Neuronal morphology in MeCP2 mouse models is intrinsically variable and depends on age, cell type, and Mecp2 mutation

I-Ting J. Wang 1, Arith-Ruth S. Reyes 1, Zhaolan Zhou *

Department of Genetics, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104, USA

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A B S T R A C T

Rett Syndrome (RTT), a progressive neurological disorder characterized by developmental regression and loss of motor and language skills, is caused by mutations in the X-linked gene encoding methyl-CpG binding protein 2 (MECP2). Neurostructural phenotypes including decreased neuronal size, dendritic complexity, and spine density have been reported in postmortem RTT brain tissue and in Mecp2 animal models. How these changes in neuronal morphology are related to RTT-like phenotype and MeCP2 function, and the extent to which restoration of neuronal morphology can be used as a cellular readout in therapeutic studies, however, remain unclear. Here, we systematically examined neuronal morphology in vivo across three Mecp2 mouse models representing MeCP2 loss-of-function, partial loss-of-function, and gain-of-function mutations, at developmental time points corresponding to early- and late-symptomatic RTT-like behavioral phenotypes. We found that in Mecp2 loss-of-function mouse models, dendritic complexity is reduced in a mild, age-dependent, and brain region-specific manner, whereas soma size is reduced consistently throughout development. Neither phenotype, however, is altered in Mecp2 gain-of-function mice. Our results suggest that, in the cell types we examined, the use of dendritic morphology as a cellular readout of RTT phenotype and therapeutic efficacy should be cautioned, as it is intrinsically variable. In contrast, soma size may be a robust and reliable marker for evaluation of MeCP2 function in Mecp2 loss-of-function studies.

Introduction

Rett Syndrome (RTT) is a neurological disorder that is caused by mutations in the X-linked gene encoding methyl-CpG binding protein 2 (MECP2) (Amir et al., 1999). It primarily affects young girls, with a prevalence of 1 in 10,000 to 15,000 live births. One striking feature of RTT is the time course at which clinical symptoms appear. After 6–18 months of apparently normal development, affected individuals enter a period of developmental stagnation, characterized by microcephaly, growth arrest and hypotonia. This stage is followed by a period of developmental stagnation, characterized by microcephaly and growth arrest (Chahrour and Zoghbi, 2007). Phenotypic variability is present within the classic RTT profile, where affected girls differ by clinical severity (Chahrour and Zoghbi, 2007). This variability may be attributed to random X-chromosome inactivation and the variety of MECP2 mutations, including missense, nonsense, deletion, and insertion mutations, that have been identified in RTT patients (Bienvenu and Chelly, 2006). In vitro biochemical studies have demonstrated that the majority of these mutations lead to MeCP2 loss-of-function (Kriaucionis and Bird, 2003). Notably, genetic studies have also identified patients carrying duplication or triplication of MECP2 (Bienvenu and Chelly, 2006). These patients are classified under MECP2 duplication syndrome and bear a similar but distinct clinical profile to that of classical RTT (Chahrour and Zoghbi, 2007). Mice carrying two-fold levels of Mecp2 expression also show behavioral abnormalities (Collins et al., 2004; Luikenhuis et al., 2004), highlighting the importance of MeCP2 dosage and the detrimental effect of Mecp2 gain-of-function.

Histological analysis of postmortem RTT brain tissue has revealed morphological phenotypes including decreased cellular size with increased cell-packing density (Bauman et al., 1995), decreased dendritic complexity (Armstrong, 2005; Belichenko et al., 1994), and decreased spine density (Belichenko et al., 1994; Chapleau et al., 2009). Interestingly, these changes are selective, as decreased dendritic complexity has been observed in the motor, frontal, and inferior temporal cortices, but not in the visual cortex or hippocampus (Armstrong et al., 1995). Moreover, these changes appear to be cortical layer-specific, even within the same cortical area (Armstrong et al., 1995). No evidence of neuronal degeneration or atrophy has been identified (Armstrong, 2005; Jellinger et al., 1988), indicating that these morphological changes are a consequence of impaired neuronal development or structural maintenance, rather than neurodegeneration. What remains unclear, however, is how mutations in MECP2 lead to these changes in neuronal...
morphology and whether they contribute to RTT disease pathology or are secondary consequences of long-term illness.

To investigate the pathogenic mechanisms underlying RTT, several mouse models harboring different Mecp2 loss-of-function mutations have been developed and characterized (Brendel et al., 2011; Chen et al., 2001; Goffin et al., 2012; Guy et al., 2011; Jentarra et al., 2010; Pelka et al., 2006; Shahbazian et al., 2002). Although the mouse models recapitulate several RTT clinical features, each Mecp2 mutation confers a discrete behavioral profile, supporting the link between heterogeneity in MECP2 mutations and phenotypic variability in RTT. Similarly, underlying cellular structure in Mecp2 mutant mice differs across Mecp2 mutation (Belichenko et al., 2008, 2009b). While reduced dendritic complexity, soma size, and spine density are commonly identified in Mecp2 mutant mice (Belichenko et al., 2009a,b; Fukuda et al., 2005; Kishi and Macklis, 2004; Robinson et al., 2012; Stuss et al., 2012; Tropea et al., 2009), these structural phenotypes also differ by cellular subtype and developmental time point (Chapleau et al., 2012; Fukuda et al., 2005). The extent to which these factors influence cellular structure, however, is not well understood, as the use of different techniques and Mecp2 mouse models have made direct comparisons across studies difficult (Belichenko et al., 2008, 2009a; Chapleau et al., 2009; Cohen et al., 2011; Fukuda et al., 2005; Jentarra et al., 2010; Kishi and Macklis, 2004; Metcalf et al., 2006; Moretti et al., 2006; Robinson et al., 2012; Stuss et al., 2012; Zhou et al., 2006).

