the motor cortex for the basal (by 9%) and apical dendrites (by 18%) (Table 1). Mecp2J mice also had thinner dendrites, with an average width that was significantly reduced for basal and apical oblique dendrites in CA1 (by 14% and 9%) and in the basal and apical oblique dendrites of the motor cortex (by 10% and 13%) (Table 1). Changes in dendrite width were noted in comparing Mecp2-mutant mice with each other and in comparing the WT mice. The width of dendrites was greater in WT for Mecp2B than in WT for Mecp2J for outer molecular layer dendrites in the FD (P = 0.004), and lower for dendrites in CA1 (P < 0.01; Table 1). Comparing the mutants, the widths of

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**TABLE 2. Dendritic Spine Morphology in Brain Regions**

<table>
<thead>
<tr>
<th>Structure</th>
<th>Genotype</th>
<th>Area of spine head (μm²)</th>
<th>Length of spine neck (μm)</th>
<th>Dendrites with spineless spots (%)</th>
<th>Dendrites with long spines (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fascia dentata</td>
<td>WT for Mecp2B</td>
<td>0.34 ± 0.01²</td>
<td>0.42 ± 0.01²</td>
<td>29.3</td>
<td>18.6</td>
</tr>
<tr>
<td></td>
<td>Mecp2B</td>
<td>0.31 ± 0.01³</td>
<td>0.47 ± 0.01³</td>
<td>63.6</td>
<td>31.9</td>
</tr>
<tr>
<td></td>
<td>Mecp2J</td>
<td>0.33 ± 0.01</td>
<td>0.38 ± 0.01</td>
<td>22.4</td>
<td>18.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.314 ± 0.005³</td>
<td>0.41 ± 0.01</td>
<td>24.4</td>
<td>31.2</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>CA1 basal</td>
<td>WT for Mecp2B</td>
<td>0.34 ± 0.01²</td>
<td>0.41 ± 0.01²</td>
<td>46.3</td>
</tr>
<tr>
<td></td>
<td>Mecp2B</td>
<td>0.32 ± 0.01³</td>
<td>0.45 ± 0.02⁴</td>
<td>75.9</td>
<td>20.7</td>
</tr>
<tr>
<td></td>
<td>Mecp2J</td>
<td>0.36 ± 0.005³</td>
<td>0.34 ± 0.01</td>
<td>23.9</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.34 ± 0.005³</td>
<td>0.38 ± 0.01³</td>
<td>68.4</td>
<td>26.3</td>
</tr>
<tr>
<td>Motor cortex</td>
<td>Layers II–III basal</td>
<td>WT for Mecp2B</td>
<td>0.39 ± 0.01²</td>
<td>0.82 ± 0.02</td>
<td>35.1</td>
</tr>
<tr>
<td></td>
<td>Mecp2B</td>
<td>0.40 ± 0.01³</td>
<td>0.88 ± 0.02</td>
<td>61.2</td>
<td>57.6</td>
</tr>
<tr>
<td></td>
<td>Mecp2J</td>
<td>0.42 ± 0.01</td>
<td>0.81 ± 0.02</td>
<td>20.6</td>
<td>38.2</td>
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<tr>
<td></td>
<td></td>
<td>0.37 ± 0.01³</td>
<td>0.90 ± 0.02³</td>
<td>43.8</td>
<td>43.8</td>
</tr>
</tbody>
</table>

¹The number of mice used was as follows: WT for Mecp2B/Mecp2B/WT for Mecp2J/Mecp2J = 3/3/3/3.
²P < 0.05, significantly different between WT mice.
³P < 0.05, significantly different from WT mice.
⁴P < 0.05, significantly different between mutant mice.

