Hippocampal synaptic plasticity is impaired in the Mecp2-null mouse model of Rett syndrome

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Introduction

Rett syndrome is an X-linked neurodevelopmental disorder caused by mutations in the gene encoding the transcriptional repressor methyl-CpG-binding protein 2 (MeCP2). Here we demonstrate that the Mecp2-null mouse model of Rett syndrome shows an age-dependent impairment in hippocampal CA1 long-term potentiation induced by tetanic or theta-burst stimulation. Long-term depression induced by repetitive low-frequency stimulation is also absent in behaviorally symptomatic Mecp2-null mice. Immunoblot analyses from behaviorally symptomatic Mecp2-null mice reveal altered expression of N-methyl-D-aspartate receptor subunits NR2A and NR2B. Presynaptic function is also affected, as demonstrated by a significant reduction in paired-pulse facilitation. Interestingly, the properties of basal neurotransmission are normal in the Mecp2-null mice, consistent with our observations that the levels of expression of synaptic and cytoskeletal proteins, including glutamate receptor subunits GluR1 and GluR2, PSD95, synaptophysin-1, synaptobrevin-2, synaptotagmin-1, MAP2, βIII-tubulin and NF200, are not significantly altered. Together, these data provide the first evidence that the loss of MeCP2 expression is accompanied by age-dependent alterations in excitatory synaptic plasticity that are likely to contribute to the cognitive and functional deficits underlying Rett syndrome. © 2005 Elsevier Inc. All rights reserved.

Keywords: LTP; LTD; Mental retardation

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Available online on ScienceDirect (www.sciencedirect.com).

0969-9961/$ - see front matter © 2005 Elsevier Inc. All rights reserved. doi:10.1016/j.nbd.2005.07.005
that these studies were limited by the relatively small number of human samples, and that the disturbances in excitatory neurotransmitter levels may be related to some of the clinical manifestations of Rett syndrome, such as seizures and abnormal EEG activity. Indeed, it remains entirely an open question whether the morphological abnormalities observed in postmortem human tissue are the cause or consequence of neurodevelopmental defects in Rett syndrome.

Mutations in the X-linked gene encoding the transcriptional repressor methyl-CpG-binding protein 2 (MeCP2) are responsible for a majority (up to 90%) of Rett syndrome cases (Amir et al., 1999). MeCP2 binds specifically to CpG-methylated DNA templates (Free et al., 2001) and is thought to inhibit gene transcription by recruiting co-repressor and histone deacetylase complexes and altering the organizational structure of genomic DNA (Nan et al., 1998). Genetically engineered mice that lack MeCP2, or express a truncated form of MeCP2 that very closely mimic a common mutation in humans, recapitulate many key features of the clinical Rett syndrome phenotype. Targeted knockouts mimic a common mutation in humans, recapitulate many key features of the clinical Rett syndrome phenotype. Targeted knockouts of Mecp2, or express a truncated form of Mecp2 that very closely mimic a common mutation in humans, recapitulate many key features of the clinical Rett syndrome phenotype. Targeted Mecp2 gene deletion only in neurons leads to a full neurological phenotype, suggesting that the behavioral phenotype of Rett syndrome could be explained solely by MeCP2 dysfunction in neurons (Chen et al., 2001).

Since the very recent availability of the Mecp2-null mice, studies focused on molecular, anatomical or behavioral studies of the knockout model have provided key insights to the consequences of the loss of Mecp2 expression, yet none have indicated what functional aspects of neurotransmission and synaptic plasticity are affected by such a loss. Indeed, given the widespread speculation or even the assumption that the molecular, biochemical or morphological changes would lead to alterations in synaptic transmission and plasticity, it is a critical missing piece in understanding the puzzle of Rett syndrome to elucidate what functional changes in fact result from the loss of Mecp2. In fact, neurons have been shown to be exquisitely capable of homeostatically compensating or regulating the efficacy of neuronal communication in the face of drastic changes in morphology or activity (for review, see Turrigiano and Nelson, 2004), and so, in the absence of electrophysiological experiments, it would not be straightforward to predict what changes in synaptic properties would result in the Mecp2 knockout brain.