For example, reduced spine density has been reported in motor cortex layer II/III and V pyramidal neurons in 3-week-old Mecp2-null mice (Belichenko et al., 2009a,b), and in layer V at 8 weeks (Tropea et al., 2009), while no change in spine density has been reported in the somatosensory cortex layer II/III pyramidal neurons in 8-week-old Mecp2-null mice (Kishi and Macklis, 2004). Whether these dissimilar findings are consequences of brain region-specific or age-dependent regulation of neuronal morphology is difficult to evaluate, as studies have used different imaging techniques, including Golgi staining, neuron dye labeling, and fluorescent reporters, and different Mecp2 mutant mice (Chen et al., 2001; Guy et al., 2001). Indeed, Golgi staining has revealed reduced spine density in hippocampus CA1 of 12-week-old Mecp2-null mice (Robinson et al., 2012), but Dil labeling shows decreased spine density in the same cells at 1 week, and not at 2 or 7 weeks (Chapleau et al., 2012). A comprehensive and systematic analysis using a single experimental method, therefore, is needed to clarify how age, brain region, and Mecp2 mutation influence RTT-related neuronal morphology.

Importantly, recent studies have shown that RTT-like symptoms can be ameliorated through the reintroduction of Mecp2 in mice (Giacometti et al., 2007; Guy et al., 2007; Liy et al., 2011; Robinson et al., 2012). The reversibility of RTT phenotypes has stimulated wide interest in developing cellular assays to measure Mecp2 function. Morphological phenotypes such as spine density, dendritic outgrowth, and soma size have been used as readouts of therapeutic efficacy upon restoration of Mecp2 (Giacometti et al., 2007; Robinson et al., 2012) or IGF-1 treatment in mice and induced pluripotent stem cells (iPSCs) (Marchetto et al., 2010; Tropea et al., 2009). Thus, understanding the relationship between Mecp2 function, neuronal structure, and RTT-like phenotype, as well as identifying robust and reproducible morphological phenotypes as readouts of Mecp2 function, are imperative.

In this study, we systematically analyzed neuronal morphology in three Mecp2 mouse models, Mecp2–/−, Mecp2T158A/+ and Mecp2T58I, that represent the spectrum of MECP2 mutations contributing to the phenotypic heterogeneity of RTT and MECP2 duplication syndrome. The Mecp2–/− mice lack Mecp2 and demonstrate the most severe behavioral phenotype, representing Mecp2 loss-of-function (Guy et al., 2001). The Mecp2T158A/+ mice, mimicking a common RTT patient missense mutation at the Threonine 158 residue, demonstrate a moderately severe behavioral phenotype, representing Mecp2 partial loss-of-function (Goffin et al., 2012). Lastly, the Mecp2T58I mice express two-fold levels of MeCP2 and are believed to model Mecp2 duplication syndrome, representing Mecp2 gain-of-function (Collins et al., 2004). Importantly, although neuronal morphology in Mecp2 loss-of-function models has been individually studied (Belichenko et al., 2008, 2009a; Chapleau et al., 2009; Fukuda et al., 2005; Kishi and Macklis, 2004; Moretti et al., 2006; Robinson et al., 2012; Stuss et al., 2012), a direct comparison of morphological phenotypes in Mecp2 mouse models across the RTT phenotypic spectrum, including the less severe partial loss-of-function Mecp2 mutations and Mecp2 duplication, has yet to be conducted.

Given the progressive nature of RTT-like phenotypic onset, we focused on early and late developmental time points representative of none-to-mild behavioral phenotype (“early” time point) and overt behavioral phenotype (“late” time point) in each mouse model. By using the same experimental method to analyze neuronal morphology in vivo across these mouse models and developmental time points, we aimed to address the following questions in this study: 1) The effect of Mecp2 loss- or gain-of-function on the development of neuronal morphology, 2) The relationship between RTT-like behavioral symptomatic severity and underlying neuronal morphology, and 3) The brain region or cell type-specific effects of Mecp2 mutation on neuronal morphology.

We found that Mecp2 loss-of-function reduces dendritic complexity in a brain region-specific manner that correlates with the onset of behavioral phenotype. The degree of changes in dendritic complexity, however, was mild, domain-specific, and dependent on Mecp2 mutation. In contrast, a significant decrease in soma size upon Mecp2 loss-of-function persisted throughout development and across both Mecp2 loss-of-function mutations. Mecp2 gain-of-function, however, did not affect dendritic outgrowth or soma size, even after onset of gain-of-function behavioral phenotypes, suggesting that changes in cellular structure may not be effective readouts of therapeutics targeted toward MECP2 duplication syndrome. The subtlety of changes in dendritic outgrowth and the dependence on Mecp2 mutation, developmental time point, and brain region raise caution in using dendritic complexity as a cellular readout of RTT-like phenotype. Changes in soma size, however, are reproducible and robust, suggesting that soma size may be a more reliable marker in assessing MeCP2 function and therapeutic efficacy in RTT studies.

Materials and methods

Animal husbandry

Experiments were conducted in accordance to the ethical guidelines of the National Institutes of Health and with an approved animal protocol from the Institutional Animal Care and Use Committee of the University of Pennsylvania. To obtain male mice carrying Mecp2 mutations and Thy1-GFP/M transgene, female mice heterozygous for Mecp2 mutations Mecp2+/− (Guy et al., 2001), Mecp2+1.1bsd (Mecp2T158A/+ (Goffin et al., 2012)), or Mecp2T58I (Collins et al., 2004) were crossed with Thy1-GFP/M reporter mice (Feng et al., 2000). Mecp2−/− and Mecp2T58I/+ were maintained on a C57BL/6 background (Charles River). Mecp2T58I were maintained on a mixed FVB/C57BL/6 background. Mice were genotyped using a PCR-based strategy for Mecp2 and Thy1-GFP/M, as detailed by the Jackson Laboratory.