**TABLE 3. Summary of Parameters That Differ in Wild-Type and Mecp2 Mutant Mice by Types**

<table>
<thead>
<tr>
<th>Type</th>
<th>Spine density</th>
<th>Width of dendrites</th>
<th>Dendritic swelling</th>
<th>Area of spine head</th>
<th>Length of spine neck</th>
<th>Spineless pots</th>
<th>Long spines</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FD, MML</td>
<td>Basal CA1, apical MC</td>
<td>FD, CA1</td>
<td>FD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Basal MC</td>
<td>Basal CA1, apical MC</td>
<td>FD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Apical oblique MC</td>
<td>MML, apical MC</td>
<td>CA1,MC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Basal, apical oblique MC</td>
<td>MML</td>
<td>CA1,MC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>CA1</td>
<td>FD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>FD, MML, OML</td>
<td>FD</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>7</td>
<td>CA1/MC</td>
<td>FD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1For the definition of types, see Table S1. Abbreviations: FD, fascia dentata; IML, inner molecular layer of FD; MC, motor cortex; MML, middle molecular layer of FD; OML, outer molecular layer of FD. Note that types 1–6 indicate WT for Mecp2B = WT for Mecp2J and types 7–12 indicate WT for Mecp2B = WT for Mecp2J.
Dendritic spine density changes in Mecp2-mutant mice. Dendrites from LY-injected neurons were imaged by confocal microscopy in the fascia dentata in WT for Mecp2B (a,b), Mecp2B (c–e), WT for Mecp2J (f,g), and Mecp2J (h–j) mice. Note that spine density is decreased in some dendrites and increased in others in Mecp2-mutant mice compared with WT. Scale bar = 5 μm in j (applies to a–j).
Figure 2.
Quantitative analysis of the frequency distribution of spine density (a,b), spine head areas (c,d), and spine neck lengths (e,f) in the fascia dentata of WT (empty bars) and Mecp2-mutant mice (black bars).
Figure 3.
Spines on the basal dendrites of CA1 pyramidal neurons in WT for Mecp2B (a,b), Mecp2B (c–e), WT for Mecp2J (f,g), and Mecp2J (h–j) mice. Dendrites from LY-injected neurons were imaged by confocal microscopy. Note that spine density is decreased and regions of dendritic swelling are increased in Mecp2-mutant mice. Scale bar = 5 μm in j (applies to a–j).
Figure 4.
Quantitative analysis of the frequency distribution of spine density (a,b), spine head areas (c,d), and spine neck lengths (e,f) in the basal dendrites of CA1 pyramidal neurons in WT (empty bars) and Mecp2-mutant mice (black bars).
Dendrites were lower for CA1 apical oblique and apical in the motor cortex in Mecp2B than in Mecp2J mice ($P < 0.01$; Table 1).

Dendritic swelling, as defined by 20% variation in widths of dendrite (see Materials and Methods), was seen in both WT and Mecp2-mutant mice. However, in Mecp2-mutant mice in comparison to WT mice, the number of dendrites with swellings was increased in all regions examined (Table 1, Fig. 7c–f). The FD had on average 1.1-fold higher frequency of swollen dendrites in Mecp2B mice and a 1.4 higher frequency in Mecp2J. The most marked increases were detected in the middle molecular layer for Mecp2B (by 3.2 times) and in the inner molecular layer for Mecp2J (by 2.6 times) (Table 1).

Figure 5. Spines on the basal dendrites of layers II–III pyramidal neurons of the motor cortex in WT for Mecp2B (a,b), Mecp2B (c–e), WT for Mecp2J (f,g), and Mecp2J (h–j) mice. Dendrites from LY-injected neurons were imaged by confocal microscopy. Irregularities in dendritic width, spine density, neck length, and head size are obvious in Mecp2-mutant mice. Scale bar = 5 μm in j (applies to a–j).
Figure 6.
Quantitative analysis of the frequency distribution of spine density (a,b), spine head areas (c,d), and spine neck lengths (e,f) in the basal dendrites of layers II–III pyramidal neurons of motor cortex in WT (empty bars) and MeCP2-mutant mice (black bars).
CA1 area of the hippocampus also showed significantly increased dendritic swellings in the mutants (−1.7 more in Mecp2B and −2.0 more in Mecp2J). The findings in layers II–III of the motor cortex were similar, with a −2.5 increase in Mecp2B and a −1.6 increase in Mecp2J (Table 1).

Changes in dendritic spine morphology in Mecp2-mutant mice

To characterize further synaptic morphology in Mecp2-mutant mice, we examined in detail the size of spine heads, length of spine neck, and frequency of spineless spots. In both mutants, there was a significant decrease in the average area of spine heads in all regions examined, except for the motor cortex in Mecp2B mice (Table 2, Figs. 2c,d, 4c,d, 6d). For Mecp2B mice, the decrease in spine head area was −10% in the FD, whereas that in CA1 basal was −6%; these changes were significant (Table 2, FD: P < 0.001; CA1 basal: P = 0.01). The frequency diagram of spine head size pointed to an overall decrease in both regions (Figs. 2c, 4c). For Mecp2J, there was a significant decrease in spine head size in the FD (by −5%; P = 0.047), in CA1 basal (by −6%; P = 0.01), and in layers II–III basal dendrites in the motor cortex (by −12%, P < 0.001) (Table 2). Comparing the WT mice, spine head area for the WT for Mecp2B mice showed an increase relative to those for the Mecp2J mice in the FD (P = 0.03; Table 2) and a decrease in CA1 (P = 0.01; Table 2) and in the motor cortex (P < 0.01; Table 2). Comparing the mutants, however, the area of spine heads in CA1 was smaller in Mecp2B than in Mecp2J mice (P = 0.01; Table 2) and significantly larger in the motor cortex (P = 0.01; Table 2).

