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NORMAL MITRAL CELL DENDRITIC DEVELOPMENT IN THE SETTING OF Mecp2 MUTATION

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Abstract—Rett syndrome (RTT) is an autism spectrum disorder caused by mutation in the gene encoding methyl CpG binding protein 2 (*MECP2*). Evidence to date suggests that these disorders display defects in synaptic organization and plasticity. A hallmark of the pathology in RTT has been identified as decreased dendritic arborization, which has been interpreted to represent abnormal dendritic formation and pruning during development. Our previous studies revealed that olfactory axons display defective pathfinding and targeting in the setting of *Mecp2* mutation. In the present work, we use *Mecp2* mutant mouse models and the olfactory system to investigate dendritic development. Here, we demonstrate that mitral cell dendritic development proceeds normally in mutant mice, resulting in typical dendritic morphology at early postnatal ages. We also failed to detect abnormalities in dendritic inputs at symptomatic stages when glomeruli from mutant mice appear smaller in area than the wild type (WT) (6 weeks postnatally). Collectively, these findings suggest that the initial defects in glomeruli impairment seen with *Mecp2* mutation do not result from abnormal dendritic development. Our results using the olfactory system indicate that dendritic abnormalities are not an early feature in the abnormalities incurred by *Mecp2* mutation.

Key words: olfaction, Rett syndrome, mitral cell, dendrite development, Mecp2, autism spectrum disorder.

Rett syndrome (RTT) is one of the few Autism Spectrum Disorders (ASDs) whose cause is identified as a single gene mutation (Amir et al., 1999). Mutations in the gene encoding the transcriptional regulator methyl CpG binding protein-2 (MeCP2) result in a series of clinical phenotypes that characterize RTT. These include disruptions in motor and cognitive development, seizures, motor stereotypies, and features of autism (Chahrour and Zoghbi, 2007). While MeCP2 expression has been found in many tissues, it is the absence of functional protein in the brain that appears to be primarily responsible for the clinical features of RTT (LaSalle et al., 2001; Shahbazian et al., 2002). Thus, significant efforts have focused on investigating the role of MeCP2 in the developing nervous system.

Studies using *in vitro* and *in vivo* models of MeCP2 dysfunction indicate that MeCP2 plays a role in neuronal maturation (Kishi and Macklis, 2004; Matarazzo et al., 2004; Palmer et al., 2008; Smrt et al., 2007), axonal and dendritic morphology (Ballas et al., 2009; Belichenko et al., 2009a, b; Cusack et al., 2004; Jugloff et al., 2005; Larimore et al., 2009; Maezawa et al., 2009; Tropea et al., 2009; Wood et al., 2009), axonal guidance (Degano et al., 2009), synaptic formation and plasticity (Asaka et al., 2006; Chao et al., 2007; Dani et al., 2005; Fukuda et al., 2005; Moretti et al., 2006), and regulation of neurotransmission (Chao et al., 2010; Dani et al., 2005; Medrihan et al., 2008; Monteggia and Kavalali, 2009; Calfa et al., 2011). These results suggest that MeCP2 participates in the formation of neuronal circuits at different levels, that is, neurite growth and complexity, pathfinding, targeting, synaptogenesis, and synaptic plasticity, implicating MeCP2 as having a critical role during the establishment and maintenance of neural connectivity. However, the timing and causal relationships of these defects to the resultant pathological findings and impairments remain unclear.

Anatomical observations of postmortem brain samples from RTT patients show striking abnormalities in dendritic arbors from cortical and hippocampal neurons (Armstrong et al., 1995; Bauman et al., 1995; Belichenko et al., 1994; Chapleau et al., 2009). Likewise, a number of studies have explored this in mouse models with varying results, depending on the anatomical region and/or age analyzed (Belichenko et al., 2009a,b; Moretti et al., 2006). Based on these findings, it has been postulated that the synaptic impairment present in RTT results largely from dendritic abnormalities incurred during dendritic development. The contribution of abnormal dendritic development has also been implicated in autism and other mental retardation disorders, suggesting a causal link (Fiala et al., 2002; Kaufmann and Moser, 2000). However, it is still unclear how MeCP2 dysfunction leads to dendritic impairment during development. Whether the dendritic abnormalities are the primary or secondary defect is unknown.

We have taken advantage of the olfactory system as a neurodevelopmental model (Cohen et al., 2003; Kim et al., 2005; Matarazzo et al., 2004; Matarazzo and Ronnett,

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Abbreviations: EPL, external plexiform layer; LOT, lateral olfactory tract; MC, mitral cells; MECP2, methyl CpG binding protein 2; OB, olfactory bulb; OMP, olfactory marker protein; OSNs, olfactory sensory neurons; PFA, paraformaldehyde; RTT, Rett syndrome; WT, wild type.