Immunohistochemistry

Mice were anesthetized with 1.25% Avertin (2,2,2-Tribromoethanol (wt/vol), transcardially perfused with 0.1 M phosphate buffered saline (PBS) for 1 min, then with 4% paraformaldehyde in 0.1 M PBS (wt/vol) for 10 min, and post-fixed in 4% paraformaldehyde in 1 h at 4 °C. Immunohistochemistry was performed on 200-μm coronal free-floating sections sliced using a Leica VT1000S vibratome. Tissues were rinsed 2 × 5 min with 0.1 M PBS and were blocked with a solution containing 5% normal goat serum, 0.25% Triton X-100 and 0.05% sodium azide for 2 h at room temperature. Tissues were incubated with primary antibody...
in blocking solution (chicken antibody to GFP, Aves Labs, 1:1000) overnight at 4 °C. Fluorescence detection was performed using secondary antibody to chicken conjugated to Alexa Fluor-488 (1:1000, Invitrogen) for 2 h at room temperature. Sections were counterstained with DAPI (1:1000, USB) to visualize DNA.

Confocal microscopy

GFP-labeled cells and their entire dendritic tree were detected spanning the depth of the 200-μm slices. For examination of dendritic complexity, somatosensory cortex layer V pyramidal neurons extending their apical dendrites to the pial surface and their basal dendrites into deep layer VI and hippocampal CA1 pyramidal neurons extending their basal dendrites into the stratum oriens and their apical dendrites through the stratum radiatum and stratum lacunosum-moleculare were imaged. Primary somatosensory cortex barrel field between Bregma coordinates −0.6 to −1.34 was analyzed, using the fimbria as an anatomical landmark. Rostral hippocampus between Bregma coordinates −1.34 to −1.94 was analyzed. For examination of soma size, cells located within 50 μm from the surface of the slice with intact somas were imaged and analyzed. Images of apical and basal dendrites were acquired separately with a Leica confocal microscope and Leica AF6000 imaging software using oil immersion 20 × and 40 × objective lenses. Each image was saved as a z-stacked series of optical sections. Four slices per mouse were imaged: 5–10 neurons for layer V somatosensory cortex per mouse and 7–10 neurons for hippocampal CA1 region per mouse were analyzed. All imaging was performed blind to genotype.

Quantitative analysis of neuronal morphology

Z-projection images of individual neurons were compressed into a max projection and dendrites were traced manually using Imagej software (National Institutes of Health) and NeuronJ, an Imagej plugin for neurite tracing and quantification (Meijering et al., 2004). Dendritic complexity was measured by performing Sholl analysis (Sholl, 1953), counting the number of dendritic crossings through a series of concentric circles centered at the soma and spaced at 20 μm intervals. Soma size was examined in Leica AF6000 by taking an area measurement of somas outlined manually. All quantification was performed blind to genotype.

Statistics

Data are reported as mean ± SEM, and statistical analysis was performed using GraphPad Prism 5.0. Sholl analysis was analyzed by two-way ANOVA with Bonferroni correction, with the independent (genotype and Sholl radius) and dependent variables (number of dendritic crossings). Soma size was analyzed by Student’s unpaired t-test with Bonferroni correction where necessary.

Results

Neuron imaging strategy

To minimize experimental manipulation and variation in our analysis of neuronal morphology in Mecp2 mutant mice, we took advantage of a genetic labeling approach that has been successfully utilized in other

Fig. 1. Thy1-GFP/M reporter imaging strategy. (a) Somatosensory cortex layer V pyramidal neurons are labeled with GFP throughout the cell body and dendritic tree in Thy1-GFP/M reporter mice. Scale bar = 250 μm. (b) Hippocampus CA1 pyramidal neurons are labeled with GFP throughout the cell body and dendritic tree in Thy1-GFP/M reporter mice. Scale bar = 250 μm. (c) Somatosensory cortex layer V pyramidal neurons extend their apical dendrites to the pial surface (arrow) and their basal dendrites deep into layer VI. Scale bar = 50 μm. (d) Basal dendrites of somatosensory cortex layer V pyramidal neurons, quantified by Sholl analysis, measuring number of intersections between concentric circles drawn around the cell soma (Sholl radii) and dendritic branches. Scale bar = 50 μm. (e) Apical dendrites of somatosensory cortex layer V pyramidal neurons, quantified by Sholl analysis. Scale bar = 50 μm.
RTT morphological studies, a Thy1-GFP/M reporter line (Belichenko et al., 2009a; Cohen et al., 2011). In this line, a sparse population of neurons intrinsically expresses GFP throughout the cell body and dendritic tree in various brain regions (Figs. 1a, b), allowing for direct visualization of individual neurons (Feng et al., 2000). To visualize neurons in MeCP2 mutant mice, we crossed male Thy1-GFP/M reporter mice to female MeCP2 heterozygotes (MeCP2−/+; MeCP2T158A/+; or MeCP2T85I). Given the confounding effects of mosaic MeCP2 expression in females from random X-chromosome inactivation, all experiments were performed using male littermates with the following genotypes: MeCP2−/−; Thy1−/−; MeCP2−/−; Thy1−/−; MeCP2T158A; Thy1−/−; MeCP2−/−; Thy1−/−; MeCP2T85I; Thy1−/−; MeCP2−/−; Thy1−/−; or MeCP2T85I. For simplicity, we will refer to MeCP2−/−; Thy1−/−; MeCP2−/−; Thy1−/−; MeCP2T158A; Thy1−/−; MeCP2−/−; Thy1−/−; MeCP2T85I as WT and MeCP2 mutant mice expressing the Thy1−/−; MeCP2−/−; Thy1−/−; MeCP2T158A; Thy1−/−; MeCP2−/−; Thy1−/−; MeCP2T85I transgene as MeCP2−/−, MeCP2T158A or MeCP2T85I.

