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Bdnf Overexpression in Hippocampal Neurons Prevents Dendritic Atrophy Caused by Rett-Associated *MECP2* Mutations

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Abstract

The expression of the methylated DNA-binding protein MeCP2 increases during neuronal development, which suggests that this epigenetic factor is crucial for neuronal terminal differentiation. We evaluated dendritic and axonal development in embryonic day-18 hippocampal neurons in culture by measuring total length and counting branch point numbers at 4 days *in vitro*, well before synapse formation. Pyramidal neurons transfected with a plasmid encoding a small hairpin RNA (shRNA) to knockdown endogenous *Mecp2* had shorter dendrites than control untransfected neurons, without detectable changes in axonal morphology. On the other hand, overexpression of wildtype (wt) human *MECP2* increased dendritic branching, in addition to axonal branching and length. Consistent with reduced neuronal growth and complexity in Rett syndrome (RTT) brains, overexpression of human *MECP2* carrying missense mutations common in RTT individuals (R106W or T158M) reduced dendritic and axonal length. One of the targets of MeCP2 transcriptional control is the *Bdnf* gene. Indeed, endogenous *Mecp2* knockdown increased the intracellular levels of BDNF protein compared to untransfected neurons, suggesting that MeCP2 represses *Bdnf* transcription. Surprisingly, overexpression of wt *MECP2* also increased BDNF levels, while overexpression of RTT-associated *MECP2* mutants failed to affect BDNF levels. The extracellular BDNF scavenger TrkB-Fc prevented dendritic overgrowth in wt *MECP2*-overexpressing neurons, while overexpression of the *Bdnf* gene reverted the dendritic atrophy caused by *Mecp2*-knockdown. However, this effect was only partial, since *Bdnf* increased dendritic length only to control levels in mutant *MECP2*-overexpressing neurons, but not as much as in *Bdnf*-transfected cells. Our results demonstrate that MeCP2 plays varied roles in dendritic and axonal development during neuronal terminal differentiation, and that some of these effects are mediated by autocrine actions of BDNF.

Keywords

MeCP2; Rett; dendrite; axon; pyramidal neuron; BDNF; hippocampus

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Introduction

Neurodevelopmental disorders caused by genetic disruptions or chemical/physical insults during brain development affect neuronal terminal differentiation, i.e. axon and dendrite growth and branching, as well as synaptogenesis, thus impairing synaptic transmission and plasticity leading to altered network connectivity (Chechlacz and Gleeson, 2003; Johnston et al., 2001). Reduced dendritic length and branching have been demonstrated in many disorders associated with mental retardation (Kaufmann and Moser, 2000). One disorder associated with impaired brain development is Rett syndrome (RTT), a disease that affects nearly 1:15,000 females worldwide (Hagberg et al., 1985). Early development appears normal in individuals with RTT until approximately 6-18 months of age, when physical, motor and social-cognitive behavior enter a period of regression (Hagberg, 2002; Percy and Lane, 2005). Indeed, the neuropathology of RTT reveals several areas of abnormal brain development. Head circumference is decelerated, and postmortem observations of RTT brains demonstrate a reduction in brain weight (Armstrong et al., 1999; Jellinger et al., 1988; Schultz et al., 1993). Microscopically, brain autopsy material from RTT patients revealed impaired dendritic growth and complexity of pyramidal cells in the frontal and motor cortices, as well as in the subiculum (Armstrong et al., 1995), and reduced levels of MAP-2, a dendritic protein involved in microtubule stabilization (Kaufmann et al., 2000; Kaufmann et al., 1995; Kaufmann et al., 1997). These observations support the notion that RTT is a disorder caused by impaired dendritic, axonal and eventually synapse formation and maturation, leaving afflicted individuals in a state of arrested brain development (Armstrong, 2002; Johnston et al., 2001; Johnston et al., 2003; Kaufmann et al., 2005; LaSalle, 2004; Naidu, 1997; Philippart, 2001).

The majority of RTT cases are associated with mutations in a gene located on the X chromosome that encodes methyl-CpG-binding protein 2 (MeCP2) (Amir et al., 1999; Bienvenu et al., 2000; Van den Veyver and Zoghbi, 2001; Wan et al., 1999). MeCP2 is a nuclear protein that binds DNA specifically to A/T rich sites in close proximity to methylated CpG (cytidine-phospodiester-guanosine) islands. After binding to these methylated CpG sites, MeCP2 recruits the mSin3a co-repressor, which contains histone deacetylase complexes, thereby altering the structure of genomic DNA and regulating the transcription of specific target genes (Jones et al., 1998; Klose et al., 2005; Nan and Bird, 2001; Nan et al., 1997; Nan et al., 1998). DNA methylation is a common mechanism of silencing genes during cellular differentiation, and MeCP2 may play a critical role in neuronal terminal differentiation by reading this epigenetic code. Furthermore, MeCP2 expression in cortical neurons increases during brain development (Akbarian et al., 2001; Cohen et al., 2003, Jung, 2003 #21226; LaSalle et al., 2001; Shahbazian et al., 2002; Zoghbi, 2003). The developmental increase in its expression strongly suggests that MeCP2 plays a critical role in neuronal terminal differentiation, i.e. the development of axons, dendrites and dendritic spines leading to proper synapse formation and maturation (Cassel et al., 2004; Kaufmann et al., 2005; Kishi and Macklis, 2004; Matarazzo et al., 2004; Matarazzo and Ronnett, 2004; Mullaney et al., 2004). Missense mutations in *MECP2* identified in RTT patients cluster in the methyl-binding domain (MBD) and transcriptional repressor domain (TRD) of the protein, suggesting that they represent loss-of-function mutations. Indeed, many of these mutations on the MBD of MeCP2 (like R106W and T158M used in the present studies; see below), reduce the affinity of MeCP2 for methylated DNA, thus impairing its ability to localize to heterochromatin and repress transcription in gene reporter assays (Kudo et al., 2001; Kudo et al., 2003).

To extend our knowledge of its role in neuronal terminal differentiation in the context of RTT, we manipulated MeCP2 expression levels in primary cultures of embryonic day-18 hippocampal neurons, a well-established experimental model for studies of neuronal differentiation (Banker and Goslin, 1998; Craig and Banker, 1994). At the time of plating, dissociated neurons were transfected with expression cDNA plasmids encoding either: (1) a

small hairpin RNA (shRNA) interfering sequence to knockdown endogenous *Mecp2* expression; (2) wildtype (wt) human *MECP2*; (3) one of two of the most common missense mutations in *MECP2* found in RTT patients, R106W or T158M (www.mecp2.org.uk). Quantitative analyses of dendritic and axonal morphology (total length and number of branch points) were performed after 4 days *in vitro*, when a single defined axon and several dendrites are well defined (Dotti et al., 1988), and before synapse formation (Ziv and Smith, 1996). Considering that *Bdnf* (the gene coding for the neurotrophin brain-derived neurotrophic factor, BDNF) is a prominent gene shown to be regulated by MeCP2 (Abuhatzira et al., 2007; Chahrour et al., 2008; Chang et al., 2006; Chen et al., 2003; Klein et al., 2007; Martinowich et al., 2003; Ogier et al., 2007; Wang et al., 2006), we estimated intracellular BDNF protein levels by quantitative immunocytochemistry. Lastly, we tested whether *Bdnf* overexpression or extracellular BDNF scavenging with TrkB-Fc could revert the morphological effects of manipulations of MeCP2 levels. Our results demonstrate that MeCP2 plays varied roles in dendritic and axonal development during neuronal terminal differentiation, and that some of these effects are mediated by autocrine actions of BDNF.

Material and Methods

Cell Culture of Dissociated Hippocampal Neurons

All animal procedures followed national and international ethical guidelines, and were reviewed and approved by the IACUC at UAB on an annual basis. Hippocampal neurons were cultured from embryonic day-18 (E18) Sprague-Dawley rat embryos (Charles River, Wilmington, MA) as previously described (Moore et al., 2007). Briefly, both hippocampi were dissected and neurons were re-suspended in Neurobasal medium containing B-27 supplement with penicillin–streptomycin and L-glutamine (InVitrogen; Carlsbad, CA). Dissociated neurons were plated on glass cover slips coated with poly-L-lysine (Sigma Aldrich, St. Louis, MO) and cultured in Neurobasal medium (B-27 supplement, penicillin–streptomycin, L-glutamine) at 37°C in a 5% CO_2 incubator.

Transfections of Cultured Neurons

Primary hippocampal neuron cultures were transfected using electroporation (Gresch et al., 2004). Approximately 30min after isolation of both hippocampi, 2,000,000 dissociated neurons were centrifuged and re-suspended in 100μl of rat neuron Nucleofector solution (Amaxa Biosystems; Cologne, Germany). Three micrograms of cDNA per expression plasmid was added in a 2mm electroporation cuvette. After electroporation following the manufacturer protocol, Neurobasal medium was added and neurons were plated at a density of 50,000 to 70,000 cells per well in a 12-well plate at 37°C in 5% CO_2 for 4 days *in vitro* (div).

Expression cDNA plasmids encoding small hairpin RNA (shRNA) interfering sequences were obtained from Origene (Rockville, MD). shRNA interfering sequences consist of a 29-base pair target gene-specific sequence, a 7-base pair loop, followed by a 29-base pair reverse complementary sequence. The expression plasmids encoding shRNA sequences are under the control of the human U6 promoter. The *MECP2*-specific shRNA sequence AATGAGACAGCAGTCTTATGCTTCCAGAA (sequence #2), reduced MeCP2 protein levels by 65%, estimated by quantitative Western blot analyses of PC12 cells co-transfected with human wt *MECP2* and shRNA plasmids (Supplemental Fig. 1A). Consistently, MeCP2 expression levels were 55% lower in hippocampal neurons transfected with the same shRNA plasmid (sequence #2) than in untransfected neighboring neurons, as estimated by quantitative MeCP2 immunofluorescence (see below and Supplemental Fig. 1B, D). Another shRNA sequence, TCAATAACAGCCGCTCCAGAGTCAGTAGT, which did not affect MeCP2 expression, was used as a control for off-target effects.

To overexpress MeCP2 protein levels, expression cDNA plasmids encoding human wildtype (wt) *MECP2* were used as previously described (Kudo, 1998; Kudo et al., 2001; Kudo et al., 2003). These plasmids represent the originally isolated form of MeCP2, where the start methionine is in exon 2, known as MeCP2β (Kriaucionis and Bird, 2004) or MeCP2A (Mnatzakanian et al., 2004). Expression plasmids encoding two of the most common missense mutations in *MECP2* (R106W and T158M) were constructed by site directed mutagenesis using PCR, where wt *MECP2* was used as a template (Kudo et al., 2001). MeCP2 was tagged with enhanced green fluorescent protein (eGFP), which allowed the identification of transfected cells. Finally, the MeCP2-eGFP construct was cloned into an expression vector under control of the cytomegalovirus (CMV) promoter. These expression plasmids produced a twofold increase in MeCP2 protein expression compared to untransfected neighboring neurons, as measured by quantitative MeCP2 immunofluorescence (Supplemental Figure 1C, D). A cDNA plasmid expressing BDNF-GFP under the control of the CMV promoter was used to overexpress *Bdnf* in cultured neurons (kindly provided by Dr. Kojima); this GFP-tagged BDNF retains biological activity (Kojima et al., 2001).