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2004; Palmer et al., 2008; Simpson et al., 2002, 2007). The primary neurons in the olfactory circuit are olfactory sensory neurons (OSNs), which are bipolar receptor cells that receive input from the external environment (e.g. odorants) at their apical dendrites and extend axons to synapse in the olfactory bulb (OB). In the OB, OSN axons establish excitatory synapses with the dendrites of mitral cells (MC) in a neuropil structure termed the glomerulus. Thus, the olfactory circuit is relatively simple, linear, and has a laminar organization, permitting visualization of OSNs, mitral and supporting cells, as well as the synaptic structure by immunohistochemical analysis. We have shown that neuronal maturation is disrupted in *Mecp2* null and *Mecp2*$^{308/y}$ mutant mice (Matarazzo and Ronnett, 2004; Palmer et al., 2008). Additionally, we found axonal guidance defects in these mouse models during initial synaptogenesis, followed by a chronic defect in the size of glomeruli, the site of excitatory synapses in the OB (Degano et al., 2009; Matarazzo and Ronnett, 2004; Palmer et al., 2008). The current studies extend this work by considering the dendritic component of this synapse.

Here, we examined the dendrites of mitral cells in the OB, the targets of the OSN, during early development to determine whether the maturation and morphology of these dendrites are affected by MeCP2 dysfunction. Our results show that even though the primary olfactory synapses (glomeruli) are abnormal when MeCP2 is absent or mutated, the process of maturation and the morphology of dendrites in those synapses are comparable with that seen in wild type (WT) mice. Our findings suggest that defects in dendritic formation are not the primary event in the development of synaptic defects. These results have implications for the pathogenesis of RTT and ASDs, as well as strategies for the development of therapeutic interventions.

EXPERIMENTAL PROCEDURES

Animals

All experimental protocols were approved by the Johns Hopkins University Institutional Animal Care and Use Committee, and all applicable guidelines from the National Institute of Health "Guide for the Care and Use of Laboratory Animals" were followed. Female Mecp2 heterozygous mice were provided by Dr. Rudolf Jaenisch (*Mecp2 KO mice*, Chen et al., 2001) and Mecp2 hemizygous mice were obtained by crossing heterozygotes with wild-type male BALB/c mice. WT and KO male littermates were used for our experiments.

129/SvEv wild-type and Mecp2$^{308/Y}$ mice were provided by Dr. Huda Zoghbi (Shahbazian et al., 2002). Male wild-type and mutant mice (*Mecp2 MT mice*) were obtained by crossing those pairs. In all cases, male mice were used exclusively to circumvent complications associated with X-chromosome inactivation, as *Mecp2* is found at loci Xq28. Three to six animals per genotype per experiment were used for the current study except where noted.

Tissue preparation

Tissue was harvested and processed after cardiac perfusion of WT and mutant animals using ice-cold phosphate buffer (0.1 M PB) and 4% paraformaldehyde (PFA) fixative (Sigma, St Louis, MO, USA). Tissue was then stored in PFA at 4 °C until use. After dye injection and diffusion, horizontal sections of the OB were cut at 50 μm using a vibratome 1500 (vibratome, Bannockburn, IL, USA). For immunohistochemistry, after overnight fixation in PFA, tissue was transferred to sucrose for 24 h, followed by cryo-embedding in Tissue-Tek® O.C.T.™ (Sakura Finetek, USA Inc, Torrance, CA, USA) and stored at −80 °C until further use. Coronal sections of the OB were cut at 50 μm using an HM500M Cryostat (MICROM International GmbH, part of Thermo Fisher Scientific Inc., Waltham, MA, USA).

Carbocyanine dye injection

The OB was positioned with the cortical region of the brain attached (this assists in the manipulation of the OB) and the ventral side face up exposing the lateral olfactory tract (LOT). Using insect pins (Fine Science Tools, CA, USA) a small piece of DiI crystal (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) (Molecular Probes Invitrogen Corporation, CA, USA) was placed superficially in the LOT. The placement was within approximately 1 mm of the OB. Both bulbs were labeled. Brains were re-introduced into 4% PFA and kept in the dark at 32 °C until the dye had diffused completely through the OB (1–3 weeks). Diffusion time was determined as 1 week plus 1 day per day of age (i.e. for a P4 animal, transport time=7 days+4 days old=11 days).

Data analysis

To assess dendritic maturation, morphological characterizations were assigned as described by Malun and Brunjes (1996). We categorize MC dendrite maturation as immature (a), having multiple processes extending toward the glomerular layer; less immature (b), having multiple processes but with one visually thicker process, all extending into glomeruli; less mature (c), having one or two thin processes and one thick processes with an arborized tuft; or mature (d), having one thick primary dendrite with a specialized tuft at its end and the extension of secondary processes laterally in the external plexiform layer (EPL). Appropriately labeled sections were viewed and imaged using confocal microscopy (Zeiss, Thornwood, NY, USA). Z-series collection consisting of 1 μm confocal sections were reconstructed and used for morphological analysis. Only mitral cells isolated from neighboring cells and having processes that could be visibly traced from the glomeruli to the soma were used for analysis. Chi-square analysis was used to determine statistical significance.