Previous studies have shown that disruption of MeCP2 results in deficits in neuronal organization, dendritic complexity and synaptic connectivity in the somatosensory cortex of both postmortem RTT tissue and mice (Cohen et al., 2011; Dani and Nelson, 2009; Kaufmann et al., 2000). We therefore analyzed neuronal morphology in layer V pyramidal neurons of the somatosensory cortex. These neurons are distinct in their cellular architecture, characterized by apical and basal dendritic trees and a pyramid-shaped soma (Spruston, 2008), and thus can be consistently identified. We measured dendritic complexity and soma size in layer V pyramidal neurons extending their apical dendrites to the pial surface and their basal dendrites into deep layer VI (Fig. 1c).

Dendritic complexity in MeCP2 loss-of-function mice

The majority of mutations in MECP2 leading to RTT are loss-of-function mutations (Chahrour and Zoghbi, 2007). Therefore, we first assessed neuronal morphology in MeCP2-null mice (MeCP2−/−) that lack both MeCP2 transcript and MeCP2 protein, and represent one of the most phenotypically severe MeCP2 mutant mice (Guy et al., 2001). Like RTT patients, MeCP2−/− mice show no initial RTT-like phenotype, but begin to develop aberrant gait and reduced mobility around 3 postnatal weeks. By 8 postnatal weeks, MeCP2−/− mice display overt RTT-like phenotypes including hindlimb clasping, tremor, and irregular breathing. We therefore chose to investigate neuronal morphology upon MeCP2 loss-of-function at P30 and P60, time points representative of early-symptomatic and late-symptomatic RTT-like phenotype, respectively. At the early-symptomatic P30 time point, Sholl analysis of somatosensory cortex layer V pyramidal neurons revealed a statistically significant decrease in dendritic complexity in MeCP2−/− mice relative to WT littermates (WT: n = 24 neurons, MeCP2−/−: n = 19 neurons; p < 0.0001) (Fig. 2a). Post hoc tests revealed that this decrease was most pronounced in MeCP2−/− mice in both basal and proximal apical dendrites 140–160 μm from soma and in the distal apical tuft 700 μm from soma. As RTT-like behavioral phenotypes in P30 MeCP2−/− mice are mild but present, these data suggest that decreased dendritic complexity accompanies RTT-like behavioral phenotype.

At the late-symptomatic P60 time point, MeCP2−/− mice also showed reduced dendritic complexity (WT: n = 36 neurons; MeCP2−/−: n = 31 neurons; p < 0.0001) (Fig. 2b). Although mild, these decreases were more widespread than that of P30 and most pronounced in the basal arbor 100–140 μm from soma and in the distal apical arbor 680–700 μm from soma, where the significant reduction in the apical tuft is likely a reflection of reduced cortical thickness and brain size in these mice (Kishi and Macklis, 2004). Given the overt RTT-like behavioral phenotypes in P60 MeCP2−/− mice, the mild reduction in dendritic complexity is surprising but consistent with reports of normal cortical lamination and organization in 7–10 week-old MeCP2−/− mice (Belichenko et al., 2008; Guy et al., 2001; Metcalf et al., 2006) and unaffected dendritic length and complexity from in vitro studies using the same mice (Chao et al., 2007). Therefore, as excitatory input onto proximal dendrites comes primarily from local or adjacent collaterals, while input onto distal dendrites comes from more distant cortical or thalamic projections (Spruston, 2008), our data indicate that the mild but widespread reduction in dendritic complexity across domains results in altered total input onto MeCP2−/− layer V excitatory neurons, both in early-symptomatic and late-symptomatic MeCP2−/− mice.

Dendritic complexity in MeCP2 T158A partial loss-of-function mice

One of the most common MECP2 missense mutations occurs at Threonine 158, converting it to methionine (T158M) or alanine (T158A). We recently developed and characterized an MeCP2 T158A knockin mouse and found that MeCP2T158A mice show a similar but
delayed progression of RTT-like behavioral phenotypes relative to that of Mecp2−/− y mice, where phenotypes begin to develop at 4–5 weeks and are overt by 13 weeks (Goffin et al., 2012). At P60, Mecp2T158A/y mice express milder locomotor, anxiety, and motor coordination phenotypes compared to age-matched Mecp2−/− y mice, indicating that Mecp2 T158A is a partial loss-of-function mutation. In addition, the MeCP2 T158A protein is expressed but is less stable and shows reduced binding to methylated DNA (Goffin et al., 2012). To investigate neuronal morphology upon Mecp2 partial loss-of-function, we measured dendritic complexity at P30, prior to onset of RTT-like phenotypes, and P90, when RTT-like phenotypes are overt by 13 weeks (Gof −781; n = 32 neurons; Mecp2T158A/y; n = 40 neurons; p < 0.05; Fig. 4b). Post hoc tests revealed that this difference was specific to the basal dendritic arbor, and does not reflect a decrease in dendritic complexity, but rather, is a result of a shift towards the soma, as peak branching in Mecp2T158A/y mice is similar to that of controls (Fig. 4c). This shift toward the soma may reflect reduced hippocampal volume, which has been reported in Mecp2 loss-of-function mice (Belichenko et al., 2008). The age-dependent and domain-specific decrease in dendritic complexity in Mecp2T158A/y mice, therefore, is specific to layer V somatosensory cortex and absent in CA1 hippocampus, consistent with RTT postmortem studies (Armstrong et al., 1995). Together, these data support age-dependent and brain region-specific regulation of dendritc outgrowth upon Mecp2 loss-of-function.