Because excitatory neurotransmission and plasticity in the CA1 region of the rodent hippocampus have been extremely well studied due to historical interests in its potentially central role in learning and memory, it provides an invaluable general framework for examining and understanding the role of MeCP2 in glutamatergic synapses in virtually all central nervous system networks. Disruptions in activity-dependent functions of glutamatergic neurotransmission are likely to play a central role in the pathophysiology of epilepsy, movement disorders, brainstem mechanisms that control respiration, as well as in cognitive deficits—all present to varying degrees in patients with Rett syndrome. In the present study, we sought to determine whether the loss of MeCP2 gives rise to impairment of synaptic function and plasticity in the CA1 region of the hippocampus. We found age-dependent changes in the maintenance of both long-term potentiation and depression (LTP and LTD) consistent with our findings of altered expression of NMDA-type glutamate receptors in the Mecp2-null mouse model of Rett syndrome. In contrast to these changes, we found basal synaptic properties and markers to be normal. Thus, disruption of activity-dependent synaptic plasticity, but surprisingly not basal neurotransmission, may contribute to abnormal functions of neural circuits throughout the brain subserving the myriad of autonomic and motor functions that are so severely affected in Rett syndrome. Moreover, since the neuroanatomical deficits observed in Rett syndrome are shared by a host of other mental retardation syndromes with genetic bases, our observations on the functional consequences of morphological reorganization and neurodevelopmental arrest in Rett syndrome may provide useful insight or comparison for syndromes such as Coffin-Lowry, Rubenstein-Taybi, Fragile X and Down’s syndromes.

Materials and methods

Mecp2-null mice

We used two available Mecp2-null mouse models generated by the Cre LoxP recombination system to delete only exon 3 of Mecp2 (Chen et al., 2001) (obtained from the Mutant Mouse Resource Center at University of California, Davis) or to delete exons 3 and 4 of Mecp2 (Guy et al., 2001) (obtained from Jackson Laboratories, Bar Harbor, ME). Both lines were back-crossed for at least 8 generations to a C57Bl/6 background to eliminate potential strain-dependent differences. Statistical comparisons of the electrophysiological findings revealed no significant differences observed between the two types of mice; thus, all electrophysiological data reported here represent both genetic strains of mice. Wild-type mice were obtained from the same litters. Mice were killed by cervical dislocation and hippocampal slices prepared as outlined below. All experimental protocols were approved by Yale University Institutional Animal Care or University Health Network Animal Use Committees in accordance with guidelines established by the National Institutes of Health and the Canadian Council on Animal Care. Mecp2-null animals were identified by PCR from tail DNA, paired with wild-type littermates and examined for hindlimb reflex impairment biweekly.

Electrophysiology

Hippocampal slices were prepared from presymptomatic (3–5 week old) and symptomatic (6–10 week old) male Mecp2-null mice and age-matched wild-type littermate controls. Animals were anesthetized with halothane (Sigma, St. Louis, MO, USA) and killed by decapitation. The brain was quickly removed and submerged in ice-cold artificial cerebrospinal fluid (ACSF) solution (125 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, 1.25 mM NaH2PO4, 2 mM CaCl2, 25 mM glucose, 26 mM NaHCO3). The hippocampi were dissected and 350 μm transverse slices were cut from the middle of the hippocampal formation with a vibratome (Leica VT1000S). Slices were allowed to recover for a minimum of 1 h at room temperature in a submerged chamber containing ACSF bubbled with 95% O2 and 5% CO2. Slices were transferred to a recording chamber, held submerged between two nylon nets, and constantly perfused at a rate of 2 ml/min with oxygenated ACSF containing 100 μM picrotoxin (Sigma, St. Louis, MO, USA) at room temperature. The recording chamber was mounted on an upright fixed stage microscope (Olympus BX50WI). A cut was made between CA1 and CA3 to prevent the propagation of epileptiform activity. Schaffer collaterals were stimulated ortho-
dromically using bipolar tungsten electrodes (FHC, Bowdoinham, ME). Test stimuli (50 µs pulses) were applied at low frequency (0.05 Hz) at a stimulus intensity that elicited a fEPSP amplitude that was 33% of maximum. All LTP induction stimulations were at the same intensity used to establish baseline responses. Synaptic responses were recorded extracellularly in the stratum radiatum of CA1 (50–100 µm from the stratum pyramidale) with glass microelectrodes (0.5–1 MΩ) filled with 2 M NaCl. Field potentials were amplified using a DP-301 differential amplifier (Warner Instrument Corp., Hamden, CT, USA), digitized at 10 kHz and analyzed with pClamp 9.0 software (Axon Instruments, Union City, CA, USA). Field excitatory postsynaptic potential (fEPSP) magnitude was measured using the initial fEPSP slope (between 10% and 90% points on the rising slope). Data points were normalized to the mean baseline value, and expressed as mean ± SEM. Percent LTD or LTP were calculated as the average of normalized fEPSP slopes at 50 min post tetanus. Data were analyzed using repeated measures ANOVA. In all cases, \( P < 0.05 \) was considered statistically significant.