The necks of spines also differed between Mecp2-mutant and WT mice. In all regions, we detected increases in the length of spine necks, and in most cases the changes were significant. In Mecp2B mice, significantly longer necks were detected in the FD (by −12%; P = 0.001; Table 2), whereas in Mecp2J mice necks were longer by −11% (P = 0.02) in CA1 basal dendrites and by −12% (P = 0.003) in the basal dendrites of the motor cortex (Table 2). The frequency distribution of spine neck lengths revealed an overall increase in the FD, CA1, and motor cortex (Figs. 2e, 4f, 6f). Our studies pointed to the presence of markedly swollen regions contained many short cytoskeletal fragments, compared with WT littermates (Figs. 9a–d). Over-}

Disorganization of axons in Mecp2-mutant mice

Significant alterations in dendrites and spines suggested that Mecp2-mutant mice might also show changes in axonal morphology. Axons were imaged by inserting a tiny crystal of Dil into the white matter of the motor cortex. Figure 8 shows representative images of Dil-labeled axons in layers II–III and layers V–VI of the motor cortex. In Mecp2B motor cortex, axons demonstrated a distribution that was more diffuse with respect to orientation than in WT mice (Fig. 8b,d). Whereas axons in the WT mice showed a relatively uniform orientation, in Mecp2B mice, axonal trajectories were quite variable. Moreover, the number of labeled axons was increased in these mice (Fig. 8b,d). We quantified the number of Dil-labeled axons by using densitometry; there was a significant increase (by −33%, P = 0.001) in Mecp2B mice (Fig. 8e). We also measured, with respect to the pial surface, the angles of orientation of Dil-labeled axons in the motor cortex. Axons in WT mice were often oriented vertically (i.e., were oriented perpendicular to the pia; Fig. 8a,c,f), whereas axons in Mecp2B mice showed a more disperse orientation (Fig. 8b,d,f). These data are evidence for the involvement of both axons and dendrites in a deficiency in MeCP2 function.

Ultrastructural abnormalities in dendrites and axons in Mecp2-mutant mice

By achieving increased resolution, EM analyses were used to confirm and extend dendritic and axonal phenotypes. Dendrites and axons in the hippocampus and cortex were examined in WT and Mecp2-mutant mice at age 21 days (Figs. 9, 10). Consistent with light microscopic findings, there were dilations and constrictions in the dendrites of Mecp2-mutant mice that were markedly increased in frequency compared with WT littermates (Figs. 9a,b, 10a,b). The cytoplasm of swollen regions contained many short cytoskeletal fragments, including apparently damaged microtubules (Fig. 9b–d). Overall, microtubules in dendrites were less preserved in Mecp2B vs. WT (Fig. 9c,d), and Mecp2B mice were more affected than Mecp2J mice. Approximately 40% of the dendrites in Mecp2B and −10% of the dendrites in Mecp2J showed what appeared to be a decreased density of microtubules as well as exam-
Figure 7.
Detailed morphologic abnormalities of dendrites and dendritic spines in the fascia dentata (a,c,g), CA1 hippocampus (b,d,e), and motor cortex (f,h–k) of MeCP2-mutant mice. Dendrites from LY-injected neurons were imaged by confocal microscopy. Note spineless spots on dendrites (a,b), dendritic swelling (c–f), and spines with elongated spine neck (g–i) demarcated by arrows. j and k illustrate different spine density on the different branches of basal dendrites from the same neuron. Scale bar = 5 μm in a–k.
Figure 8. Analysis of axonal distribution in motor cortex. Confocal images of Dil-labeled axons in layers II–III (a,b) and layers V–VI (c,d) in WT (a,c) and Mecp2B (b,d) mice. The total density of axons (e) is increased and the angles of axons (f) are more widely distributed in Mecp2B mice (black bar) than in WT mice (empty bar). Density is expressed as percent of axons occupying total area of image. Scale bar = 50 µm in d (applies to a–d).
Figure 9.
Ultrastructural analysis of dendrites and axons in the CA1 hippocampus of WT for Mecp2B (a,c,e,g) and Mecp2B (b,d,f,h) mice. Note dendritic swelling in b (arrows designate dendritic borders), alteration of microtubules in a dendrite (d), disruption of myelin sheaths in an axon (ax, f), and enlargement of mitochondrion and cristae membranes (h; arrows point to mitochondria). Scale bar = 0.5 μm in a–h.
amples in which there was apparent fragmentation of these structures. In Mecp2-mutant mice, myelin sheaths appeared to be disrupted (Fig. 9f), and axons were less myelinated (Fig. 10d); also, lower numbers of myelinated axons were found. Mitochondria appeared to be enlarged in Mecp2-mutant mice (Figs. 9h, 10f). An analysis of mitochondrial cross-sectional area in dendrites in the hippocampus of WT and Mecp2B mice showed a significant difference between them (WT for Mecp2B/H11549 0.07 ± 0.01 μm²/n, n = 5; Mecp2B/H11550 0.14 ± 0.01 μm², n = 5; P = 0.003). The ratio of maximum/minimum mitochondrial radius showed that mitochondria in Mecp2B were elongated (WT for Mecp2B/H11549 1.49 ± 0.06, n = 5; Mecp2B/H11550 2.04 ± 0.12, n = 5; P = 0.03).