Immunofluorescence

After 4 days *in vitro*, hippocampal neurons were fixed for 20min with 4% paraformaldehyde in 100mM phosphate buffer (PB). Cells were permeabilized on ice for 10min with 4% formaldehyde and 0.25% Triton, washed three times with 100mM PB saline (PBS), and then blocked with 10% bovine serum albumin for 1hr at 37°C. Primary and secondary antibodies were diluted in PBS and 3% normal horse serum and incubated overnight at 4°C, or for 2hrs at room temperature. Cover slips were washed 3 times for 10min and once with Milli-Q water, before they were dried and mounted with Vectashield and DAPI (Vector Laboratories, Burlingame, CA). The following antibodies were used: rabbit anti-MeCP2 (Millipore Corporation, Billerica, MA); rabbit anti-BDNF (Sigma); anti-mouse cascade blue; and anti-rabbit Texas red (Santa Cruz Biotechnology; Santa Cruz, CA). Mouse anti-actin and anti-α-tubulin were obtained from the Developmental Studies Hybridoma Bank under the auspices of NIH-NICHD and maintained by the Department of Biological Sciences, University of Iowa.

Quantitative Immunofluorescence

After 4 days *in vitro*, primary cultures were stained for BDNF or MeCP2 immunocytochemistry. Cells were incubated with primary antibodies overnight and an anti-Texas-red conjugated secondary antibody (Santa Cruz). Digital images were acquired using an Olympus IX70 epifluorescence microscope with a 100X (1.35 NA) oil-immersion objective, and using a Retiga 1300-cooled charge-coupled device (CCD) monochromatic camera (Qimaging, Surrey, British Columbia). Images were deconvolved using an empirical point-spread function of 0.1073μm in IPLab software (Scanalytics, BD Biosciences Bioimaging; Rockville, MD). To allow direct comparison of immunofluorescence intensities, the field of view always included more than one neuron and at least one of them was either transfected (i.e. expressing GFP for visualization) or untransfected (i.e. positive for DAPI, negative for GFP). Thus, BDNF or MeCP2 levels could be directly and quantitatively compared between transfected and untransfected neurons within the same field of view (neighboring cells). In rare circumstances, when a transfected cell was not in close proximity to an untransfected neuron, the closest untransfected neighboring cell was used for quantification. CCD camera exposure times were kept constant when taking images of separate fields of view.

Quantitative Analyses of Neuronal Morphology

Neuronal morphology was measured as described previously (Moore et al., 2007). After 4 days *in vitro*, embryonic hippocampal pyramidal neurons are polarized and extend a single axon and several dendrites that are highly branched. For quantitative morphological analyses,

neurons were co-transfected with eGFP or stained with an anti-α-tubulin antibody. Digital images were acquired using an Olympus IX70 epifluorescence microscope with a 40X (1 NA) oil-immersion objective using a Retiga 1300-cooled CCD monochromatic camera (Qimaging). The length and branch points of the axons and dendrites were measured by tracing along each neuronal projection using ImageJ software (National Institutes of Health).

Statistical Analyses

Data were analyzed statistically using unpaired Student's t test or a one-way analysis of variance (ANOVA) followed by Tukey's procedure for multiple comparisons using Prism (GraphPad; San Diego, CA). $P < 0.05$ was considered significant. Data are presented as mean ± standard error of the mean (SEM). Compromise Power Analyses were performed to determine the statistical power given the number of observations (n), the mean, and the standard deviation of the mean (SD), using G*Power (Erdfelder et al., 1996). These power analyses yielded values of Statistical Power (1-β; where β is the Type-II error) larger than 0.95 (i.e., 95% confidence of accepting the null hypothesis when is true).

Results

We studied the role of MeCP2 on neuronal terminal differentiation using hippocampal neurons from 18 day-old rat embryos maintained in dissociated primary cell culture. Pyramidal neurons in this culture preparation undergo a well-characterized morphological differentiation of axons and dendrites (Banker and Goslin, 1998; Craig and Banker, 1994). Quantitative analyses of dendritic and axonal morphology in pyramidal neurons transfected with eGFP or stained with anti-α-tubulin antibodies were performed after 4 days *in vitro*, when a single defined axon and several dendrites are well defined (Supplemental Fig. 2) (Dotti et al., 1988). At this time, synapses have not formed yet (Ziv and Smith, 1996), allowing the isolation of MeCP2's role in neuronal differentiation from the well-known contribution of synaptically driven neuronal activity to this process (Cline and Haas, 2008). The morphological differentiation of dendrite and axons was estimated by measuring their total length and counting all their branch points in every neuron.

1. Endogenous *Mecp2* Knockdown Reduced Dendritic Length Without Affecting Axonal Morphology

In PC12 cells, *Mecp2* knockdown using antisense oligonucleotides prevented neurite outgrowth induced by nerve growth factor (NGF) (Cusack et al., 2004). We first demonstrated that shRNA-mediated knockdown caused a 55% decrease in MeCP2 protein levels, as measured by Western blots from PC12 cells and quantitative immunocytochemistry of cultured hippocampal pyramidal neurons ($p<0.05$; Supplemental Fig. 1). After 4 days *in vitro, Mecp2* knockdown caused a significant reduction of dendritic length (control shRNA sequence = 105.2 ± 7.54μm, n=5 *vs Mecp2* shRNA = 50.43 ± 5.11μm, n=20; $p<0.001$; Fig. 1A, C), without affecting the number of dendritic branch points per neuron (control shRNA = 3 ± 0.55 branch points/neuron, n=5 *vs. Mecp2* shRNA = 3.46 ± 0.37 branch points/neuron, n=20; p=0.573; Fig. 1A, C). On the other hand, neither axonal length (control shRNA = 139.7 ± 18.81μm length, n=5 *vs. Mecp2* shRNA = 99.68 ± 24.89μm length, n=5; p=0.236) nor the number of axonal branch points were significantly affected by *Mecp2* knockdown (shRNA control = 2.6 ± 0.51 branch points/neuron, n=5 *vs. Mecp2* shRNA = 2.2 ± 0.73 branch points/neuron, n=5; p=0.668; Fig. 1A, B).

2. Overexpression of Wildtype Human *MECP2* Increased Dendritic Branching, as well as Axonal Length and Branching

Quantitative immunocytochemistry of cultured pyramidal neurons demonstrated that expression plasmids using the CMV promoter caused a twofold increase in MeCP2 protein

levels ($p<0.05$; Supplemental Fig. 1). These levels of immunolabeled MeCP2 reflect both the exogenously transfected protein plus the endogenously expressed MeCP2 (i.e. the endogenous *Mecp2* gene was not manipulated). After 4 days *in vitro*, wt *MECP2* overexpression did not affect dendritic length (GFP control = 108.5 ± 26μm, n=20 *vs.* wt *MECP2* = 101.5 ± 10.6μm, n=18; p=0.05; Fig. 2A, C), but significantly increased the number of dendritic branch points (GFP control = 3.7 ± 0.53 branch points/neuron, n=20 *vs.* wt *MECP2* = 6 ± 0.78 branch points/neuron, n=18; p=0.02; Figure 2A, C). On the other hand, both axonal length (GFP control = 147.1 ± 20μm, n=5 *vs.* wt *MECP2* = 253.6 ± 10.79μm, n=5; p=0.002; Fig. 2A, B) and the number of branch points (GFP control = 2.4 ± 0.24 branch points/neuron, n=5 *vs.* wt *MECP2* = 4.2 ± 0.37 branch points/neuron, n=5; p=0.004; Fig. 2A, B) were significantly increased after wt *MECP2* overexpression.

3. Overexpression of Missense *MECP2* Mutations Common in Rett Syndrome Reduced Dendritic Length and Branching, as well as Axonal Length

Next, we transfected E18 hippocampal pyramidal neurons with expression plasmids encoding two different single-point missense mutations in human *MECP2*, R106W and T158M, which are located in the methyl-binding domain (MBD) of the MeCP2 protein and reduce its transcriptional repression activity (Kudo et al., 2001; Kudo et al., 2003). Of relevance to Rett syndrome, these are two of the most common missense mutations in *MECP2* found in RTT patients (www.mecp2.org.uk). As in the previous experiments overexpressing wildtype human *MECP2*, the endogenous *Mecp2* gene was not manipulated.

In contrast to the effects of wildtype *MECP2*, overexpression of either *MECP2* mutation significantly reduced both dendritic length (GFP control = 108.5 ± 26μm *vs.* R106W = 43.26 ± 4.25μm, n=20; p=0.02; *vs.* T158M = 51.69 ± 7.7μm, n=20; p=0.04; Fig. 2A, C) and the number of dendritic branch points (GFP control = 3.7 ± 0.53 branch points/neuron, n=20 *vs.* R106W = 2.1 ± 0.34 branch points/neuron, n=20; p=0.01; *vs.* T158M = 2 ± 0.37 branch points/neuron, n=20; p=0.01; Fig. 2A, C). Axonal length was also significantly reduced in neurons overexpressing the R106W mutation (GFP control = 147.1 ± 20μm, n=5 *vs.* R106W = 81.67 ± 7.03μm, n=5; p=0.04; Fig. 2A, B), but not the T158M mutation (T158M = 84.78 ± 18.85μm, n=5; p=0.053; Fig. 2A, B). On the other hand, neither *MECP2* mutation affected the number of axonal branch points (GFP control = 2.4 ± 0.24 branch points/neuron, n=5 *vs.* R106W = 2.8 ± 0.86 branch points/neuron, n=5; p=0.678; T158M = 1.8 ± 0.58 branch points/neuron, n=5; p=0.371; Fig. 2A, B).

4. Either Endogenous *Mecp2* Knockdown or Overexpression of Wildtype *MECP2* – but not of Rett-Associated *MECP2* Mutations – Increased Intracellular BDNF Levels in Hippocampal Neurons

Considering that BDNF is a powerful modulator of dendritic, axonal and synaptic development (McAllister et al., 1999; Vicario-Abejon et al., 2002), the observation that *Bdnf* was a target of MeCP2 transcriptional repression (Chen et al., 2003; Martinowich et al., 2003) led to a so-called “BDNF hypothesis of Rett syndrome” (Amaral et al., 2007). Indeed, increased BDNF levels reverted some crucial RTT-like phenotypes in *Mecp2* knockout mice (Chang et al., 2006; Ogier et al., 2007). However, whether MeCP2 represses or activates *Bdnf* gene transcription is still unclear (Cohen et al., 2008; Sun and Wu, 2006) and the specific mechanism/s and final BDNF protein levels may dependent on neuronal subtypes (Abuhatzira et al., 2007; Chahrour et al., 2008; Klein et al., 2007). In any case, deregulation of *Bdnf* transcription and BDNF protein levels by RTT-associated *MECP2* mutations could have a profound impact on neuronal development.

To estimate intracellular BDNF protein levels in cultured hippocampal neurons after either rat *Mecp2* knockdown or human *MECP2* overexpression, we performed quantitative BDNF

immunocytochemistry. As a benchmark for comparisons, transfection with a *Bdnf*-GFP expression plasmid under the control of the CMV promoter (Kojima et al., 2001) resulted in a 2.2-fold increase in BDNF immunofluorescence intensity, compared to an untransfected neighboring neuron (untransfected neurons 1649.83 ± 99 AU BDNF immunofluorescence intensity *vs.* BDNF-transfected neurons 3428 ± 62.72 AU BDNF immunofluorescence intensity; 2.19 ± 0.27 fold increase in immunofluorescence intensity, n=6; p=0.01; Fig. 3). Consistent with the function of a transcriptional repressor, endogenous *Mecp2* knockdown caused a significant 1.5-fold increase in BDNF immunofluorescence levels (untransfected neurons 2140.83 ± 303.41 AU BDNF immunofluorescence intensity *vs.* shRNA *Mecp2*-transfected neurons 3150.5 ± 302.57 AU BDNF immunofluorescence intensity; 1.52 ± 0.08 fold increase in immunofluorescence intensity, n=6; p=0.002; Fig. 3). Surprisingly, overexpression of human wildtype *MECP2* also resulted in a significant 1.4-fold increase in BDNF immunofluorescence levels (untransfected neurons 2120 ± 154.94 AU BDNF immunofluorescence intensity *vs.* wt *MECP2* transfected neurons 2912.38 ± 139.22 AU BDNF immunofluorescence intensity; 1.41 ± 0.09 fold increase in immunofluorescence intensity, n=8; p=0.003; Fig. 3). These observations are consistent with BDNF mRNA levels observed in cultured cortical neurons from *Mecp2* null mice in the absence of neuronal activity (i.e. in TTX) (Chen et al., 2003) or in cultured cortical neurons overexpressing wt *MECP2* (Klein et al., 2007).