Immunohistochemistry

Tissue sections were permeabilized in 0.2% Triton-X in phosphate buffer saline (PBST). After washing sections in PBS, sections were blocked in 4% normal donkey serum and incubated overnight at 4 °C in anti-olfactory marker protein (OMP) at 1:3000 (Wako, Richmond, VA, USA); in anti-MAP2 protein at 1:1000 (AbChem, Cambridge, MA, USA). Following overnight incubation, sections were rinsed and incubated with FITC-conjugated donkey anti-mouse 2° antibody (1:50); Cy-3-conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at room temperature. Sections were washed and mounted using Mowiol (Calbiochem, San Diego, CA, USA).

Neurite length

Neurite length for individual mitral cells was measured using Pascal analysis software (Zeiss, Thornwood, NY, USA). Only mitral cells in category "D" or fully mature, exhibiting a single dendrite and corresponding tuft were used for analysis. Measurements were made from the base of the cell body to the initial bifurcation of the tuft. Student *t*-test was used for statistical analysis.

Sholl analysis

A modified version of Sholl analysis was performed using the NIH ImageJ software (http://rsbweb.nih.gov/ij) and the Sholl Analysis Plugin (http://www-biology.ucsd.edu/labs/ghosh/software). The base of the bifurcation of individual tufts was chosen as the center of concentric circles used for the analysis. Because of differences in size and shape of individual tufts, the total number of intersections per tuft was summed over all concentric circle radii for WT, KO, or MT mice. Statistical analysis was performed using the Student *t*-test.

RESULTS

MC dendrite maturation proceeds normally with MeCP2 deficiency or dysfunction

Our previous results obtained using the olfactory system show that *Mecp2* mutation causes a transient delay in neuronal maturation and defective axonal targeting, which is followed by a persistent defect in primary olfactory synapses in the OB (Matarazzo and Ronnett, 2004; Palmer et al., 2008). Considering the prevailing hypothesis that defects in dendritic structural development lead to synaptic defects, we evaluated whether *Mecp2* mutations alter the rate of maturation of MC dendrites, the primary target of OSN axons. In the OB, the maturation of MCs has been well characterized (Malun and Brunjes, 1996; Matsutani and Yamamoto, 2000; Blanchart et al., 2006). MC soma appears by E15, when the initial OSN axon segment is visible. By postnatal day 1 (P1), mitral cell axons have bundled to form the LOT, which extends to the cortex where the axons terminally differentiate. However, MC dendrites do not mature until P7. At P1, MCs extend many thin dendrites, and by P4 one of these dendrites has a thick shaft that visually stands out from the others. By P7, there is one remaining dendrite with an arborized tuft at its end and secondary processes extending into the EPL (Malun and Brunjes, 1996; Matsutani and Yamamoto, 2000).

To assess MC dendritic morphology, we stained MCs by retrograde labeling of the LOT in *Mecp2* KO, *Mecp2* MT, and WT mice. Using this procedure, we were able to label approximately the same number of MCs in *Mecp2* KO and *Mecp2* MT mice compared with WT (data not shown). We categorized MC dendrite maturation as immature (a), having multiple processes extending toward the glomerular layer; less immature (b), having multiple processes but with one visually thicker process, all extending into glomeruli; less mature (c), having one or two thin processes and one thick processes with an arborized tuft; and mature (d), having one thick primary dendrite with a specialized tuft at its end and the extension of secondary processes laterally in the EPL (Fig. 1). In newborn rodents, the majority of MC dendrites are "category A," displaying many thin processes. By P7–P10, the majority of MC dendrites are "category D," projecting a single dendrite with a tuft (Matsutani and Yamamoto, 2000; Imamura and Greer, 2009). We used this metric of maturation to assess whether disruptions in MeCP2 function directly affected the maturation of MCs. In P7 *Mecp2* KO mice, we found that the percent of MC in category D (70%) was not significantly different from WT controls (74%) (Fig. 1A). Similarly, using *Mecp2* MT mice, we found that the percent of mature MC dendrites at P7 not significantly different from those of corresponding WTs (78% in *Mecp2* MT; 72% in WT) (Fig. 1B). These results suggest that although *Mecp2* mutation affects neuronal maturation and axonal targeting, maturation of MC dendrites proceeds normally and in a timely manner despite MeCP2 dysfunction.