Mitochondrial cristae also appeared to be enlarged (Figs. 9h, 10f). Of note, the cell bodies in the hippocampus and cortex were free of swollen vacuoles and electron-dense bodies; dendrites were free of dystrophic neurites and of vacuoles. There was no disruption of mitochondria and endoplasmic reticulum, and few multivesicular bodies and laminated structures were seen. In general, Mecp2B mice showed fewer normal-appearing dendrites and axons in the hippocampus and cortex than Mecp2J mice. These data confirm the presence of marked changes in dendrites and axons in Mecp2-mutant mice.

**DISCUSSION**

Given earlier reports in subjects with RTT (Armstrong, 1992; Belichenko and Dahlstrom, 1995a; Belichenko et al., 1994; Armstrong et al., 1995), and recent anatomical findings in Mecp2-mutant mice (Belichenko et al., 2008), we expected to find changes in synaptic and axonal structure in mice mutant for the Mecp2 gene. Unexpected was the extent of these changes, their diversity of type, and the variable extent to which individual neurons were affected. The picture that emerges is of a process in which individual brain regions and, indeed, individual neurons are differentially affected by a de-
iciency in MeCP2. The pathological signatures that we detected are consistent with differential circuit involvement within the maturing brain. It is reasonable to assert that it is this complex pattern of circuit failure that is responsible for the corresponding clinical picture. Defining the pathogenesis of MECP2 mutations will require studies to decipher the actions of MeCP2 within individually affected circuits and to discern how mutations impact their structure and function.

MeCP2 is a protein with a complex set of functions that are carried out, at the highest level, in postmitotic, mature neurons (Kishi and Macklis, 2004; Dragich et al., 2007; Bird, 2008). Subsequently, the neurological picture of MeCP2 dysfunction would be expressed in the neuronal circuits in which such neurons participate. This seems to be the case clinically, as the changes in function emerge only after birth. Given the diversity of MeCP2 functions and its widespread pattern of expression, it is not surprising that a striking variety of symptoms is seen clinically and that these change character over time. Therefore, we hypothesized that to understand the neurobiology of RTT it would be necessary to examine in detail the consequences of MeCP2 mutation at both regional and subregional levels. We recently reported findings on the volume and shape of a number of brain regions (Belichenko et al., 2008). Herein we have described dendritic and axonal phenotypes (Tables 1–3, S1). We discovered that changes in dendritic and axonal morphology similar to those found in RTT would be detected in MeCP2-mutant mice. Indeed, significant alterations from WT were detected in both dendrites and axons in the Mecp2B and Mecp2J mouse brains. The changes were widespread, involving the hippocampus (CA1 and FD) and motor cortex (Tables 1–3, S1). We examined a large number of morphological parameters. At 3 weeks of age, most (33 of 41) parameters were significantly altered in Mecp2B mice; fewer (23 of 39) were abnormal in Mecp2J mice (Table S1). Striking changes were evident in the density and size of the dendritic spines, width of dendrites, and frequency of dendritic swelling, and density, and orientation of axons.