We next examined whether Rett-associated *MECP2* mutations affected intracellular BDNF protein levels. Overexpression of the R106W mutation did not change intracellular BDNF protein levels (untransfected neurons 3056.5 ± 211.6 AU BDNF immunofluorescence intensity *vs.* R106W-transfected neurons 3486.33 ± 258.5 AU BDNF immunofluorescence intensity; 1.18 ± 0.14 fold change in immunofluorescence intensity, n=6; p=0.310; Fig. 3). Likewise, the levels of BDNF protein were not affected by overexpression of the T158M mutation (untransfected neurons 2122.58 ± 96.72 AU BDNF immunofluorescence intensity *vs.* T158M-transfected neurons 1925.67 ± 112.77 AU BDNF immunofluorescence intensity; 0.92 ± 0.07 fold change in immunofluorescence intensity, n=12; p=0.232; Fig. 3). These observations demonstrate that in contrast to the overexpression of wildtype *MECP2* or the knockdown of endogenous *Mecp2*, RTT-associated *MECP2* mutations do not affect BDNF protein levels, suggesting that these mutations may represent a “toxic gain-of-function” and not a simple “loss-of-function”.

Considering the well-characterized effects of BDNF on dendritic and axonal growth (McAllister et al., 1999; Vicario-Abejon et al., 2002), and the changes in BDNF protein levels after *Mecp2* knockdown or *MECP2* overexpression (Fig. 3), we next tested whether the increased axonal and dendritic length and branching in wildtype *MECP2*-overexpressing neurons resulted from elevated BDNF levels. In addition, we setout to determine if *Bdnf* overexpression could rescue the neuronal atrophy observed after endogenous *Mecp2* knockdown or expression of RTT-associated *MECP2* mutations. In order to answer these questions, we first confirmed the consequences of *Bdnf* overexpression on axonal and dendritic length and complexity. It had been previously shown that the BDNF-GFP fusion protein encoded by an expression plasmid under control of the CMV promoter preserves biological activity (Kojima et al., 2001). Indeed, overexpression of BDNF-GFP in hippocampal pyramidal neurons for 4 days *in vitro* significantly increased dendritic length (GFP control = 108.5 ± 26μm, n=20 *vs.* BDNF-GFP = 267.7 ± 20.27μm, n=19; p<0.001; Supplemental Fig. 3A, C), and the number of dendritic branch points (GFP control = 3.7 ± 0.53 branch points/neuron, n=20 *vs.* BDNF-GFP = 5.8 ± 0.83 branch points/neuron, n=19; p=0.02; Supplemental Fig. 3A, C). On the other hand, BDNF-GFP overexpression only increased the number of axonal branch points (GFP control = 2.4 ± 0.24 branch points/neuron, n=5 *vs.* BDNF-GFP = 4.2 ± 0.37 branch points/neuron, n=5; p=0.02; Supplemental Fig. 3A, B), without affecting axonal length (GFP

control = 147.1 ± 20μm, n=5 *vs.* BDNF-GFP = 155.9 ± 11.37μm, n=5; p=0.711; Supplemental Fig. 3A, B).

The soluble and membrane impermeable fusion protein TrkB-Fc inhibits BDNF-induced signaling by binding extracellular BDNF, thus preventing the activation of endogenous membrane-bound receptors (Shelton et al., 1995). Such "extracellular BDNF scavenging" prevents the effects of either exogenously applied or endogenously released BDNF (Amaral and Pozzo-Miller, 2007; Chen et al., 1999; Figurov et al., 1996; Kang et al., 1997; McAllister et al., 1997; Shimada et al., 1998; Tyler and Pozzo-Miller, 2001; Tyler et al., 2006). Application of TrkB-Fc (20μg/mL) to GFP-expressing control neurons for 4 days *in vitro* did not affect neither dendritic length/branching (54.5±4.88μm of dendritic length; 2.35 ± 0.47 branch points; n=17), or axonal length/branching (143.14 ± 9.79μm of axonal length; 2.0 ± 0.44 branch points, n=5; p>0.05 *vs.* GFP; Supplemental Fig. 3A-C), suggesting that release of endogenous BDNF is limited during this initial developmental period (i.e. before synapse formation). On the other hand, TrkB-Fc (20μg/mL) prevented the increase in dendritic length (TrkB-Fc + BDNF-GFP = 43.56 ± 6.5μm, n=9; p<0.001 *vs.* BDNF-GFP see above) and number of branch points (TrkB-Fc + BDNF-GFP = 0.11 ± 0.11 branch points/neuron, n=9; p<0.001 *vs.* BDNF-GFP see above) caused by BDNF-GFP expression (Supplemental Fig. 3A-C). In addition, TrkB-Fc (20μg/mL) prevented the increase in the number of axonal branch points in BDNF-GFP expressing neurons (TrkB-Fc + BDNF-GFP = 0.6 ± 0.25 branch points/neuron, n=5; p<0.001 *vs.* BDNF-GFP see above; Supplemental Fig. 3A-C).

Having confirmed that BDNF-GFP overexpression increases, and TrkB-Fc decreases, dendritic and axonal development in hippocampal neurons, we setout to determine whether the observed changes in neuronal morphology caused by wildtype human *MECP2* overexpression or endogenous *Mecp2* knockdown, as well as by expression of RTT-associated *MECP2* mutations resulted from misregulated BDNF levels.

5. The BDNF Scavenger TrkB-Fc Prevented the Increase in Dendritic Branching Caused by Wildtype Human *MECP2* Overexpression

Scavenging extracellular BDNF with TrkB-Fc allowed testing whether axonal and dendritic overgrowth caused by *MECP2* overexpression was mediated by increased BDNF levels. Indeed, TrkB-Fc (20μg/mL) prevented the increase in the number of dendritic branch points observed in neurons overexpressing wt *MECP2* (wt *MECP2* + TrkB-Fc = 2.6 ± 0.58 branch points/neuron, n=17 *vs.* wt *MECP2* = 6 ± 0.78 branch points/neuron, n=18; p<0.01; Figure 4A, C). Intriguingly, TrkB-Fc did not prevent the increase in axonal length (wt *MECP2* + TrkB-Fc = 246 ± 42.10μm, n=5 *vs.* wt *MECP2* = 253.6 ± 10.79μm, n=5; p>0.05) or the number of axonal branch points (wt *MECP2* + TrkB-Fc = 4.6 ± 0.51 branch points/neuron, n=5 *vs.*wt *MECP2* = 4.2 ± 0.37 branch points/neuron, n=5; p>0.05) caused by wt *MECP2* overexpression (Fig. 4A, B). These results suggest that enhanced BDNF signaling is responsible for the enhanced dendritic branching caused by wt *MECP2* overexpression, while other factors participate in *MECP2*-induced axonal overgrowth.

6. *Bdnf* Overexpression Fully Rescued Dendritic Growth in *Mecp2* Knockdown Neurons, while its Effect in Cells Expressing Rett-Associated Mutant *MECP2* was Only Partial

Considering the potent effects of BDNF in dendritic and axonal growth (McAllister et al., 1999; Vicario-Abejon et al., 2002), we next tested whether *Bdnf* overexpression could rescue the dendritic atrophy caused by shRNA-mediated knockdown of endogenous *Mecp2*. Indeed, *Bdnf* overexpression significantly increased dendritic length in neurons where *Mecp2* was knockdown with shRNA (*Mecp2* shRNA + BDNF-GFP = 275.5 ± 34.81μm, n=6 *vs.* control shRNA = 105.2 ± 7.54μm, n=5; p<0.001; Fig. 5A, C). In fact, the effect of *Bdnf* overexpression on dendritic length was significantly larger in *Mecp2* knockdown neurons (162% increase),

than in control GFP neurons (147% increase; p>0.05). Furthermore, *Bdnf* overexpression significantly increased axonal length and branching after shRNA-mediated *Mecp2* knockdown, despite the lack of effect of *Mecp2* knockdown in these axonal features (Fig. 5A, B). Axonal length was 202.1 ± 14.33μm in neurons co-transfected with BDNF-GFP and *Mecp2* shRNA (n=5), compared to 139.7 ± 18.81μm in shRNA control cells (n=5; p=0.03). Neurons co-transfected with BDNF-GFP and *Mecp2* shRNA had an average of 4.2 ± 0.37 branch points/neuron (n=5), compared to 2.6 ± 0.51 branch points/neuron in shRNA control cells (n=5 p=0.04).

Lastly, we tested if *Bdnf* overexpression could rescue the dendritic atrophy caused by overexpression of RTT-associated *MECP2* mutations. Indeed, *Bdnf* overexpression prevented the decrease in dendritic length caused by the R106W *MECP2* mutation: dendritic length was 127.8 ± 14.77μm in neurons co-transfected with BDNF-GFP and R106W (n=11), compared to 43.26 ± 4.25μm in neurons expressing R106W alone (n=20; p<0.05). *Bdnf* overexpression also prevented the dendritic atrophy caused by the T158M mutation (T158M + BDNF-GFP = 99.17 ± 16.62μm, n = 21 *vs.* T158M = 51.69 ± 7.7μm, n=20; p<0.05) (Fig. 6A, C). However, *Bdnf* overexpression did not prevent the reduction in dendritic branch points resulting from the expression of the R106W *MECP2* mutation (R106W + BDNF-GFP = 2.46±0.91 branch points/neuron, n=11 *vs.* R106W = 2.1 ± 0.34 branch points/neuron, n=20; p>0.05) (Fig. 6A, C). Furthermore, *Bdnf* overexpression did not prevent the reduction in dendritic branch points caused by the T158M mutation (T158M + BDNF-GFP = 2.46 ± 0.43 branch points/neuron, n=21 *vs.* T158M = 2 ± 0.37 branch points/neuron, n=20; p>0.05; Figure 6A, C). It's important to note that the effects of *Bdnf* overexpression in neurons expressing RTT-associated *MECP2* mutations were only partial, i.e. dendritic length was comparable to that of neurons expressing only GFP, but not to that of neurons expressing BDNF-GFP (compare GFP alone *vs.* BDNF-GFP in Fig. 5). Lastly, *Bdnf* was only able to rescue dendritic impairments, because axonal atrophy was not prevented by co-transfection of *MECP2* mutations and *Bdnf* (Fig. 6A, C).

Discussion

Here, we presented several new observations regarding the role of MeCP2 in the terminal differentiation of hippocampal neurons, specifically the growth and branching of their dendrites and axons. First, endogenous *Mecp2* knockdown reduced dendritic length without affecting axonal morphology. Second, overexpression of wildtype human *MECP2* increased dendritic branching, as well as axonal length and branching. Third, overexpression of missense *MECP2* mutations common in Rett syndrome reduced dendritic length and branching, as well as axonal length. It is important to note that the endogenous *Mecp2* gene was not manipulated in these wildtype or mutant *MECP2* overexpression experiments. Fourth, either endogenous *Mecp2* knockdown or overexpression of wildtype *MECP2* – but not of Rett-associated *MECP2* mutations – increased intracellular BDNF levels in hippocampal neurons. Fifth, the BDNF scavenger TrkB-Fc prevented the increase in dendritic branching caused by wildtype human *MECP2* overexpression. Lastly, *Bdnf* overexpression fully rescued dendritic growth in *Mecp2* knockdown neurons, while its effect in cells expressing Rett-associated mutant *MECP2* was only partial. Taken together, these results demonstrate that MeCP2 plays varied roles in dendritic and axonal development during neuronal terminal differentiation, and that some of these effects are mediated by autocrine actions of BDNF.