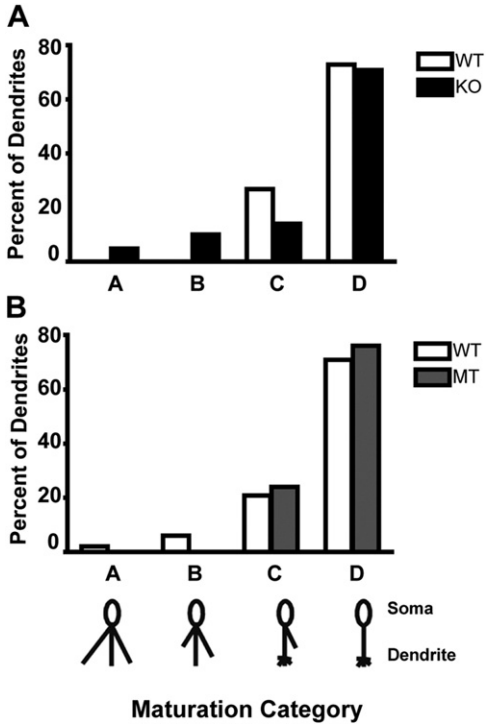

Fig. 1. Dendritic development is preserved in mitral cells from *Mecp2* KO and MT mice. Mitral cells dendrites were identified by DiI retrograde labeling and images of individual cells were acquired by confocal microscopy and reconstructed. The extent of dendritic maturation for individual cells was determined according to the criteria established by Malun and Brunjes (1996), as shown at the bottom of the figure. The percentage of cells in each category was then calculated for each genotype. (A) Maturational analysis of mitral cell dendrites at P7 in WT and *Mecp2* KO mice and (B) in *Mecp2* MT mice and corresponding WT. The total number of cells analyzed per group was 30–40. No significant differences in dendritic maturation were observed among the groups (chi-square analysis, GraphPad, USA).

Dendritic length is not affected by *Mecp2* mutation

Since the majority of MCs have reached maturity by P7, we used this pre-symptomatic age to assess other aspects of a potential delay in the developmental process. Therefore, we evaluated whether MeCP2 deficiency or dysfunction affect dendritic outgrowth at this stage of development.

Selecting only MCs that were labeled "category D" (fully mature) in the OB from P7 mice, we measured the length of mature MC dendrites from the base of their soma to the first bifurcation in their corresponding tuft (Fig. 2A). Adjustments for changes in OB size were not necessary because there are no significant differences in OB volume among the RTT models at this early time point, P7 (Matarazzo et al., 2004; Palmer et al., 2008). The results show

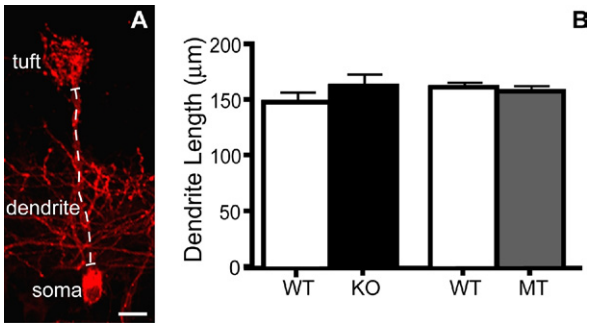

Fig. 2. Dendritic length is not affected by *Mecp2* deficiency or dysfunction. Dendrites from mitral cell visualized by DiI retrograde labeling that could be traced from the glomeruli to the soma were used for analysis. Dendritic length from fully mature mitral cells (category D) was measured in the OB from *Mecp2* MT, *Mecp2* KO, and respective WT mice at postnatal day 7. (A) Dendritic length was calculated from the base of the mitral cell soma to the first bifurcation in their corresponding tuft. Scale Bar: 20 μm. (B) Each bar represents the average length from 30 to 40 mitral cell dendrites per genotype, that was similar between each mutant and its respective WT group (Mann–Whitney *t*-test). Scale Bar: 20 μm.

that dendrite length was similar in MC apical dendrites from WT, *Mecp2* KO or MT mouse models (Fig. 2B). These results indicate that during early postnatal development, dendritic outgrowth appears unaffected by *Mecp2* mutations, and suggest that defects in synaptic structures might not be because of intrinsic dendritic abnormalities.

Mitral cell tuft morphology is normal in *Mecp2* null and mutant animals

Studies examining the effect of RTT mutations in patients report that dendritic arborizations are reduced in postmortem brains (Armstrong et al., 1995; Bauman et al., 1995), and this has also been reported for certain brain regions from *Mecp2* null mice (Chen et al., 2001; Kishi and Macklis, 2004). Thus, it is plausible that while MC dendrite maturation and length are unaffected by *Mecp2* mutation, the complexity of dendritic arborizations may be abnormal in the MCs of *Mecp2* KO and MT mice. To explore this possibility, we assessed MCs tufts, the arborized structures of their primary dendrites that innervate the glomeruli.

We compared the morphology of MC tufts from *Mecp2* KO and MT mice at P7 with their corresponding WT control. Tufts from MC labeled with DiI that reached the glomerular layer were reconstructed and analyzed using confocal microscopy.

Figs. 3 and 4 illustrate representative MC tufts from *Mecp2* KO and *Mecp2* MT, in comparison with the respective WTs. We observed that there were no aberrantly projecting processes or visible differences in the complexity and structural formation among the different groups (Figs. 3 and 4). We also applied quantitative morphological analyses of individual tufts to Figs. 3 and 4 using a modified Sholl analysis as described in the Experimental Procedures. Sholl analysis did not show significant differences between *Mecp2* WT and *Mecp2* KO (180.4±29.65 vs. 141.0±11.12 N, $P=0.2486$; Fig. 3) as well as *Mecp2* WT vs. *Mecp2* MT (128.4±24.04 vs. 83.60±18.78, $P=0.1801$, Fig. 4). These data confirm that *Mecp2* mutation has little effect on MC dendritic complexity and targeting in the OB during early postnatal development, suggesting that no intrinsic defects on early structural development occur with *Mecp2* KO or with the *Mecp2*308 mutation.