Morphological changes involving dendrites and axons in MeCP2-mutant mice

The importance of studying the morphology of dendrites, dendritic spines, and axons is that these neuronal structures conduct and receive electrical inputs needed for neurotransmission between different neurons (Li and Sheng, 2003). Changes in synaptic plasticity followed by poor learning and memory have been linked to morphological changes in axons, dendrites, and spines (Ikegaya et al., 2001b; Kaufmann and Moser, 2000; Murthy et al., 2001; Yuste and Bonhoeffer, 2001; Fiala et al., 2002; Tanaka et al., 2008). We described several dendritic phenotypes in Mecp2-mutant mice (Tables 3, S1). The most striking change was an increased frequency of dendritic swelling in all regions examined in both Mecp2-mutant mice. The average number of dendritic swellings was 1.4 times higher in Mecp2 vs. WT mice. EM studies confirmed the presence of swollen dendrites and showed that they contain many short cytoskeletal fragments and damaged microtubules. Dendritic swellings were similar to those reported in earlier studies in RTT (Comford et al., 1994). The underlying cause of the dendritic swellings we detected is unknown.

In principle, three kinds of changes can be envisioned: local changes in dendritic water content due to excessive activation of ion channels; local dysregulation of the underlying cytoskeleton of the dendrite; or the accumulation within dendrites of debris or the products of neurodegeneration. Although the data are insufficient to prove the case, our observations are most consistent with the first possibility in that by EM we detected significant decreases in the density of staining and in the concentration of organelles within enlarged dendritic shafts. This phenotype resembles that present in cultured hippocampal dendrites in response to excessive synaptic activation produced by high concentrations of N-methyl-D-aspartate (NMDA) exposure (Ikegaya et al., 2001a). In Mecp2B mice, whereas in general synaptic properties of hippocampal neurons were preserved, altered basal inhibitory rhythms were speculated to predispose to hippocampal network hyperexcitability (Zhang et al., 2008). It is interesting to note that in mice expressing a truncated allele of MeCP2 (Mecp2308/308), basal synaptic transmission was enhanced, whereas paired-pulse facilitation was decreased, suggesting enhanced probability of glutamate release (Moretti et al., 2006).

With respect to the observed cytoskeletal and microtubular abnormalities seen by EM, it is of interest that expression of stathmin-like 2 (STM2) was decreased in the cerebellum of Mecp2B and Mecp2J mice (Jordan et al., 2007). STM2 encodes the neuronal growth-associated protein SCG10 (superior cervical ganglia neural-specific 10 protein). In neurons, SCG10 modulates microtubule dynamics by acting at both ends of microtubules (Manna et al., 2007). In addition, SCG10 binds to microtubules along their entire lengths, and SCG10 protein levels are found in a stoichiometric relationship to tubulin subunits (Bondallaz et al., 2006). Thus, it is not hard to imagine that reduced SCG10 levels in Mecp2-mutant brains could affect microtubule dynamics and stability.

Focusing on dendritic spines, the area of spine heads was significantly reduced, and changes in spine density were detected in several brain regions (Tables S3, S1). Interestingly, involvement of spines differed from region to region, from one neuron to the next, and within the dendrites of an individual neuron. Some dendrites showed decreased density, whereas for others near-normal or increased density was present. Because spines mark the sites of synaptic contacts, we conclude that marked changes must exist in synaptic connectivity in Mecp2-mutant mice. Moreover, although the changes are widespread, differences in the severity of changes, between and within regions, suggest that an underlying selectivity exists in the severity of involvement in different types of neural circuits.

Whatever the cause for the changes detected, their presence may well be correlated with abnormal synaptic transmission, as reported in Mecp2-mutant mice (Asaka et al., 2006) and with deficiencies of learning and memory (Stearns et al., 2007). In both Mecp2B and Mecp2J mice, induction of LTP and LTD were significantly decreased (Asaka et al., 2006). Inability to induce LTP in these mice may serve as the cellular correlate of reduced cognitive ability (Stearns et al., 2007). This reduction in synaptic plasticity was also accompanied by a decrease in paired-pulse facilitation, suggesting presynaptic terminal abnormalities.