Antibodies

All antibodies used in this study were obtained from commercial sources. Monoclonal anti-glial fibrillary acidic protein (anti-GFAP #G3839), monoclonal anti-β-III tubulin (#T8660) and monoclonal anti-neurofilament 200 (anti-NF200 #N0142) were obtained from Sigma-Aldrich (St. Louis, MO); monoclonal anti-microtubule associated protein 2 (anti-MAP2 #1284959) was obtained from Roche (Basel, Switzerland); monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH MAB374), monoclonal anti-glutamate receptor subunit 1 (anti-GluR1, MAB1504) and monoclonal anti-glutamate receptor subunit 2 (anti-GluR2, MAB397), were obtained from Chemicon (Long Beach, CA); polyonal NMDA receptor subunit 2A (anti-NR2A #6870-1059) and polyonal NMDA receptor subunit 2B (anti-NR2B #6870-1109) were obtained from BioGenes (Kingston NH); anti-NMDA receptor subunit 1 (anti-NR1 #454570) was obtained from CalBiochem (La Jolla, CA); monoclonal anti-synaptobrevin-protein 2 (#I04 211, clonal lane 10.1), anti-synaptophysin-1 (#I01 001) and anti-synaptotagmin-1 (#I05 001) were obtained from Synaptic Systems Inc., Gottingen, Germany; anti-postsynaptic density 95 kDa protein (anti-PSD95 #610495) and anti-neuronal nitric oxide synthase (anti-nNOS #610308) were obtained from BD Bioscience (Palo Alto, CA).

Gel electrophoresis and immunoblotting

Mice were sacrificed by decapitation, the brain was removed from the skull, chilled on an ice-cold dissection disc and paired hippocampi were rapidly isolated and frozen on dry ice. Hippocampal tissue was then homogenized in immunoblot lysis buffer (150 mM NaCl, 1mM EDTA, 20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 1% (v/v) Nonidet P-40), supplemented with the protease inhibitors aprotinin (100 µg/ml), leupeptin (100 µg/ml) and phenylmethylsulfonyl fluoride (2 mM). To prepare lysates, DNA was sheared by sequential passage through 18, 20 and 25 gauge needles, and the homogenate spun at 12,000 × g for 5 min to remove insoluble material. Aliquots of the soluble fraction were frozen and stored until use in liquid nitrogen. Protein concentrations were determined by the Folin method (Sigma-Aldrich). Equivalent amounts of protein from each sample were heated in SDS sample buffer, and resolved by electrophoresis on a 5% acrylamide stacking gel and 10% resolving acrylamide gel in tris–glycine Laemelli running buffer. The proteins were transferred electrophoretically to nitrocellulose in standard transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol), and prehybridized overnight at 4°C in blocking solution (Tris-buffered saline containing 0.05% (v/v) Tween-20 (TBST) containing 5% (w/v) nonfat dried milk). Blots were incubated with primary antibody diluted in blocking solution overnight at 4°C. The following antibodies and dilutions were used: Anti-NR1 (1/2,000), anti-NR2A (1/1000), anti-NR2B (1/1000), anti-GluR1 (1/4,000), anti-synaptotagmin-1 (1/4,000), anti-synaptobrevin-II (1/4000), anti-synaptophysin-1 (1/1000), anti-NF200 (1/750), anti-β-III tubulin (1/800), anti-MAP2 (1/500), anti-PSD95 (1/500), anti-nNOS (1/200). After washing in TBST (3X-20 min/wash), goat anti-mouse HRP secondary antibody (1/5000 dilution; Jackson Immunoresearch, West Grove, PA) was applied, and incubated for 90 min at room temperature. After extensive washing in TBST, specific immunoreactivity was visualized using Lumiglo enhanced chemiluminescence (New England Biolabs, Mississauga, Ontario) with Biomax film (Eastman Kodak, Rochester, NY). Prior to semi-quantitative comparison, serial dilutions of protein were tested with the stated dilution of primary antibody to confirm if each condition generated a linear optical density relation. Conditions that met the linear requirements were selected for use in the final assays. Molecular masses of detected products were estimated by their migratory proximity to prestained protein markers (MBI Fermentes, Burlington, ON).