Dendritic inputs are not affected in olfactory synapses from symptomatic *Mecp2* mutant OB

Our previous studies have shown that in both *Mecp2* KO and MT mice, glomerular volume, which is the site of synapses between OSNs axons and MC dendrites, although similar at P2 weeks, is smaller in mutant mice when the animals become symptomatic (~4–6 postnatal weeks) (Matarazzo et al., 2004; Palmer et al., 2008). Considering our present study that MC dendritic maturation proceeds normally in the absence of functional MeCP2, we considered whether MC dendrites are affected at 6 weeks when symptoms begin and glomerular defects are becoming apparent (Matarazzo et al., 2004; Palmer et al., 2008).

Immunostaining of OB sections with MAP2, a dendritic marker, shows that the dendritic compartments that form the glomerular structure show no apparent differences

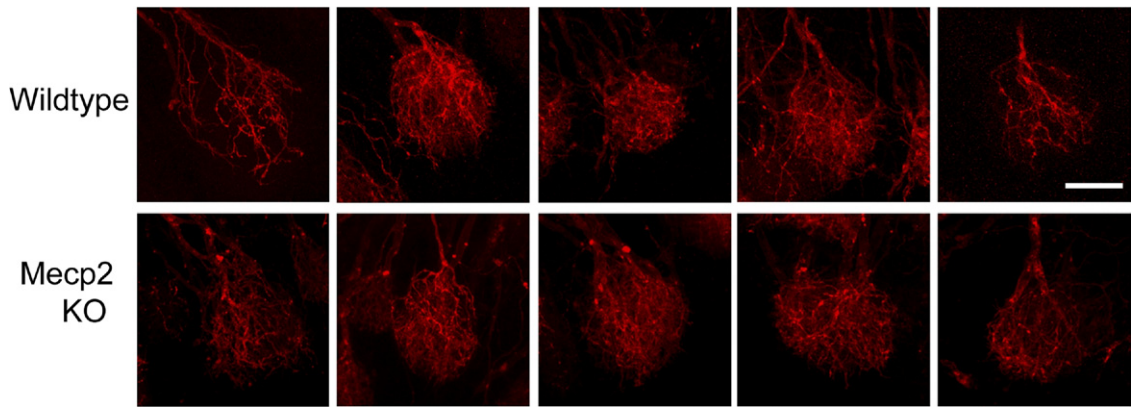

Fig. 3. Mitral cell dendritic tufts appear normal in *Mecp2* null mice. Representative images of DiI labeled-dendritic tufts in OB mitral cells from WT (top panels) and *Mecp2* KO mice (bottom panels). Mitral cell tufts were analyzed at P7 by confocal microscopy and reconstructed. *Mecp2* KO mice show no gross differences with respect to control. Scale Bar: 20 μm.

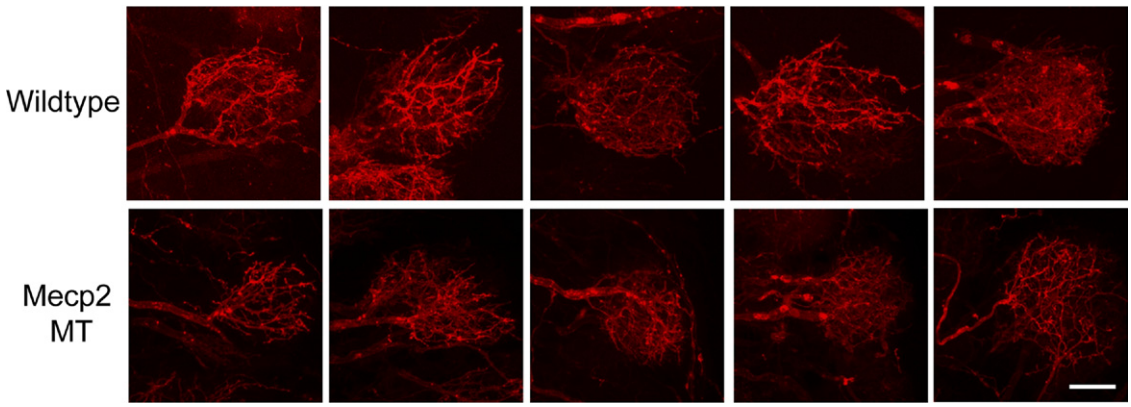

Fig. 4. Mutant *MeCP2* does not affect mitral cell dendritic arbors. Images of DiI-labeled tufts in the OB from WT (top panels) and *Mecp2* MT mice (bottom panels) at P7. Confocal microscopy analysis of the dendritic arbors showed no differences between mutant and WT dendrites. Scale Bar: 20 μm.

between mutants and WT animals (Fig. 5). Thus, although glomeruli from symptomatic *Mecp2* mutant mice appear smaller, as reported earlier (Matarazzo et al., 2004; Palmer et al., 2008), the dendritic distribution in individual glomeruli is consistent in appearance with previous results from WT mice (Kasowski et al., 1999). These observations suggest that in the absence of functional MeCP2, dendritic alterations are not the primary defect leading to abnormal neural connectivity.