**Soma size is regulated throughout development by MeCP2**

As both reduced dendritic outgrowth and soma size are believed to contribute to RTT patient microcephaly and reduced soma size has been reported in RTT postmortem tissue (Bauman et al., 1995), we also measured soma size in our MeCP2 mutant mice at both early- and late-symptomatic time points. In striking contrast to the age-dependence and brain region-specificity of changes in dendritic complexity, we found that changes in soma size persisted throughout development and were consistent between MeCP2 loss-of-function mutations. Soma size in somatosensory cortex layer V pyramidal neurons was reduced in both P30 early-symptomatic MeCP2−/− y mice (WT: 282.9 ± 4.0 μm², n = 124 neurons; MeCP2−/− y; 237.6 ± 4.7 μm², n = 103 neurons; p < 0.01) and in P60 late-symptomatic MeCP2−/− y mice (WT: 287.9 ± 4.4 μm², n = 126 neurons; MeCP2−/− y; 237.2 ± 4.4 μm², n = 104 neurons; p < 0.01; Fig. 5a), relative to WT littermates, indicating that MeCP2 loss-of-function leads to reduced soma size during both early and late development of RTT-like phenotypes. Similarly, soma size was reduced in both P30 pre-symptomatic MeCP2T158A/y mice (WT: 290.6 ± 3.9 μm², n = 115 neurons; MeCP2T158A/y; 251.3 ± 4.6 μm²,
n = 121 neurons; p < 0.01) and in P90 late-symptomatic Mecp2T158A/y mice (WT: 311.1 ± 5.0 μm², n = 8 neurons; Mecp2T158A/y: 262.6 ± 3.3 μm², n = 106 neurons; p < 0.01) relative to WT littermates (Fig. 5b), indicating that reduced soma size may even precede onset of RTT behavioral phenotypes and is a consequence of Mecp2 loss-of-function. Moreover, we previously reported that hippocampal CA1 pyramidal neuron soma size is decreased in both pre- and post-symptomatic Mecp2T158A/y mice (Goffin et al., 2012). The persistent reduction in soma size in Mecp2 loss-of-function mouse models throughout development and across different cell types contrasted with the age-dependent and brain region-specific changes in dendritic complexity suggests that MeCP2 regulates dendritic outgrowth and soma size through distinct mechanisms and that soma size may be a more reproducible and reliable cellular marker for MeCP2 function than dendritic complexity.

Fig. 4. Dendritic complexity in hippocampus CA1 of Mecp2T158A/y mice. (a) Sholl analysis of hippocampal CA1 pyramidal neurons in P30 WT (27 neurons from 5 mice) and Mecp2T158A/y mice (n = 33 neurons from 5 mice) show no change in dendritic complexity in Mecp2T158A/y mice relative to WT. Two-way ANOVA, p > 0.05. Bars represent mean ± sem. (b) Sholl analysis of hippocampal CA1 pyramidal neurons in P90 WT (n = 32 neurons from 5 mice) and Mecp2T158A/y mice (n = 40 neurons from 5 mice) show altered dendritic complexity in Mecp2T158A/y mice relative to WT specifically in the basal dendritic arbor. Two-way ANOVA with Bonferroni correction, p < 0.0001 (interaction); *p < 0.05, **p < 0.01. (c) Sholl analysis of basal dendritic complexity of hippocampal CA1 pyramidal neurons in P90 WT and Mecp2T158A/y mice show no difference in peak number of crossings in Mecp2T158A/y mice relative to WT.

Fig. 5. Soma size is regulated throughout development by MeCP2 function. (a) Somatosensory cortex layer V pyramidal neuron soma size is reduced in Mecp2−/− mice relative to WT at both P30 (WT: n = 124 neurons from 4 mice; Mecp2−/−: n = 103 neurons from 4 mice) and P60 (WT: n = 126 neurons from 4 mice; Mecp2−/−: n = 104 neurons from 4 mice). **p < 0.01, unpaired two-tailed Student t-test with Bonferroni correction. Bars represent mean ± SEM. (b) Somatosensory cortex layer V pyramidal neuron soma size is reduced in Mecp2T158A/y mice relative to WT at both P30 (WT: n = 115 neurons from 4 mice; Mecp2T158A/y: n = 121 neurons from 4 mice) and P90 (WT: n = 81 neurons from 4 mice; Mecp2T158A/y: n = 106 neurons from 4 mice). **p < 0.01, unpaired two-tailed Student t-test with Bonferroni correction.
Dendritic complexity in Mecp2 gain-of-function mice

As the importance of MeCP2 dosage has been highlighted though the link between gain-of-function MECP2 mutations and neurodevelopmental disorder (Chahrour and Zoghbi, 2007), we next examined a transgenic mouse line expressing two-fold levels of MeCP2 (Mecp2\textsuperscript{gfv}) to determine how Mecp2 gain-of-function affects neuronal morphology (Collins et al., 2004). Importantly, neuronal morphology has yet to be described in seizures, ataxia, and premature death (Collins et al., 2004). We therefore behavior by 20 postnatal weeks and after 30 postnatal weeks, exhibit seizures, ataxia, and premature death (Collins et al., 2004). We therefore examined a transgenic mouse line expressing two-fold levels of MeCP2 (WT: n = 101 neurons from 2 mice; Mecp2\textsuperscript{gfv}: n = 19 neurons; p > 0.05) (Fig. 6a) and P140 (WT: n = 23 neurons; Mecp2\textsuperscript{gfv}: n = 23 neurons; p > 0.05) (Fig. 6b). These data are in contrast to the reduced dendritic complexity observed in late-symptomatic Mecp2 loss-of-function animals (Figs. 2b, 3b), and suggest a separate mechanism of dendritic outgrowth regulation in Mecp2 gain-of-function that is not coupled to behavioral phenotype.