The expression of *MECP2*/*Mecp2* in humans and rodents increases during neuronal development and maturation (Akbarian et al., 2001; Cohen et al., 2003, Jung, 2003 #21226; LaSalle et al., 2001; Shahbazian et al., 2002; Zoghbi, 2003). Consistently, the development of axons and dendrites has been shown to be affected by MeCP2 levels (Cassel et al., 2004; Kaufmann et al., 2005; Kishi and Macklis, 2004; Matarazzo et al., 2004; Matarazzo and

Ronnett, 2004; Mullaney et al., 2004). Furthermore, cultured hippocampal neurons from *Mecp2* null mice formed fewer excitatory autapses than wildtype neurons, while neurons from *MECP2* overexpressing mice (~twofold the endogenous levels) showed a higher density of excitatory autapses than wildtype neurons after 2 weeks *in vitro* (Chao et al., 2007). Together with the present results, these observations suggest that overexpression of wildtype *MECP2* enhances synapse formation because it promotes dendritic growth during early neuronal development. In addition, this interpretation predicts that endogenous *Mecp2* knockdown, or the expression of Rett-associated *MECP2* mutations will result in reduced dendritic spine density, an issue investigated in the companion manuscript (Chapleau *et al.*, manuscript NBD-08-380 under revision after first review). It is important to note that the only published study of the consequences of mutant *MECP2* expression on dendritic and axonal morphology described increased dendritic and axonal branching (not length) in embryonic day-15 cortical neurons overexpressing $MeCP2^{293}$, a truncated protein caused by an arginine to nonsense mutation in RTT individuals (Jugloff et al., 2005). This discrepancy with our observations (reduced dendritic length and branching, as well as axonal length after mutant *MECP2* overexpression) may result from different brain regions (cortex *vs.* hippocampus), developmental ages (E15 *vs.* E18), when were neurons transfected (5 days *in vitro vs.* 30min after dissociation), the duration of mutant *MECP2* overexpression (24hs *vs.* 96hs), or the method of transfection (lipofection *vs.* electroporation).

The altered molecular pathway(s) that contribute to the pathogenesis of Rett syndrome (RTT) remain unknown and under exhaustive investigation. Numerous gene expression profiles have been conducted on samples from Rett patients or from *Mecp2*-deficient mouse models (Ballestar et al., 2005; Chahrour et al., 2008; Colantuoni et al., 2001; Delgado et al., 2006; Deng et al., 2007; McGill et al., 2006; Nuber et al., 2005; Tudor et al., 2002), with each report arriving to different conclusions regarding deregulated protein(s) in RTT through either direct or indirect interactions with MeCP2. One prominent and quite consistent gene target of MeCP2 transcriptional regulation is *Bdnf* (Caballero and Hendrich, 2005; Chahrour et al., 2008; Chen et al., 2003; Klose and Bird, 2003; Martinowich et al., 2003; Wade, 2004). Chen *et al.* (Chen et al., 2003) first demonstrated that MeCP2 represses *Bdnf* transcription by directly binding to mouse *Bdnf* promoter IV, which is activated by neuronal activity and Ca^{2+} influx through voltage-gated Ca^{2+} channels (Tao et al., 1998). In the absence of neuronal activity (i.e. in the presence of TTX), exon IV mRNA transcripts were twofold higher in cultured cortical neurons from *Mecp2* null mice than in neurons from wildtype mice (Chen et al., 2003). However, it was later found that BDNF protein levels measured by ELISA were found to be lower in brain samples from *Mecp2* null mice compared to wildtype mice at 6-8 weeks of age (Chang et al., 2006). Given that *Bdnf* mRNA and protein levels are tightly regulated by neuronal activity, the reduced firing frequency observed in cortical neurons from *Mecp2* null mice (Dani et al., 2005) may be the cause of impaired activity-dependent BDNF protein synthesis, as estimated by BDNF protein ELISA measurements (Chang et al., 2006; Ogier et al., 2007; Wang et al., 2006). In addition, *MECP2* overexpression in cultured cortical neurons resulted in elevated *Bdnf* mRNA levels, likely via a homeostatic regulatory loop that includes the CREB-induced microRNA132, which negatively regulates MeCP2 levels (Klein et al., 2007). Thus, the functional significance and the specific mechanism(s) of the interaction between BDNF and MeCP2 remain unknown and hotly debated (Cohen et al., 2008; Sun and Wu, 2006). At the clinical level, the role of neurotrophins such as BDNF in Rett syndrome is also unclear. Initial studies described lower levels of NGF in blood serum or cerebral spinal fluid of RTT patients compared to non-RTT individuals, but no differences in BDNF levels (Lappalainen et al., 1996; Riikonen, 2003; Riikonen and Vanhala, 1999; Vanhala et al., 1998). However, a recent study reports that *BDNF* mRNA levels are reduced in brain samples from RTT patients (Abuhatzira et al., 2007).

Considering the powerful actions of BDNF on dendritic, axonal and, eventually, synaptic development, (McAllister et al., 1999; Vicario-Abejon et al., 2002), and the observations that increasing BDNF protein levels reverted some crucial RTT-like phenotypes in *Mecp2* knockout mice (Chang et al., 2006; Ogier et al., 2007), a "BDNF hypothesis of Rett syndrome" has been proposed (Amaral et al., 2007). Consistent with this hypothesis, the membrane impermeable TrkB-Fc fusion protein, which scavenges extracellular BDNF (Shelton et al., 1995), prevented the increase in dendritic complexity caused by overexpression of wildtype *MECP2* in hippocampal neurons. It remains to be tested whether a similar manipulation of extracellular BDNF levels prevents the effect of *MECP2* overexpression reported in cortical dendrites (Jugloff et al., 2005) and hippocampal excitatory autapses (Chao et al., 2007). Despite the current debate regarding the functional interaction between BDNF and MeCP2 and their role in Rett syndrome (Cohen et al., 2008; Sun and Wu, 2006), our current knowledge indicates that MeCP2 acts both directly and indirectly to control the expression of *Bdnf*. Indeed, our present results show that both knockdown of endogenous *Mecp2* as well as overexpression of wildtype *MECP2* increased intracellular BDNF protein levels. Whether these elevated intracellular BDNF levels are accompanied by enhanced activity-dependent release and autocrine/paracrine signaling remains to be determined. For example, neurons of the nodose ganglion from *Mecp2*-null mice show lower total BDNF levels than wildtype neurons, but are able to release it at wildtype levels (Wang et al., 2006). Intriguingly, overexpression of RTT-associated missense mutations did not affect intracellular BDNF protein levels, which may or may not be released appropriately by neuronal activity. Overall, our observations support the view that the developmental increase in *MECP2*/*Mecp2* expression controls BDNF protein levels, which in turn participate in the growth and maturation of dendritic architecture, and that Rett-associated *MECP2* mutations impair neuronal terminal differentiation by deregulating BDNF levels and/or its activity-dependent release.

The observations that: (1) *Bdnf* overexpression rescued the hypoactivity in wheel running exhibited by *Mecp2* knockout mouse, and the low frequency of action potential firing observed in their cortical neurons (Chang et al., 2006); and (2) treatment of *Mecp2* null mice with AMPAkines (which increases BDNF mRNA and protein levels) rescued the irregular respiratory patterns exhibited by *Mecp2* null mice (Ogier et al., 2007), prompt us to test whether *Bdnf* overexpression could rescue the stunted dendritic growth caused by either endogenous *Mecp2* knockdown or overexpression of mutant *MECP2*. Indeed, *Bdnf* was able to increase dendritic length in neurons after *Mecp2* knockdown, whose dendrites reached average lengths comparable to neurons transfected with *Bdnf* alone. Intriguingly, the effect in neurons expressing Rett-associated *MECP2* mutations was only partial, because it increased dendritic length only to control levels observed in GFP-expressing neurons, without the enhanced overgrowth produced by *Bdnf* overexpression. Despite this partial effect, these results demonstrate that BDNF is able to revert the dendritic atrophy caused by Rett-associated *MECP2* mutations.

In addition to *Bdnf*, the search for gene targets of MeCP2 has yielded a few intriguing candidates (Bienvenu and Chelly, 2006). For example, the genes encoding *CRH*, corticotropin-releasing hormone (McGill et al., 2006), *FXYD1* – a transmembrane modulator of the Na^+/K^+-ATPase (Deng et al., 2007) and the *inhibitors of differentiation ID1-4* (Peddada et al., 2006) are all upregulated in *Mecp2* null mice and in Rett patients, in addition to participating in neuronal and dendritic development. Recently, a more targeted study identified 2,582 genes symmetrically misregulated in the hypothalamus of *Mecp2* null and *MECP2* overexpressing mice, suggesting that they represent primary targets (Chahrour et al., 2008). Unexpectedly, 85% of them seem to be activated by MeCP2 because they were up-regulated in *MECP2* overexpressing and down-regulated in *Mecp2* null mice (Chahrour et al., 2008). The contribution of any of these genes in our results, as well as any potential interactions with *Bdnf* transcription and translation remains unknown.

DNA methylation is a common mechanism of silencing genes during cellular differentiation, and MeCP2 may play a critical role in neuronal terminal differentiation by reading this epigenetic code. Indeed, gene methylation is required for NGF to induce neurite outgrowth in PC12 cells (Persengiev and Kilpatrick, 1996), a signature of their differentiation into neurons. Consistently, the expression of the *inhibitors of differentiation* genes (*ID1*, *ID2* and *ID3*) is reduced during NGF-induced PC12 differentiation, an effect sensitive to inhibition of DNA methyltransferase activity (Persengiev and Kilpatrick, 1997). Intriguingly, all four *ID* genes are targets of MeCP2 transcriptional repression, demonstrated by increased mRNA and protein levels in brain samples from *Mecp2* null mice and RTT individuals (Peddada et al., 2006). The observation that *Mecp2* null mice express lower levels of *Neurod1*, a gene target of ID proteins that is critical for neuronal development (Andres-Barquin et al., 2000; Chae et al., 2004), strongly suggest that *Mecp2*-deficient neurons are arrested in an immature stage of differentiation by the inability to properly read the epigenetic code represented by DNA methylation of gene promoters (Shahbazian and Zoghbi, 2002). In summary, our results are consistent with a role of MeCP2 in early neuronal development and differentiation, providing novel evidence supporting the involvement of BDNF in the changes caused by MeCP2 deregulation leading to prominent features of the neuropathology of Rett syndrome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Abuhatzira L, Makedonski K, Kaufman Y, Razin A, Shemer R. MeCP2 deficiency in the brain decreases BDNF levels by REST/CoREST-mediated repression and increases TRKB production. Epigenetics 2007;2:214–22. [PubMed: 18075316]

Akbarian S, Chen RZ, Gribnau J, Rasmussen TP, Fong H, Jaenisch R, Jones EG. Expression pattern of the Rett syndrome gene MeCP2 in primate prefrontal cortex. Neurobiol Dis 2001;8:784–91. [PubMed: 11592848]

Amaral MD, Chapleau CA, Pozzo-Miller L. Transient receptor potential channels as novel effectors of brain-derived neurotrophic factor signaling: Potential implications for Rett syndrome. Pharmacol Ther 2007;113:394–409. [PubMed: 17118456]

Amaral MD, Pozzo-Miller L. TRPC3 channels are necessary for brain-derived neurotrophic factor to activate a nonselective cationic current and to induce dendritic spine formation. J Neurosci 2007;27:5179–89. [PubMed: 17494704]

Amir RE, Van den Veyver IB, Wan M, Tran CQ, Francke U, Zoghbi HY. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. Nat Genet 1999;23:185–8. [PubMed: 10508514]

Andres-Barquin PJ, Hernandez MC, Israel MA. Id genes in nervous system development. Histol Histopathol 2000;15:603–18. [PubMed: 10809382]

Armstrong D, Dunn JK, Antalffy B, Trivedi R. Selective dendritic alterations in the cortex of Rett syndrome. J Neuropathol Exp Neurol 1995;54:195–201. [PubMed: 7876888]

Armstrong DD. Neuropathology of Rett syndrome. Ment Retard Dev Disabil Res Rev 2002;8:72–6. [PubMed: 12112730]

Armstrong DD, Dunn JK, Schultz RJ, Herbert DA, Glaze DG, Motil KJ. Organ growth in Rett syndrome: a postmortem examination analysis. Pediatr Neurol 1999;20:125–9. [PubMed: 10082341]

Ballestar E, Ropero S, Alaminos M, Armstrong J, Setien F, Agrelo R, Fraga MF, Herranz M, Avila S, Pineda M, Monros E, Esteller M. The impact of MECP2 mutations in the expression patterns of Rett syndrome patients. Hum Genet 2005;116:91–104. [PubMed: 15549394]

Banker, G.; Goslin, K. Culturing nerve cells. MIT Press; Boston: 1998.