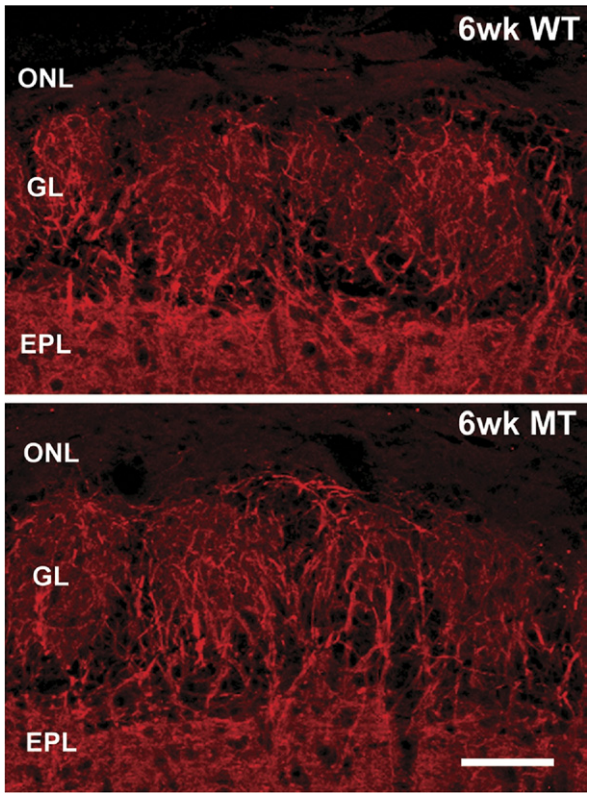

Fig. 5. Dendritic innervation is not altered in symptomatic *Mecp2* mutant mice. Coronal OB sections from 6 wk WT (top) and *Mecp2* MT mice (bottom) were stained for MAP2 to visualize dendrites. MAP2 labeled projections innervate glomerular layer similarly in WT and mutant mice. n=3 mice per genotype. Scale Bar: 50 μm. ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer.

Axonal laminar targeting is disrupted in mice expressing a truncating mutation

We have previously shown using *Mecp2* KO mice that OSNs have delayed maturation and that the axonal projections to their targets in the OB are aberrant and dysmorphic (Degano et al., 2009; Matarazzo et al., 2004). Although previous studies have hypothesized that MeCP2 deficiency results in disruptions in synaptogenesis (Fukuda et al., 2005; Matarazzo et al., 2004), it is hypotheses favoring disruptions in dendritic development that have been most often considered (Johnston et al., 2001; Zhou et al., 2006; Zoghbi, 2003). However, our studies and others (Belichenko et al., 2009b; Degano et al., 2009; Larimore et al., 2009; Matarazzo et al., 2004) have described defects in axonal projections that could also account for the abnormalities in synaptogenesis. We extended these studies using the MT mice.

Using coronal sections of OB from MT and WT animals, we assessed the convergence of axons from mature OSN (OMP-positive) into the neuropil of glomerular structures. We find that in MT animals, as OSN axons undergo terminal differentiation, they project outside of the neuropil at the glomerular level. In 2-week old mice, these aberrant projections are significantly more profuse in MT mice than in WT littermates (Fig. 6). Similar to our previous findings in *Mecp2* KO animals, this defect resolved at older ages. These data confirm that mutations in *Mecp2* adversely affect axonal targeting at the time of synaptogenesis and suggest that defects in axonal input might underlie synaptic alterations.

DISCUSSION

In the present study, mitral cell dendrites within the olfactory circuit were examined to assess whether *Mecp2* deficiency or mutation affected dendritic maturation or dendritic morphology during early development.