Immunoblot analysis and statistics

For each assay, \( n = 4–5 \) mutant and \( n = 4–5 \) wild-type tissues were compared within a common blot. Following their initial use, each blot was stripped and re-probed with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to provide a control for load variations between samples. The autoradiographic film pixel densities (relative optical densities, ROIs) within a common area size were measured for each of the products from an individual immunoblot, and normalized to the ROD obtained for its corresponding GAPDH. The resulting mutant and wild-type values were then compared using a nonpaired, two-tailed Student’s \( t \) test, and significant differences ascribed to \( P \) values <0.05.

Results

Altered hippocampal NMDA receptor expression in symptomatic MeCP2-null mice

Observations of significant alterations in the expression of NMDA- and AMPA-type glutamate receptors from postmortem brain samples from Rett syndrome patients (Blue et al., 1999a,b), as well as elevations in CSF glutamate levels in Rett patients compared to autistic controls (Hamberger et al., 1992; Lappalainen and Riikonen, 1996) support the prevalent hypothesis that dysfunction of excitatory neurotransmission plays a central role in the pathophysiology of Rett syndrome. However, the presence of co-morbidities and the small sample sizes of available human subjects hamper the reliability of such studies. By taking advantage of the MeCP2-null mouse model of Rett syndrome that mirrors the pathophysiology of the clinical condition, we are able to repeat
early biochemical studies, and for the first time correlate changes in neurotransmitter receptor levels with functional alterations in synaptic transmission and plasticity. Moreover, it is possible to capitalize on the delayed onset of behavioral symptoms in the Mecp2-null mice to specifically test the hypothesis that abnormalities in synaptic transmission and/or plasticity underlie the cognitive, motor and autonomic dysfunctions of Rett syndrome.

We first used immunoblot analysis to examine whether the prevalence of AMPA receptor subunits GluR1 or GluR2 differed significantly in hippocampi of age-matched Mecp2-null and wild-type populations of mice. As shown in Fig. 1A, unlike the earlier human studies, no significant differences in the expression of either AMPA receptor subunit were detected. We then examined whether the loss of Mecp2 would affect the prevalence of NMDA receptor subunits. In contrast to the preservation seen with GluR1 and GluR2, significant differences between symptomatic Mecp2-null and wild-type NR2A and NR2B subunit prevalence were detected. NR2A levels were significantly diminished, while NR2B levels were significantly increased when compared to age-matched control levels (see Fig. 1B). Although the levels of NR2A and NR2B were affected by the loss of Mecp2, the immunoreactive prevalence of the NR1 subunit did not differ between groups in either presymptomatic or symptomatic mice compared to age-matched controls (see Fig. 1C). Functional studies have demonstrated a gradual developmental replacement of the contribution of the NR2B subunit with NR2A subunits of NMDARs to the excitatory postsynaptic current (Cull-Candy et al., 2001). This shift in the NR2 subunit composition of the NMDARs is critical in determining the biophysical and pharmacological properties of the receptor, and is implicated in the ability of neural circuits to undergo experience- or activity-dependent synaptic plasticity (van Zundert et al., 2004).