Similarly, we found no changes in soma size in Mecp2\textsuperscript{gfv} mice relative to WT littermates, both prior to (P30; WT: 266.3 ± 4.3 \(\mu\text{m}^2\), n = 101 neurons; Mecp2\textsuperscript{gfv}: 272.8 ± 3.0 \(\mu\text{m}^2\), n = 110 neurons) and after the development of behavioral phenotypes (P140; WT: 268.9 ± 4.2 \(\mu\text{m}^2\), n = 110 neurons; Mecp2\textsuperscript{gfv}: 285.0 ± 4.2 \(\mu\text{m}^2\), n = 113 neurons; p > 0.05) (Fig. 6c). This finding is consistent with in vitro studies showing no effect of MeCP2 overexpression on soma size (Jugloff et al., 2005).

Discussion

RTT phenotypes are believed to originate from disruptions in neuronal circuits caused by MeCP2 dysfunction in the brain (Goffin and Zhou, 2012; Katz et al., 2012), and changes in neuronal morphology provide a cellular basis for alterations in circuit function. Given the rise in the use of morphological phenotypes as readouts of therapeutic efficacy (Marchetto et al., 2010; Tropea et al., 2009) or restoration of MeCP2 function (Giacometti et al., 2007; Robinson et al., 2012), understanding how MECP2 mutations impact the maintenance of proper neuronal structure and the relationship between RTT-like phenotype and underlying cellular morphology has become vital. Robust and reproducible RTT-related morphological phenotypes have been difficult to identify, as the use of different techniques, animal models, cell types, and developmental time points across different labs have made direct comparisons between studies difficult. Therefore, we sought to eliminate the confounds of methodological bias by using the same experimental method to analyze neuronal morphology in vivo across the development of Mecp2 loss-of-function, partial loss-of-function, and gain-of-function mouse models. Given the successful utilization of

![Fig. 6. Dendritic complexity in Mecp2\textsuperscript{gfv} mice. (a) Sholl analysis of somatosensory cortex layer V pyramidal neurons in P30 WT (n = 16 neurons from 2 mice) and Mecp2\textsuperscript{gfv} mice (n = 19 neurons from 2 mice) show no change in dendritic complexity in Mecp2\textsuperscript{gfv} mice relative to WT. Two-way ANOVA, p > 0.05. Bars represent mean ± sem. (b) Sholl analysis of somatosensory cortex layer V pyramidal neurons in P140 WT (n = 23 neurons from 2 mice) and Mecp2\textsuperscript{gfv} mice (n = 23 neurons from 2 mice) show no change in dendritic complexity in Mecp2\textsuperscript{gfv} mice relative to WT. Two-way ANOVA, p > 0.05. (c) Somatosensory cortex layer V pyramidal neuron soma size is not affected in Mecp2\textsuperscript{gfv} mice relative to WT at both P30 (WT: n = 101 neurons from 2 mice; Mecp2\textsuperscript{gfv}: n = 110 neurons from 2 mice) and P140 (P140; n = 110 neurons from 2 mice; Mecp2\textsuperscript{gfv}: n = 113 neurons from 2 mice); p > 0.05, unpaired two-tailed Student t-test.](image-url)
Thy1-GFP/M reporter mice in other cortical and hippocampal morphological studies (Belichenko et al., 2009a; Cohen et al., 2011) and the absence of added experimental manipulations for cellular visualization, we chose to cross our Mecp2 mutant mice to a Thy1-GFP/M reporter line in which neurons are intrinsically labeled with GFP in a mosaic manner throughout several brain regions, including the somatosensory cortex and hippocampus CA1 (Feng et al., 2000). In our study, we aimed to address the following three questions: 1) How does Mecp2 loss- or gain-of-function affect the development of neuronal morphology? 2) What is the relationship between RTT-like behavioral phenotypic severity and underlying neuronal morphology? 3) Are these changes in neuronal morphology brain region or cell type-specific?

To address the first question, we measured dendritic complexity and soma size in Mecp2^{−/−} and Mecp2^{7T158A/y} mice, representing Mecp2 loss-of-function and Mecp2 partial loss-of-function, respectively, and Mecp2^{+/+} mice, representing Mecp2 gain-of-function. We found decreased dendritic complexity and soma size in Mecp2 loss-of-function mice (Mecp2^{−/−}, Mecp2^{7T158A/y}) and no change in either phenotype in Mecp2 gain-of-function mice (Mecp2^{+/+}) relative to WT littermates. These data suggest that Mecp2 expression levels may play a role in the regulation of neuronal outgrowth, but increased Mecp2 expression has no structural effect. Our data also indicate that a greater reduction in Mecp2 may cause more severe morphological defects, as we observed a more widespread reduction in dendritic branching in Mecp2^{−/−} mice, which lack MeCP2 protein (Guy et al., 2001), relative to Mecp2^{7T158A/y} mice, which have reduced MeCP2 protein expression (Goffin et al., 2012).