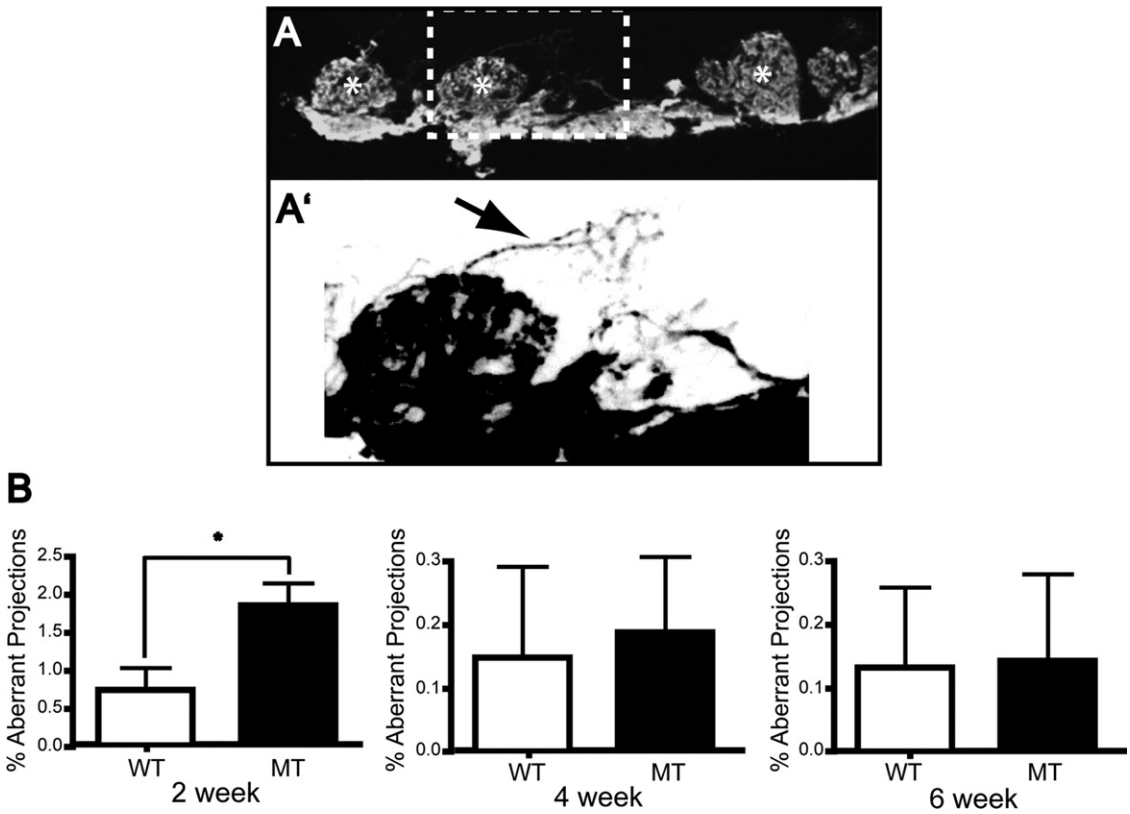

Fig. 6. Abnormal axonal targeting in *Mecp2* mutant mice. Coronal OB sections from WT and *Mecp2* mutant mice were stained with anti-OMP antibodies to visualize mature olfactory axons that innervate the OB. Arrow indicates axons overshooting the external plexiform layer in mutant mice; detail shown in (A') (inset). (B) Percentage of glomeruli that show aberrant projections in OB from WT and *Mecp2*-null mice at 2, 4, and 6 wk. Quantification was performed in three or four mice per genotype per age. From each mouse, OB sections at similar rostro-caudal levels were analyzed blind for each genotype (* $P<0.01$, Mann–Whitney *t*-test).

The lack of abnormal dendritic structural development in the setting of *Mecp2* mutation provides an alternative to the hypothesis that cell autonomous defects in dendritic structural development are a primary event in the pathology incurred by *Mecp2* mutation. Our findings suggest that axonal defects or failed communication from the dendrite to axon may contribute to or even represent the primary event leading to synaptic dysfunction in the setting of *Mecp2* mutation.

Using the olfactory system, we have previously shown that either Mecp2 deficiency or dysfunction delays the maturation of OSNs, affects the axonal targeting, and causes persistent defects in primary excitatory synapses within the OB (Degano et al., 2009; Matarazzo et al., 2004; Matarazzo and Ronnett, 2004; Palmer et al., 2008). In the present study, we extend these studies by considering the dendritic component of synapse formation. While studies leading to the development of the dendritic hypothesis relied upon post-mortem examination, we utilized retrograde labeling methods to study dendritic maturation dynamically in the OB from *Mecp2* null and *Mecp2* MT mice.

Our results indicate that although *Mecp2* mutation affects neuronal maturation, MC dendrites development proceeds in a timely manner in *Mecp2* KO and *Mecp2* MT mice. We also measured neurite length and dendritic arborization of MC tufts to assess whether defects in innervation might contribute to the smaller glomerular size present later in symptomatic mice (Matarazzo et al., 2004; Palmer et al., 2008) and to assess further whether dendritic abnormalities described in human and rodent RTT studies were also present in our development model. We found no difference in dendritic length or complexity in *Mecp2* KO or *Mecp2* MT mice compared with control mice at P7 when most MC dendrites are mature. These data confirm that *Mecp2* mutation has little effect on MC dendritic growth, complexity, or targeting in the OB during early postnatal development, indicating that no intrinsic defects in dendritic structural development occur with MeCP2 dysfunction. We also performed MAP2 immunostainings in OBs from older mice (6 weeks old) to determine whether dendritic abnormalities develop with the onset of symptoms. We found that the integrity of dendrites innervating glomeruli was retained during the onset of symptoms, suggesting that dendritic maintenance was also unaffected by *Mecp2* mutation.