In summary, we speculate that the dendritic and axonal alterations detected herein and in human studies are linked to failed synaptic plasticity in both the neocortex and the hippocampus, and with decreased learning and memory in Mecp2-mutant mice and in RTT.
Differences in severity of dendritic phenotypes of Mecp2B and Mecp2J mice

Using the same strategy to compare severity of brain morphology between Mecp2-mutant mice (Belichenko et al., 2008), we conclude that dendritic and axonal phenotypes were generally more severe in Mecp2B vs. Mecp2J mice. As summarized in Tables 3 and S1, 20 phenotypes were significantly worse in Mecp2B vs. Mecp2J mice (types 4, 6, 10, and 12), and only 9 were significantly worse in Mecp2J vs. Mecp2B (types 3, 5, 9, and 11). Examples in which Mecp2B was more severely affected included: spine density, dendritic swelling, and spineless spots on dendrites (Tables 1, 2, S1). For some phenotypes, five in total, the two mutant strains were affected equally (Table S1, types 2 and 8). Consistent with previous reports, the onset of symptoms and time of death were on average somewhat later in the Mecp2J vs. Mecp2B mice (Chen et al., 2001; Guy et al., 2001; Chang et al., 2006; Santos et al., 2007). Our previous (Belichenko et al., 2008) and current findings provide evidence that Mecp2 mutation impacts brain volume and dendritic and axonal structure and suggest that many brain regions are affected. The background strain differences, specifically the action of strain-specific modifying genes, may also contribute to the variances observed between WT mice (Tables 1, 2).

Dendritic and axonal alterations in Mecp2-mutant mice: similarities to RTT

Dendritic and axonal alterations in Mecp2-mutant mice recapitulated some of those changes seen in the RTT brain. The truncation and thickening of dendrites in RTT (Cornford et al., 1994) resemble dendritic swellings described here in Mecp2-mutant mice. Decreased numbers of spines in pyramidal neurons of the cortex in subjects with RTT were described (Belichenko et al., 1994). Here we documented similar findings in the motor cortex, CA1, and FD of Mecp2B and in CA1 of Mecp2J mice (Tables 3, S1). Regional losses of spines in the cortex of subjects with RTT were described earlier by one of the authors (Belichenko et al., 1994; Belichenko and Dahlstrom, 1995a) and were confirmed in the present study in the motor cortex, CA1, and FD of Mecp2B and in the motor cortex and CA1 in Mecp2J mice by quantitative analysis. It is interesting to note that distribution of axons followed the same pattern in the motor cortex of RTT (Belichenko et al., 1994) and in Mecp2B mice (present study). Ultrastructural studies in Mecp2-mutant mice showed that dendrites also contained larger mitochondria and altered morphology of cristae. Mitochondrial abnormalities were reported in the brain of RTT (Cornford et al., 1994) and Mecp2B mice (Kriaucionis et al., 2006). The alterations included increased size, granular inclusions (Cornford et al., 1994), and misregulation of genes encoding for the mitochondrial respiratory chain (Kriaucionis et al., 2006).

The lack of comparable studies on dendrites and axons in subjects with RTT limits our ability to compare such features as increased frequency of dendritic swelling, spines with extremely long heads, decreased thickness of dendrites, and alteration of spine morphology in Mecp2-mutant mice and RTT. Future work on detailed spine morphology in subjects with RTT will test whether similar alterations can be revealed in RTT. Poor ability to learn and retain new information in RTT (Percy, 2002) may be a consequence of dendritic and axonal abnormalities.

Toward the mechanism for abnormal neural circuits in RTT

Our findings of widespread changes in dendrites, dendritic spines, and axons in Mecp2-mutant mice support the view that RTT impacts principally the structure and function of neural circuits (Belichenko et al., 1994). One interpretation is that the disease appears to impact differentiating neurons more potently than either migrating neurons or the events that characterize earlier neuronal development. Indeed, our studies provided no evidence for changes in neuronal migration, as cortical lamination and hippocampal formation were normal (Belichenko et al., 2008). The same is true in RTT (Belichenko et al., 1994, 1997; Armstrong, 2005). Also consistent with the view that MeCP2 deficiency affects neural differentiation are our results on the trajectories of axons in the mutant mice and RTT brain. In both, we detected increased numbers of cortical axons stained with Dil and marked changes in axonal orientation. What is uncertain is how these changes arise, whether they are primary or compensatory, and which ones are most significant for clinical deficits. It can be shown, as suggested herein, that not all circuits are affected equally, future studies should focus on which circuits are predisposed, when they are affected, and the genes and gene products that are implicated. The ability to focus efforts on a well-defined subset of genes could accelerate progress toward definition of those whose dysregulation is caused by MECP2 mutations and hasten the identification of therapeutic targets.

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LITERATURE CITED

Belichenko NP, Belichenko PV, Li HH, Mobley WC, Francke U. 2006.