To our knowledge, neuronal morphology has not been studied in Mecp2 gain-of-function mouse models, and the effect of MeCP2 overexpression in vitro on neuronal morphology remains unclear due to reports of increased (Jugloff et al., 2005; Larimore et al., 2009), decreased (Zhou et al., 2006), and unchanged dendritic complexity (Chapleau et al., 2009) in cultured neurons and organotypic slices, and decreased dendritic outgrowth in Drosophila and Xenopus models (Marshak et al., 2012; Vonhoff et al., 2012). Our findings suggest that both dendritic outgrowth and soma size are reduced upon Mecp2 loss-of-function, whereas neither phenotype is affected by Mecp2 gain-of-function with two-fold MeCP2 expression. Notably, Mecp2^{+/+} mice were maintained on a mixed FVB/C57BL/6 background, and both Mecp2 loss-of-function models were maintained on a pure C57BL/6 background. Therefore, the different genetic backgrounds may contribute to the differences seen between loss-of-function and gain-of-function models. It is possible that, although two-fold expression of MeCP2 is sufficient to impair synaptic function leading to circuit defects (Chao et al., 2007; Na et al., 2012), it is not sufficient to disrupt cellular morphology in vivo. Future study of neuronal morphology in mice expressing three-fold levels of MeCP2 may address this hypothesis, as MeCP2 triplication produces more severe phenotypes both clinically and in mice (Samaco et al., 2012; Van Esch et al., 2007).

To address the second question, the relationship between RTT-like phenotypic severity and underlying neuronal morphology, we measured dendritic complexity and soma size in Mecp2^{−/−} and Mecp2^{7T158A/y} mice prior to or at the onset of RTT-like phenotypes (P30) and after development of RTT-like phenotypes. Because RTT-like behavioral progression differs across these mouse models, the ages corresponding to late-symptomatic RTT similarly differed (P60, P90, respectively). Overall, we observed a subtle age-dependent effect of Mecp2 mutation on dendritic complexity in Mecp2 loss-of-function, where progression of RTT-like behavioral phenotype correlates with underlying cellular changes. At the early-symptomatic P30 time point, only the mildly-symptomatic Mecp2^{−/−} mice showed decreased dendritic complexity, while the pre-symptomatic Mecp2^{7T158A/y} mice did not, indicating that early in the development of RTT-like phenotypes, changes in neuronal structure vary across different Mecp2 mutations and may reflect development of RTT-like behavioral phenotypes. This hypothesis is consistent with findings from a comparative study of brain morphology in two 3-week-old Mecp2 loss-of-function mouse models, in which Mecp2^{−/−} mice with a more severe RTT-like phenotype exhibited more widespread and marked changes in brain structure than Mecp2^{−/−} mice with a milder RTT-like phenotype (Belichenko et al., 2008).

Similarly, we found greater changes in dendritic complexity in late-symptomatic P60 Mecp2^{−/−} and P90 Mecp2^{7T158A/y} mice relative to that of P30. The reduction in dendritic complexity in P60 Mecp2^{−/−} mice was slightly more widespread than that of P30 Mecp2^{−/−} mice, and P90 Mecp2^{7T158A/y} showed changes in dendritic complexity that were absent at the pre-symptomatic time point. These data indicate that changes in dendritic outgrowth accompany the development of RTT-like phenotypes upon Mecp2 loss-of-function and are consistent with RTT postmortem histological data showing a correlation between the degree of morphological abnormality and RTT symptom severity at time of death (Bauman et al., 1995).

The developmental changes in dendritic complexity we identified, however, were relatively mild, compared to reports from other Mecp2 loss-of-function studies (Kishi and Macklis, 2004; Robinson et al., 2012; Stuss et al., 2012), and limited to specific dendritic domains. This is surprising, as the overt behavioral phenotypes present at both late-symptomatic time points would predict underlying cellular architecture to be severely disrupted. One factor in this subtle dendritic outgrowth phenotype may be experimental bias from the Thy1-GFP/H reporter line. While an interaction between MeCP2 and a different reporter transgene Thy1-YFP/H has been reported (Stuss et al., 2012), we did not identify GFP-labeling biases in our mice. Our selection of layer V cortical neurons extending their apical tufts to the pial surface, however, could have introduced a bias for analysis of neurons with a less severe morphological phenotype within the Mecp2 mutant neuronal network. As changes in dendritic morphology have been shown to widely differ between individual neurons within a population (Belichenko et al., 2009b), we cannot exclude the possibility that our experimental method was selective for neurons with intact dendritic structures, resulting in a mild dendritic complexity phenotype.

Data from other Mecp2 mouse models, however, indicate that changes in dendritic arborization may not reflect the severity of behavioral phenotype. While Mecp2 A140V knockin mice with no obvious RTT-like behavioral phenotypes show decreased dendritic complexity (Jentarra et al., 2010), Mecp2^{108y} mice with motor, social, and learning deficits that model an Mecp2 early truncation show no changes in dendritic complexity or post-synaptic density in cortical and hippocampal CA1 neurons, both before and after onset of RTT-like phenotypes (Moretti et al., 2006). In addition, a developmental time window may exist in which neuronal morphology is more sensitive to Mecp2 function, as previous work has shown that newborn neurons in the hippocampus are severely affected by Mecp2 loss-of-function (Smrt et al., 2007). Together, these data suggest that while decreases in dendritic arborization may accompany the development of RTT-like behavioral phenotypes in Mecp2 loss-of-function, the degree of these changes does not necessarily match the behavioral phenotypic severity and therefore, dendritic arborization may not be a robust and consistent cellular readout of RTT-like phenotype.