Bienvenu T, Carrie A, de Roux N, Vinet MC, Jonveaux P, Couvert P, Villard L, Arzimanoglou A, Beldjord C, Fontes M, Tardieu M, Chelly J. MECP2 mutations account for most cases of typical forms of Rett syndrome. Hum Mol Genet 2000;9:1377–84. [PubMed: 10814719]

Bienvenu T, Chelly J. Molecular genetics of Rett syndrome: when DNA methylation goes unrecognized. Nat Rev Genet 2006;7:415–26. [PubMed: 16708070]

Caballero IM, Hendrich B. MeCP2 in neurons: closing in on the causes of Rett syndrome. Hum Mol Genet 2005;14(Spec No 1):R19–26. [PubMed: 15809268]

Cassel S, Revel MO, Kelche C, Zwiller J. Expression of the methyl-CpG-binding protein MeCP2 in rat brain. An ontogenetic study. Neurobiol Dis 2004;15:206–11. [PubMed: 15006690]

Chae JH, Stein GH, Lee JE. NeuroD: the predicted and the surprising. Mol Cells 2004;18:271–88. [PubMed: 15650322]

Chahrour M, Jung SY, Shaw C, Zhou X, Wong ST, Qin J, Zoghbi HY. MeCP2, a Key Contributor to Neurological Disease, Activates and Represses Transcription. Science 2008;320:1224–9. [PubMed: 18511691]

Chang Q, Khare G, Dani V, Nelson S, Jaenisch R. The disease progression of Mecp2 mutant mice is affected by the level of BDNF expression. Neuron 2006;49:341–8. [PubMed: 16446138]

Chao HT, Zoghbi HY, Rosenmund C. MeCP2 controls excitatory synaptic strength by regulating glutamatergic synapse number. Neuron 2007;56:58–65. [PubMed: 17920015]

Chechlacz M, Gleeson JG. Is mental retardation a defect of synapse structure and function? Pediatr Neurol 2003;29:11–7. [PubMed: 13679116]

Chen G, Kolbeck R, Barde YA, Bonhoeffer T, Kossel A. Relative contribution of endogenous neurotrophins in hippocampal long- term potentiation. J Neurosci 1999;19:7983–90. [PubMed: 10479698]

Chen WG, Chang Q, Lin Y, Meissner A, West AE, Griffith EC, Jaenisch R, Greenberg ME. Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2. Science 2003;302:885–9. [PubMed: 14593183]

Cline H, Haas K. The regulation of dendritic arbor development and plasticity by glutamatergic synaptic input: a review of the synaptotrophic hypothesis. J Physiol 2008;586:1509–17. [PubMed: 18202093]

Cohen DR, Matarazzo V, Palmer AM, Tu Y, Jeon OH, Pevsner J, Ronnett GV. Expression of MeCP2 in olfactory receptor neurons is developmentally regulated and occurs before synaptogenesis. Mol Cell Neurosci 2003;22:417–29. [PubMed: 12727440]

Cohen S, Zhou Z, Greenberg ME. Medicine. Activating a repressor. Science 2008;320:1172–3. [PubMed: 18511680]

Colantuoni C, Jeon OH, Hyder K, Chenchik A, Khimani AH, Narayanan V, Hoffman EP, Kaufmann WE, Naidu S, Pevsner J. Gene expression profiling in postmortem Rett Syndrome brain: differential gene expression and patient classification. Neurobiol Dis 2001;8:847–65. [PubMed: 11592853]

Craig AM, Banker G. Neuronal polarity. Annu Rev Neurosci 1994;17:267–310. [PubMed: 8210176]

Cusack SM, Rohn TT, Medeck RJ, Irwin KM, Brown RJ, Mercer LM, Oxford JT. Suppression of MeCP2beta expression inhibits neurite extension in PC12 cells. Exp Cell Res 2004;299:442–53. [PubMed: 15350542]

Dani VS, Chang Q, Maffei A, Turrigiano GG, Jaenisch R, Nelson SB. Reduced cortical activity due to a shift in the balance between excitation and inhibition in a mouse model of Rett syndrome. Proc Natl Acad Sci U S A 2005;102:12560–5. [PubMed: 16116096]

Delgado IJ, Kim DS, Thatcher KN, Lasalle JM, Van den Veyver IB. Expression profiling of clonal lymphocyte cell cultures from Rett syndrome patients. BMC Med Genet 2006;7:61. [PubMed: 16859563]

Deng V, Matagne V, Banine F, Frerking M, Ohliger P, Budden S, Pevsner J, Dissen GA, Sherman LS, Ojeda SR. FXYD1 is an MeCP2 target gene overexpressed in the brains of Rett syndrome patients and Mecp2-null mice. Hum Mol Genet 2007;16:640–50. [PubMed: 17309881]

Dotti CG, Sullivan CA, Banker GA. The establishment of polarity by hippocampal neurons in culture. J Neurosci 1988;8:1454–68. [PubMed: 3282038]

Erdfelder E, Faul F, Buchner A. GPOWER: A general power analysis program. Behavior Research Methods 1996;28:1–11.

Figurov A, Pozzo-Miller LD, Olafsson P, Wang T, Lu B. Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. Nature 1996;381:706–9. [PubMed: 8649517]

Gresch O, Engel FB, Nesic D, Tran TT, England HM, Hickman ES, Korner I, Gan L, Chen S, Castro-Obregon S, Hammermann R, Wolf J, Muller-Hartmann H, Nix M, Siebenkotten G, Kraus G, Lun K. New non-viral method for gene transfer into primary cells. Methods 2004;33:151–63. [PubMed: 15121170]

Hagberg B. Clinical manifestations and stages of Rett syndrome. Ment Retard Dev Disabil Res Rev 2002;8:61–5. [PubMed: 12112728]

Hagberg B, Goutieres F, Hanefeld F, Rett A, Wilson J. Rett syndrome: criteria for inclusion and exclusion. Brain Dev 1985;7:372–3. [PubMed: 4061772]

Jellinger K, Armstrong D, Zoghbi HY, Percy AK. Neuropathology of Rett syndrome. Acta Neuropathol (Berl) 1988;76:142–58. [PubMed: 2900587]

Johnston MV, Jeon OH, Pevsner J, Blue ME, Naidu S. Neurobiology of Rett syndrome: a genetic disorder of synapse development. Brain Dev 2001;23:S206–13. [PubMed: 11738874]

Johnston MV, Mullaney B, Blue ME. Neurobiology of Rett syndrome. J Child Neurol 2003;18:688–92. [PubMed: 14649550]

Jones PL, Veenstra GJ, Wade PA, Vermaak D, Kass SU, Landsberger N, Strouboulis J, Wolffe AP. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet 1998;19:187–91. [PubMed: 9620779]

Jugloff DG, Jung BP, Purushotham D, Logan R, Eubanks JH. Increased dendritic complexity and axonal length in cultured mouse cortical neurons overexpressing methyl-CpG-binding protein MeCP2. Neurobiol Dis 2005;19:18–27. [PubMed: 15837557]

Kang H, Welcher AA, Shelton D, Schuman EM. Neurotrophins and time: different roles for TrkB signaling in hippocampal long-term potentiation. Neuron 1997;19:653–64. [PubMed: 9331355]

Kaufmann WE, Johnston MV, Blue ME. MeCP2 expression and function during brain development: implications for Rett syndrome's pathogenesis and clinical evolution. Brain Dev 2005;27:S77–S87. [PubMed: 16182491]

Kaufmann WE, MacDonald SM, Altamura CR. Dendritic cytoskeletal protein expression in mental retardation: an immunohistochemical study of the neocortex in Rett syndrome. Cereb Cortex 2000;10:992–1004. [PubMed: 11007550]

Kaufmann WE, Moser HW. Dendritic anomalies in disorders associated with mental retardation. Cereb Cortex 2000;10:981–91. [PubMed: 11007549]

Kaufmann WE, Naidu S, Budden S. Abnormal expression of microtubule-associated protein 2 (MAP-2) in neocortex in Rett syndrome. Neuropediatrics 1995;26:109–13. [PubMed: 7566447]

Kaufmann WE, Worley PF, Taylor CV, Bremer M, Isakson PC. Cyclooxygenase-2 expression during rat neocortical development and in Rett syndrome. Brain Dev 1997;19:25–34. [PubMed: 9071487]

Kishi N, Macklis JD. MECP2 is progressively expressed in post-migratory neurons and is involved in neuronal maturation rather than cell fate decisions. Mol Cell Neurosci 2004;27:306–21. [PubMed: 15519245]

Klein ME, Lioy DT, Ma L, Impey S, Mandel G, Goodman RH. Homeostatic regulation of MeCP2 expression by a CREB-induced microRNA. Nat Neurosci 2007;10:1513–4. [PubMed: 17994015]

Klose R, Bird A. Molecular biology. MeCP2 repression goes nonglobal. Science 2003;302:793–5. [PubMed: 14593157]

Klose RJ, Sarraf SA, Schmiedeberg L, McDermott SM, Stancheva I, Bird AP. DNA binding selectivity of MeCP2 due to a requirement for A/T sequences adjacent to methyl-CpG. Mol Cell 2005;19:667–78. [PubMed: 16137622]

Kojima M, Takei N, Numakawa T, Ishikawa Y, Suzuki S, Matsumoto T, Katoh-Semba R, Nawa H, Hatanaka H. Biological characterization and optical imaging of brain-derived neurotrophic factor-green fluorescent protein suggest an activity- dependent local release of brain-derived neurotrophic factor in neurites of cultured hippocampal neurons. J Neurosci Res 2001;64:1–10. [PubMed: 11276045]

Kriaucionis S, Bird A. The major form of MeCP2 has a novel N-terminus generated by alternative splicing. Nucleic Acids Res 2004;32:1818–23. [PubMed: 15034150]

Kudo S. Methyl-CpG-binding protein MeCP2 represses Sp1-activated transcription of the human leukosialin gene when the promoter is methylated. Mol Cell Biol 1998;18:5492–9. [PubMed: 9710633]

Kudo S, Nomura Y, Segawa M, Fujita N, Nakao M, Dragich J, Schanen C, Tamura M. Functional analyses of MeCP2 mutations associated with Rett syndrome using transient expression systems. Brain Dev 2001;23:S165–73. [PubMed: 11738866]

Kudo S, Nomura Y, Segawa M, Fujita N, Nakao M, Schanen C, Tamura M. Heterogeneity in residual function of MeCP2 carrying missense mutations in the methyl CpG binding domain. J Med Genet 2003;40:487–93. [PubMed: 12843318]