Although other reports describe reductions in spine density (Belichenko et al., 1994; Kishi and Macklis, 2004), as well as deficient arborization in other brain regions from *Mecp2* null mice (Ballas et al., 2009; Kishi and Macklis, 2004), those studies utilized older symptomatic animals exclusively. The present work is, to our knowledge, the first

developmental assessment of dendritic maturation/maintenance in mouse models of *Mecp2* mutation. In agreement with the present results, no dendritic abnormalities have been reported in other brain regions from the *Mecp2* MT model (Moretti et al., 2006), which challenges the hypothesis that dendritic defects underlie RTT phenotypes. Interestingly, gene expression profiles of postmortem RTT brains revealed decreased MAP2 levels respect to control patients (Kaufmann and Moser, 2000), consistent with the anatomical observations (Armstrong, 2001; Belichenko et al., 1994). However, similar studies from different RTT mouse models do not report significant differences of the dendritic marker (Ben-Shachar et al., 2009; Chahrour et al., 2008; Kriaucionis et al., 2006; Nuber et al., 2005; Urdinguio et al., 2008). Altogether, these findings suggest that although *Mecp2* mutations could cause dendritic defects at later ages or under certain conditions, it is not a prevalent finding at early developmental times in murine models. Thus, it seems unlikely that dendritic alterations constitute the primary cause for neurological phenotypes that recapitulate the human disorder and are shared by the different mouse models of RTT.

Studies in dissociated cultures from cortical and hippocampal neurons or hippocampal slices have shown that either MeCP2 deficiency or MeCP2 over expression induces dendritic defects that are cell autonomous in nature (Chapleau et al., 2009; Larimore et al., 2009; Zhou et al., 2006). However, Belichenko (2009a) analyzed the brain of *Mecp2* heterozygous mice and showed that the morphology of WT neurons was also abnormal, indicating that *Mecp2* null neurons exert paracrine pathogenic effects on the neighboring cells. In this respect, recent studies demonstrate that Mecp2 null glia or glia-conditioned medium was able to alter dendritic morphology and synapses (Ballas et al., 2009; Lioy et al., 2011; Maezawa et al., 2009; Maezawa and Jin, 2010). These data demonstrate the complexity of MeCP2's role in synaptogenesis and challenge the thought that cell-autonomous changes affect RTT neurons.

Lastly, in the present work using *Mecp2* MT mice, we described disruptions in axonal targeting as OSNs enter glomeruli in the OB (Fig. 6), furthering our previous findings in the OB of *Mecp2* KO mice (Matarazzo et al., 2004; Degano et al., 2009). The projection of OSN axons beyond the glomerular layer during development has been described by others (Tenne-Brown and Key, 1999; Chan et al., 2011). However, it is important to emphasize that in both Mecp2 mouse models these aberrant projections into the EPL take longer to resolve than in the respective WT mice (Fig. 6 and Matarazzo et al. (2004). Interestingly, we have previously demonstrated decreased semaphorin 3F function in Mecp2 null olfactory bulb (Degano et al., 2009). Considering the key role of this semaphorin during the development of the olfactory circuit (Cloutier et al., 2004), this alteration could explain the presence of OSN axons beyond the glomerular layer in Mecp2 mutant mice.

In addition to the present work, other studies indicate that *Mecp2* dysfunction has varied effects on both axons and dendrites in the CNS (Belichenko et al., 2009a,b; Larimore et al., 2009). Altogether, these findings demonstrate the occurrence of axonal defects in different brain regions and mouse models of RTT, and supports the hypothesis put forth here that the defect associated with synaptic maturation is not exclusively a result of dendritic disruption, but can be influenced by axonal disruptions. Thus, we propose that *Mecp2* mutations affect the development of neural circuits *in vivo*, including both axonal and dendritic components by means of non-cell autonomous mechanisms.

Several candidate molecules could be mediating the non-cell autonomous effect of *Mecp2* mutations. Some of the candidate genes products, including BDNF, IGF, Crh, semaphorins, and semaphorin receptors, are known to be directly or indirectly regulated by MeCP2 (Chen et al., 2003; Degano et al., 2009; Martinowich et al., 2003; McGill et al., 2006; Tropea et al., 2009) and play important roles in dendrite and axonal development and synaptic formation and maintenance. More studies that correlate biological effects with molecular analyses will be necessary to establish a mechanistic link.

CONCLUSIONS

We have further validated the olfactory system for modeling the neurodevelopmental changes occurring with Mecp2 mutations in mice and humans. Our present results in this model indicate that abnormal dendritic development is not the initial defect leading to synaptic impairment and that axonal impairment may also contribute to synaptic dysfunction in the setting of *Mecp2* mutation. These results question the longstanding hypothesis that dendritic defects solely underlie RTT phenotypes and could have valuable implications for the development of therapeutic approaches.

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