In contrast to the intrinsic variability of dendritic outgrowth in Mecp2 mice, we found that MeCP2 soma size regulation is persistent throughout development and dependent on MeCP2 function. Both Mecp2 loss-of-function mouse models, Mecp2^{−/−} and Mecp2^{7T158A/y}, showed decreased soma size at early- and late-symptomatic time points relative to WT, suggesting that soma size decreases upon Mecp2 loss-of-function but does not change with increasing RTT-like symptomatic severity. These data are consistent with findings from ESC-differentiated neurons, where neurons originating from several lines of Mecp2 loss-of-function mice show decreased soma size relative to controls throughout the course of neuronal maturation (Yazdani et al., 2012), and can be rescued upon restoration of MeCP2. Together,
these data indicate that soma size is regulated by MeCP2 function throughout development and can be an indicator of MeCP2 function.

To address the third question, whether changes in neuronal morphology are brain region or cell type-specific, we assessed neuronal morphology in a separate brain region by measuring dendritic complexity in hippocampal CA1 pyramidal neurons in pre- and post-symptomatic Mecp2T158A/− mice. In contrast to the age-dependent decrease in basal dendritic complexity we observed in somatosensory cortex layer V pyramidal neurons of Mecp2T158A/− mice, we found no age-dependent reduction in dendritic complexity of hippocampal CA1 pyramidal neurons. These data are consistent with region-specific morphological phenotypes identified in RTT postmortem brain tissue, where pyramidal neurons of the motor, frontal, and inferior temporal cortices show decreased dendritic complexity, but pyramidal neurons in the visual cortex and hippocampus exhibit no change with respect to age-matched controls (Armstrong et al., 1995; Belichenko et al., 1994). Specificity may even extend to cortical layer, as decreased dendritic arborization has been identified in both basal and apical dendrites of layer V motor cortex cells but only in basal dendrites of layer III motor cortex cells (Armstrong et al., 1995). A comparison of data from different studies also supports a cortical layer-specific regulation of neuronal morphology in mice, where neocortical layer II/III pyramidal neurons show decreased dendritic complexity in both proximal basal and apical dendrites and no change in spine density (Kishi and Macklis, 2004) but layer V pyramidal neurons show decreased dendritic complexity in only proximal basal and distal apical dendrites and decreased spine density (Stuss et al., 2012).

In contrast to the cell type-specific changes in dendritic outgrowth, multiple lines of evidence indicate that soma size is consistent across cell types. We previously reported decreased soma size in hippocampal CA1 pyramidal neurons in both pre- and post-symptomatic Mecp2T158A/− mice (Goffin et al., 2012), similar to what we observed in the somatosensory cortex in this study. In addition, decreased soma size has been reported in RTT postmortem brain tissue (Armstrong, 2005) and in the locus ceruleus (Taneja et al., 2009), hippocampus CA2 (Chen et al., 2001), layer II/III somatosensory cortex (Kishi and Macklis, 2004), layer II/III motor cortex (Robinson et al., 2012), and layer V motor cortex (Stuss et al., 2012) of different Mecp2-null mouse strains. Moreover, various lines of Mecp2-deficient ESC-derived neurons (Yazdani et al., 2012) and RTT patient-derived induced pluripotent stem cells (Marchetto et al., 2010) also show reduced soma sizes, indicating that, in contrast to dendritic arborization, a reduction in soma size upon Mecp2 loss-of-function is a phenotype that is consistent across cell types and experimental systems. Similarly, our finding that soma size is not affected upon MeCP2 gain-of-function has also been observed in vitro (Jugloff et al., 2005), indicating that the absence of a change in soma size upon Mecp2 gain-of-function is also conserved across cell types.

This consistent and robust reduction in soma size across studies, Mecp2 loss-of-function mutations, experimental systems, cell types, and developmental time points, is in sharp contrast to changes in dendritic complexity, which vary across these parameters and can be subtle and context-dependent (Stuss et al., 2012). Therefore, in accordance with a recent study evaluating spine density throughout development in Mecp2 loss-of-function mice (Chapleau et al., 2012), we caution the use of dendritic complexity as a phenotypic endpoint for therapeutic evaluation, and suggest that soma size may be a more robust and reproducible readout of MeCP2 function. Evidence supporting soma size as a reliable morphologic phenotype comes from existing rescue experiments, where activation of a quiescent Mecp2 gene in adult mice results in partial restoration of dendritic complexity and length but a full restoration of soma size (Robinson et al., 2012). In addition, postnatal neuron-specific reactivation of MeCP2 is sufficient to rescue hippocampal and cortical soma size in Mecp2-deficient mice (Giacometti et al., 2007) and neuronal size is restored by the re-expression of MeCP2 in several strains of MecP2-deficient ESC-derived neurons (Yazdani et al., 2012). Future studies are needed to understand how soma size and dendritic outgrowth are differentially regulated by MeCP2. It is conceivable that dendritic arborization is more sensitive to the cellular environment, growth factors, and homeostatic regulation, whereas soma size is more directly affected by the function of nuclear proteins such as MeCP2.

Overall, our data indicates that within a population of excitatory neurons in RTT mouse models, in vivo changes in dendritic complexity upon MeCP2 loss-of-function are brain region-specific, correlated with behavioral phenotype, and mild. In contrast, soma size is regulated throughout development and may be a reliable marker for evaluating MeCP2 function and therapeutic efficacy. These phenotypes are specific to MeCP2 loss-of-function, as in vivo morphological changes upon Mecp2 gain-of-function are absent. The use of neuronal morphology as a cellular readout of RTT phenotype and restoration of neuronal circuitry, therefore, should be cautioned, as morphological phenotypes are intrinsically variable.

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