Lappalainen R, Lindholm D, Riikonen R. Low levels of nerve growth factor in cerebrospinal fluid of children with Rett syndrome. J Child Neurol 1996;11:296–300. [PubMed: 8807419]

LaSalle JM. Paradoxical role of methyl-CpG-binding protein 2 in Rett syndrome. Curr Top Dev Biol 2004;59:61–86. [PubMed: 14975247]

LaSalle JM, Goldstine J, Balmer D, Greco CM. Quantitative localization of heterogeneous methyl-CpG-binding protein 2 (MeCP2) expression phenotypes in normal and Rett syndrome brain by laser scanning cytometry. Hum Mol Genet 2001;10:1729–40. [PubMed: 11532982]

Martinowich K, Hattori D, Wu H, Fouse S, He F, Hu Y, Fan G, Sun YE. DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. Science 2003;302:890–3. [PubMed: 14593184]

Matarazzo V, Cohen D, Palmer AM, Simpson PJ, Khokhar B, Pan SJ, Ronnett GV. The transcriptional repressor Mecp2 regulates terminal neuronal differentiation. Mol Cell Neurosci 2004;27:44–58. [PubMed: 15345242]

Matarazzo V, Ronnett GV. Temporal and regional differences in the olfactory proteome as a consequence of MeCP2 deficiency. Proc Natl Acad Sci U S A 2004;101:7763–8. [PubMed: 15128950]

McAllister AK, Katz LC, Lo DC. Opposing roles for endogenous BDNF and NT-3 in regulating cortical dendritic growth. Neuron 1997;18:767–78. [PubMed: 9182801]

McAllister AK, Katz LC, Lo DC. Neurotrophins and synaptic plasticity. Annu Rev Neurosci 1999;22:295–318. [PubMed: 10202541]

McGill BE, Bundle SF, Yaylaoglu MB, Carson JP, Thaller C, Zoghbi HY. Enhanced anxiety and stress-induced corticosterone release are associated with increased Crh expression in a mouse model of Rett syndrome. Proc Natl Acad Sci U S A 2006;103:18267–72. [PubMed: 17108082]

Mnatzakanian GN, Lohi H, Munteanu I, Alfred SE, Yamada T, MacLeod PJ, Jones JR, Scherer SW, Schanen NC, Friez MJ, Vincent JB, Minassian BA. A previously unidentified MECP2 open reading frame defines a new protein isoform relevant to Rett syndrome. Nat Genet 2004;36:339–41. [PubMed: 15034579]

Moore CD, Thacker EE, Larimore J, Gaston D, Underwood A, Kearns B, Patterson SI, Jackson T, Chapleau C, Pozzo-Miller L, Theibert A. The neuronal Arf GAP centaurin alpha1 modulates dendritic differentiation. J Cell Sci 2007;120:2683–93. [PubMed: 17635995]

Mullaney BC, Johnston MV, Blue ME. Developmental expression of methyl-CpG binding protein 2 is dynamically regulated in the rodent brain. Neuroscience 2004;123:939–49. [PubMed: 14751287]

Naidu S. Rett syndrome: a disorder affecting early brain growth. Ann Neurol 1997;42:3–10. [PubMed: 9225679]

Nan X, Bird A. The biological functions of the methyl-CpG-binding protein MeCP2 and its implication in Rett syndrome. Brain Dev 2001;23:S32–7. [PubMed: 11738839]

Nan X, Campoy FJ, Bird A. MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin. Cell 1997;88:471–81. [PubMed: 9038338]

Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN, Bird A. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature 1998;393:386–9. [PubMed: 9620804]

Nuber UA, Kriaucionis S, Roloff TC, Guy J, Selfridge J, Steinhoff C, Schulz R, Lipkowitz B, Ropers HH, Holmes MC, Bird A. Up-regulation of glucocorticoid-regulated genes in a mouse model of Rett syndrome. Hum Mol Genet 2005;14:2247–56. [PubMed: 16002417]

Ogier M, Wang H, Hong E, Wang Q, Greenberg ME, Katz DM. Brain-derived neurotrophic factor expression and respiratory function improve after ampakine treatment in a mouse model of Rett syndrome. J Neurosci 2007;27:10912–7. [PubMed: 17913925]

Peddada S, Yasui DH, LaSalle JM. Inhibitors of differentiation (ID1, ID2, ID3 and ID4) genes are neuronal targets of MeCP2 that are elevated in Rett syndrome. Hum Mol Genet 2006;15:2003–14. [PubMed: 16682435]

Percy AK, Lane JB. Rett syndrome: model of neurodevelopmental disorders. J Child Neurol 2005;20:718–21. [PubMed: 16225824]

Persengiev SP, Kilpatrick DL. Nerve growth factor induced differentiation of neuronal cells requires gene methylation. Neuroreport 1996;8:227–31. [PubMed: 9051786]

Persengiev SP, Kilpatrick DL. The DNA methyltransferase inhibitor 5-azacytidine specifically alters the expression of helix-loop-helix proteins Id1, Id2 and Id3 during neuronal differentiation. Neuroreport 1997;8:2091–5. [PubMed: 9243590]

Philippart M. Rett and Angelman's syndromes: models of arrested development. Pediatr Neurol 2001;25:288–94. [PubMed: 11704397]

Riikonen R. Neurotrophic factors in the pathogenesis of Rett syndrome. J Child Neurol 2003;18:693–7. [PubMed: 14649551]

Riikonen R, Vanhala R. Levels of cerebrospinal fluid nerve-growth factor differ in infantile autism and Rett syndrome. Dev Med Child Neurol 1999;41:148–52. [PubMed: 10210246]

Schultz RJ, Glaze DG, Motil KJ, Armstrong DD, del Junco DJ, Hubbard CR, Percy AK. The pattern of growth failure in Rett syndrome. Am J Dis Child 1993;147:633–7. [PubMed: 8506830]

Shahbazian MD, Antalffy B, Armstrong DL, Zoghbi HY. Insight into Rett syndrome: MeCP2 levels display tissue- and cell-specific differences and correlate with neuronal maturation. Hum Mol Genet 2002;11:115–24. [PubMed: 11809720]

Shahbazian MD, Zoghbi HY. Rett syndrome and MeCP2: linking epigenetics and neuronal function. Am J Hum Genet 2002;71:1259–72. [PubMed: 12442230]

Shelton DL, Sutherland J, Gripp J, Camerato T, Armanini MP, Phillips HS, Carroll K, Spencer SD, Levinson AD. Human trks: molecular cloning, tissue distribution, and expression of extracellular domain immunoadhesins. J Neurosci 1995;15:477–491. [PubMed: 7823156]

Shimada A, Mason CA, Morrison ME. TrkB signaling modulates spine density and morphology independent of dendrite structure in cultured neonatal Purkinje cells. J Neurosci 1998;18:8559–70. [PubMed: 9786964]

Sun YE, Wu H. The ups and downs of BDNF in Rett syndrome. Neuron 2006;49:321–3. [PubMed: 16446133]

Tao X, Finkbeiner S, Arnold DB, Shaywitz AJ, Greenberg ME. Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. Neuron 1998;20:709–26. [PubMed: 9581763]

Tudor M, Akbarian S, Chen RZ, Jaenisch R. Transcriptional profiling of a mouse model for Rett syndrome reveals subtle transcriptional changes in the brain. Proc Natl Acad Sci U S A 2002;99:15536–41. [PubMed: 12432090]

Tyler WJ, Pozzo-Miller LD. BDNF enhances quantal neurotransmitter release and increases the number of docked vesicles at the active zones of hippocampal excitatory synapses. J Neurosci 2001;21:4249–58. [PubMed: 11404410]

Tyler WJ, Zhang XL, Hartman K, Winterer J, Muller W, Stanton PK, Pozzo-Miller L. BDNF increases release probability and the size of a rapidly recycling vesicle pool within rat hippocampal excitatory synapses. J Physiol 2006;574:787–803. [PubMed: 16709633]

Van den Veyver IB, Zoghbi HY. Mutations in the gene encoding methyl-CpG-binding protein 2 cause Rett syndrome. Brain Dev 2001;23:S147–51. [PubMed: 11738862]

Vanhala R, Korhonen L, Mikelsaar M, Lindholm D, Riikonen R. Neurotrophic factors in cerebrospinal fluid and serum of patients with Rett syndrome. J Child Neurol 1998;13:429–33. [PubMed: 9733288]

Vicario-Abejon C, Owens D, McKay R, Segal M. Role of neurotrophins in central synapse formation and stabilization. Nat Rev Neurosci 2002;3:965–74. [PubMed: 12461553]

Wade PA. Dynamic regulation of DNA methylation coupled transcriptional repression: BDNF regulation by MeCP2. Bioessays 2004;26:217–20. [PubMed: 14988922]

Wan M, Lee SS, Zhang X, Houwink-Manville I, Song HR, Amir RE, Budden S, Naidu S, Pereira JL, Lo IF, Zoghbi HY, Schanen NC, Francke U. Rett syndrome and beyond: recurrent spontaneous and familial MECP2 mutations at CpG hotspots. Am J Hum Genet 1999;65:1520–9. [PubMed: 10577905]

Wang H, Chan SA, Ogier M, Hellard D, Wang Q, Smith C, Katz DM. Dysregulation of brain-derived neurotrophic factor expression and neurosecretory function in Mecp2 null mice. J Neurosci 2006;26:10911–5. [PubMed: 17050729]

Ziv NE, Smith SJ. Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. Neuron 1996;17:91–102. [PubMed: 8755481]

Zoghbi HY. Postnatal neurodevelopmental disorders: meeting at the synapse? Science 2003;302:826–30. [PubMed: 14593168]

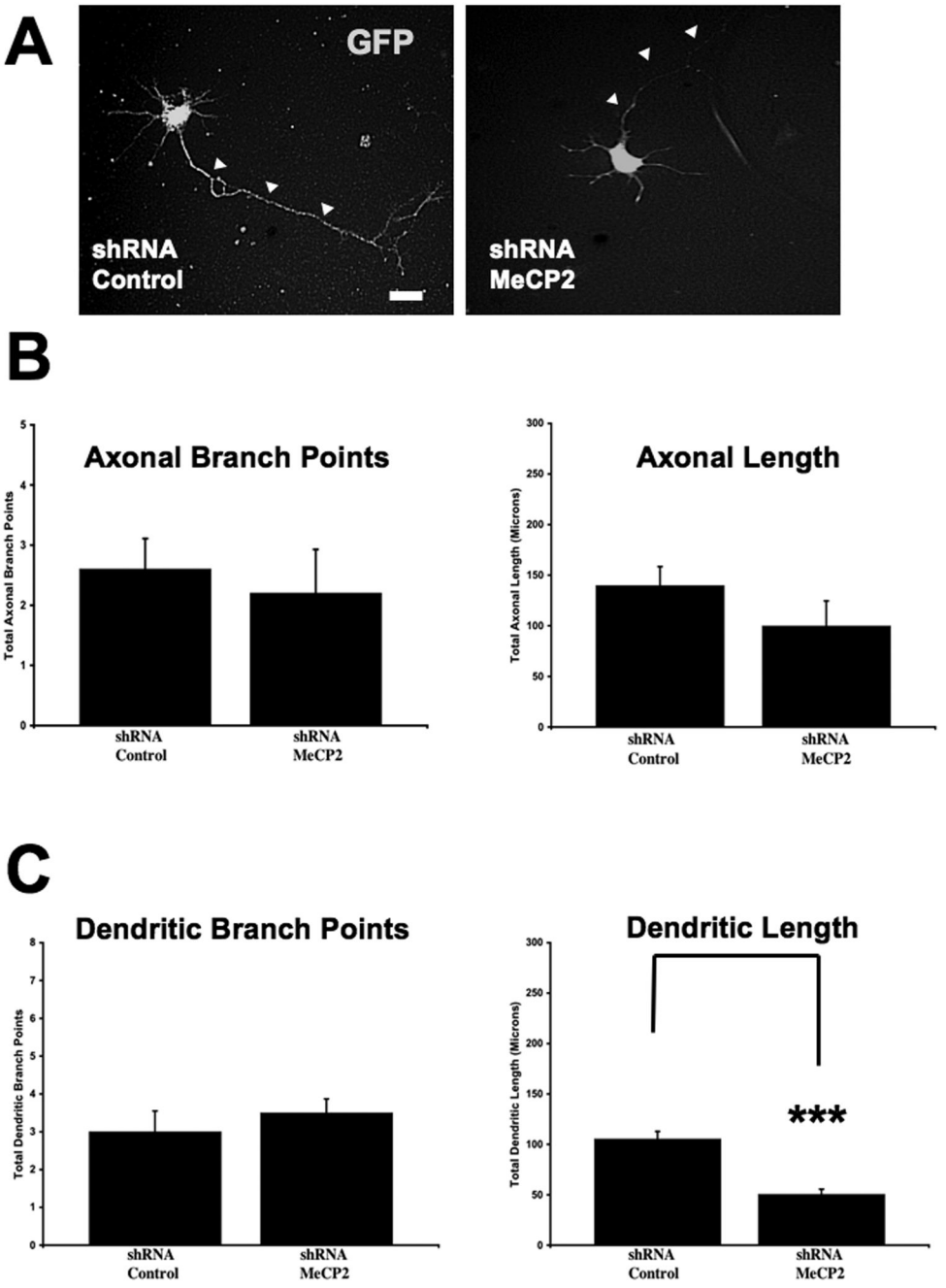

Figure 1. shRNA-mediated Knockdown of Endogenous *Mecp2* Reduced Dendritic Length in Pyramidal Hippocampal Neurons, without Affecting Axonal Morphology

A. Representative examples of 4 div hippocampal neurons transfected with a shRNA control sequence (left) or shRNA interfering sequence to knockdown endogenous *Mecp2* (right). Neurons were co-transfected with eGFP (green) to perform quantitative morphological analyses (scale bar = 10μm). **B.** Population data on axonal length and branch points. **C.** Population data on dendritic length and branch points. In this and all subsequent figures, * indicates $p<0.05$, ** indicates $p<0.01$, and *** indicates $p<0.001$, from unpaired Student's t test or one-way ANOVA (see text for details). Additionally, in this and all subsequent figures, arrowheads indicate the position of the axons.

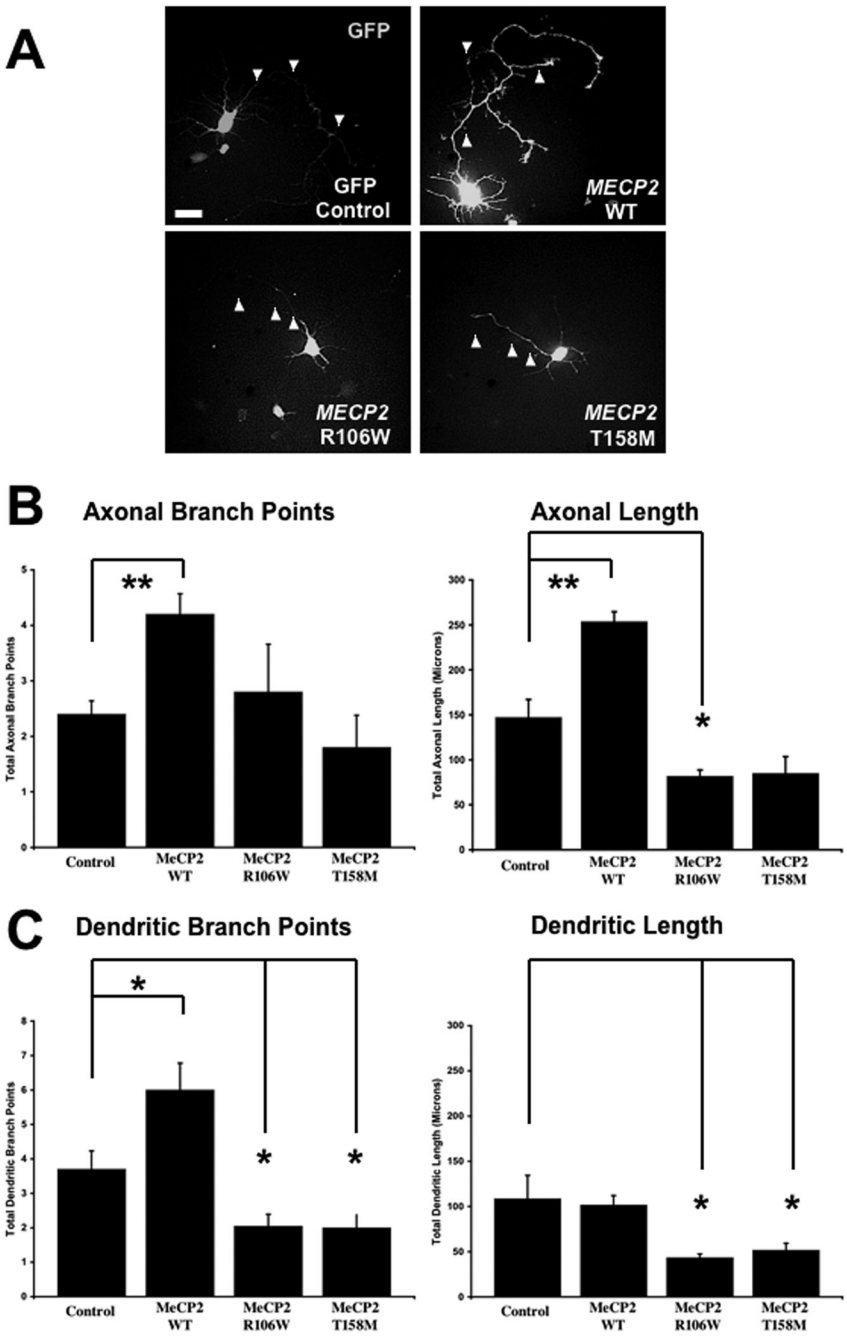

Figure 2. The Overexpression of Wildtype *MECP2* Increased Dendritic Branching and Axonal Growth, while the Overexpression of RTT-Associated *MECP2* Mutations Reduced Dendritic and Axonal Development

A. Representative examples of neurons transfected with either a control empty vector, a plasmid to overexpress wildtype human *MECP2*, or plasmids to overexpress to different missense *MECP2* mutations (R106W or T158M) commonly found in Rett syndrome patients. Neurons were co-transfected with eGFP to perform quantitative morphological analyses (scale bar = 10μm). **B.** Population data on axonal length and branch points. **C.** Population data on dendritic length and branch points.

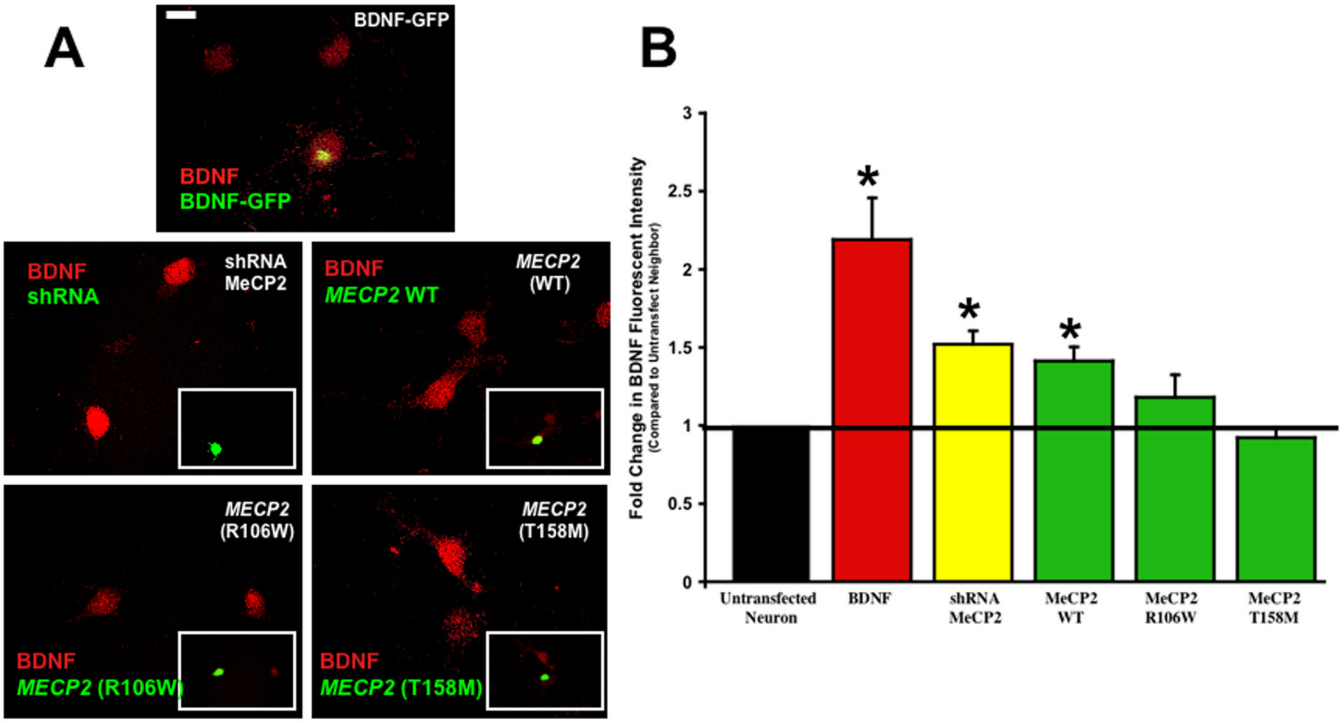

Figure 3. *Mecp2* Knockdown or Wildtype *MECP2* Overexpression Increased Intracellular BDNF Levels, while RTT-associated *MECP2* Mutants Did Not Affect BDNF Levels

A. Representative examples of BDNF immunostaining (red) in hippocampal neurons transfected with different expression plasmids (green). Neurons were transfected with expression plasmids to overexpress BDNF-GFP, wildtype *MECP2* or *MECP2* mutations tagged with GFP. Neurons were also transfected with an shRNA sequence to knockdown endogenous *Mecp2* and eGFP (scale bar = 10μm). Images fields always included two or more transfected neurons (i.e. GFP positive) and untransfected neurons (i.e. GFP negative) to perform quantitative comparisons of BDNF immunofluorescence staining. **B.** Population data on BDNF immunofluorescence intensity normalized to untransfected neurons within the same fields of view.

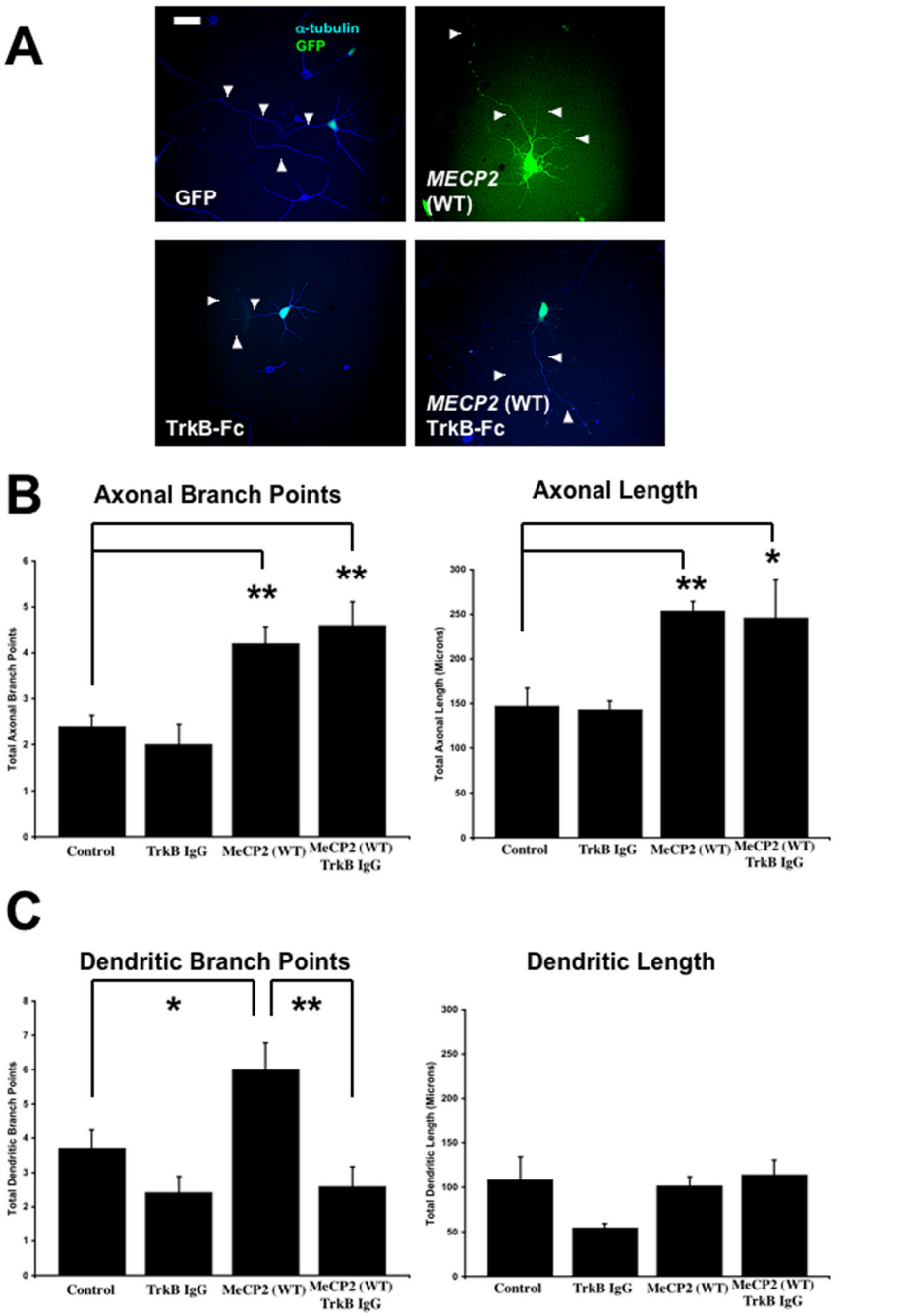

Figure 4. The BDNF Scavenger TrkB-Fc Prevented the Increase in Dendritic Branching Induced by Wildtype *MECP2* Overexpression, without Affecting Axonal Growth

A. Representative examples of hippocampal neurons co-transfected with a control GFP plasmid and a plasmid to overexpress wildtype *MECP2* (scale bar = 10μm). For quantitative morphological analysis, the neurons were co-transfected with eGFP (green) or stained with anti-α-tubulin antibodies (blue). **B.** Population data on axonal length and branch points. **C.** Population data on dendritic length and branch points.

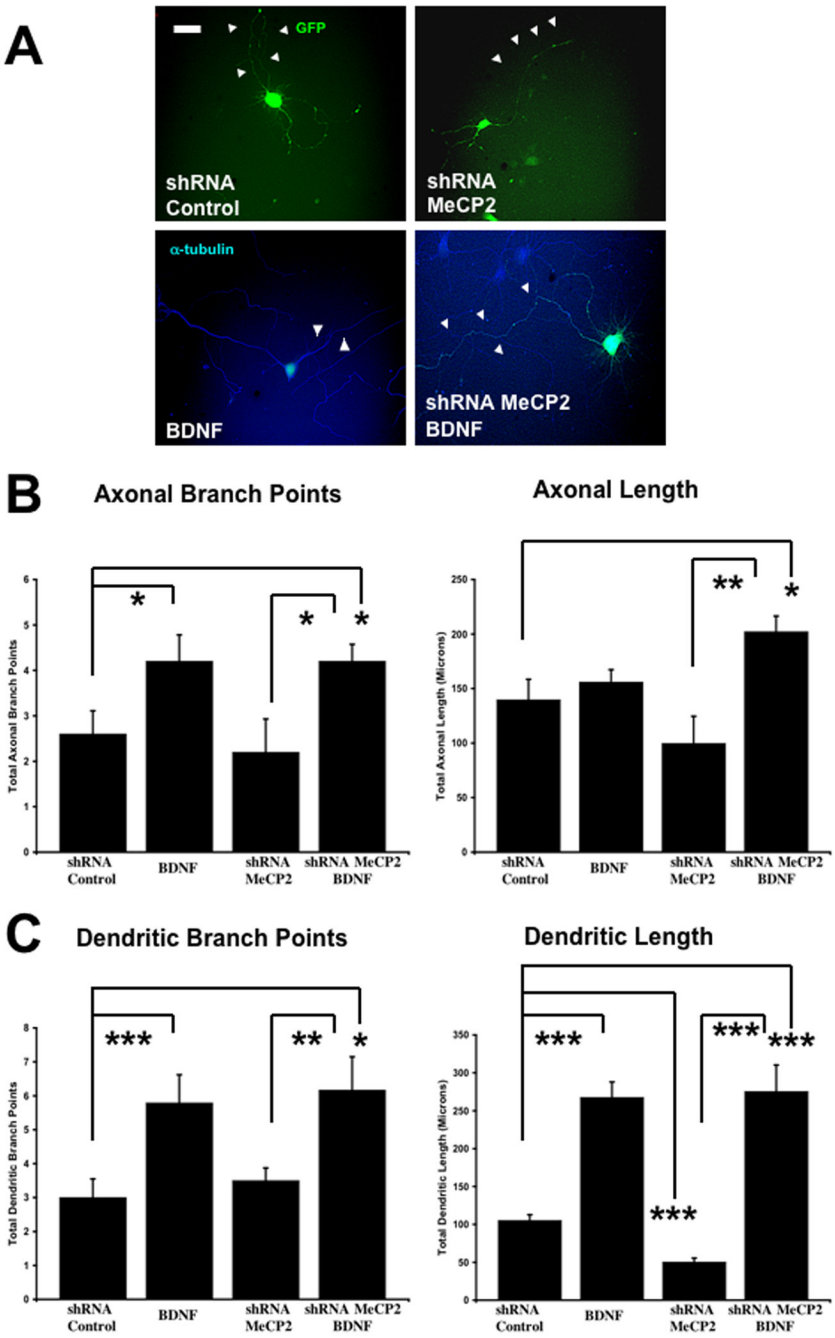

Figure 5. *Bdnf* Overexpression Rescued Dendritic Atrophy Caused by *Mecp2* Knockdown, Increasing Dendritic Length as much as in BDNF-Transfected Neurons

A. Representative examples of neurons transfected with an shRNA control plasmid, an shRNA plasmid to knockdown endogenous *Mecp2*, and a plasmid to overexpress *Bdnf* (scale bar = 10μm). For quantitative morphological analyses, neurons were co-transfected with eGFP (green) or stained with anti-α-tubulin antibodies (blue). **B.** Population data on axonal length and branch points. **C.** Population data on dendritic length and branch points.

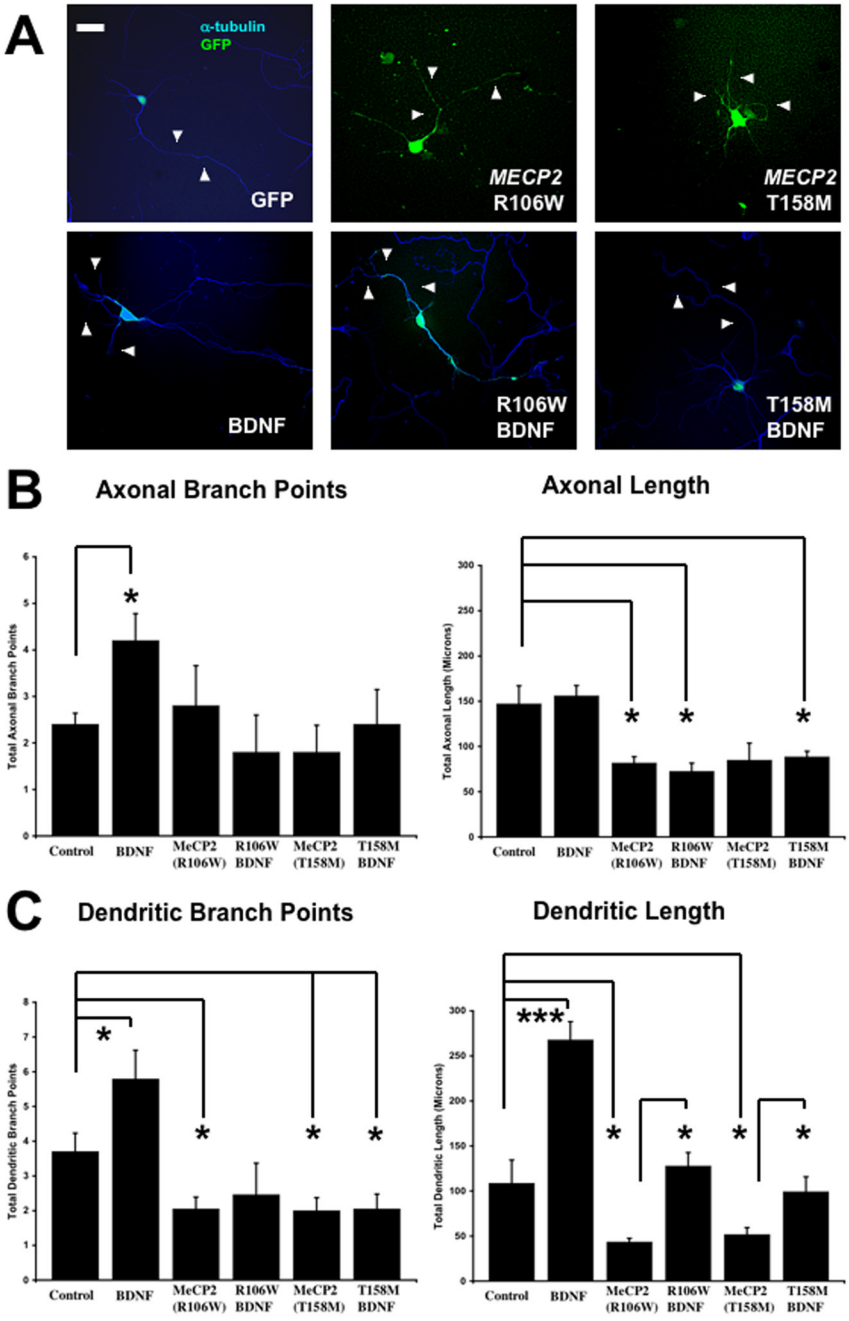

Figure 6. *Bdnf* Overexpression Partially Rescued Dendritic Atrophy in Neurons Expressing RTT-Associated *MECP2* Mutations, Increasing Dendrite Length Only to Control Levels

A. Representative examples of neurons transfected with a control GFP plasmid, plasmids to overexpress to different missense *MECP2* mutations (R106W or T158M) commonly found in Rett syndrome patients, and a plasmid to overexpress *Bdnf* (scale bar = 10μm). For quantitative morphological analyses, neurons were co-transfected with eGFP (green) or stained with anti-α-tubulin antibodies (blue). **B.** Population data on axonal length and branch points. **C.** Population data on dendritic length and branch points.