Long-term potentiation (LTP) is impaired in symptomatic Mecp2-null mice

Long-term potentiation (LTP) in the CA1 region of the hippocampus is an activity-dependent long lasting increase in the efficacy of neurotransmission that requires NMDA receptor activation, and is thought to represent a very likely cellular mechanism underlying some forms of learning and memory (Lynch, 2004). The observation that the NR2 receptor subunit expression is altered in symptomatic Mecp2-null mice, together with the prevalent hypothesis that cognitive deficits associated with mental retardation could result from abnormalities in glutamatergic synaptic plasticity (Johnston, 2004) prompted us to examine LTP in hippocampal area CA1 of Mecp2-null and wild-type mice.

We recorded at least 15 min of stable baseline fEPSPs (single stimulus delivered once every 20 seconds) recorded in the stratum radiatum of area CA1 of Mecp2-null and littermate, age-matched wild-type control hippocampal slices. Long-term potentiation (LTP) was induced by two 100-Hz trains of 100 stimuli separated by 20 s. Immediately following the tetanus, testing of synaptic strength was resumed by returning to the baseline test frequency for at least 90 min. The average amount of potentiation relative to the average baseline was determined for each slice. As shown in

Fig. 1. NMDA receptor prevalence is significantly altered in symptomatic Mecp2-null hippocampus. Normalized immunoblot protein level comparisons of symptomatic Mecp2-null hippocampal mutant (grey column) and control age-matched wild-type control tissue (white column). (A) No significant changes were observed in the expression of members of the AMPA class of glutamate receptors (GluR1, \( P = 0.8019 \); GluR2, \( P = 0.1045 \); Student’s unpaired t test, performed for all comparisons). (B) NR2B expression levels are significantly increased in hippocampal tissue of symptomatic Mecp2-null mice, and NR2A expression levels are attenuated when compared to age-matched wild-type controls (* \( P < 0.05 \)). (C) NR1 levels remain unchanged (C, \( P = 0.1759 \)). All blots were re-probed with GAPDH to normalize for load, and the cumulative data from \( n = 4 \) animal sets are presented in histogram form as normalized mean ± standard error. Representative wild-type control (wt) and Mecp2-null (null) blots are displayed to the right of each histogram. The mean age of the symptomatic subjects was 56 days, representing an average of 10 days after onset of symptoms in the test group.
Fig. 2B, we observed a dramatic reduction in the magnitude of LTP expressed in Mecp2-null slices prepared from mice >6 weeks of age, compared to age-matched wild-type controls. This difference in the magnitude of LTP maintenance was highly significant (repeated measure ANOVA at 40–60 min, \( P = 0.043 \); wild-type control animals exhibited potentiation of 161 ± 16% of baseline, 8 slices/3 mice; Mecp2-null animals exhibited potentiation of only 124 ± 7.4% of baseline, 9 slices/3 mice). The magnitude of potentiation in the first minute following the tetanic stimulation was significantly attenuated in the Mecp2-null slices (repeated measure ANOVA at 0–1 minute, \( P = 0.006 \); at 1 min posttetanus, wild-type control animals exhibited potentiation of 299 ± 19% of baseline; Mecp2-null animals exhibited potentiation of only 219 ± 18% of baseline).

The deficit in LTP expression in the Mecp2-null slices was not attributable to inadequate responses to the tetanic stimulation, since the measurements of the area of the total response to the second tetanic train did not differ between Mecp2-null and wild-type slices (data not shown; total charge transfer Mecp2-null slices 39.9 ± 7.5 mV * ms; wild-type slices 36.9 ± 8.7 mV * ms; Student’s \( t \) test, two-tailed \( P = 0.79 \)). Moreover, comparisons of the input resistance and action potential amplitudes measured by whole-cell patch clamp recordings of neurons from Mecp2-null (\( n = 4 \) mice) and littermate wild-type controls (\( n = 5 \) mice) were not significantly different, indicating normal viability of the neurons in Mecp2-null slices (data not shown).

To determine whether abnormalities in synaptic plasticity could underlie the onset of cognitive, motor and autonomic dysfunctions, we examined LTP in hippocampal slices prepared from presymptomatic 3 to 5 weeks old Mecp2-null mice. In contrast to the symptomatic mice, these younger Mecp2-null mice did not differ from their age-matched wild-type littermate controls (see Fig. 2A; repeated measure ANOVA at 40–60 min postinduction, \( P = 0.46 \); fEPSP slopes of wild-type control exhibited potentiation of 147 ± 7.4% of baseline; Mecp2-null mice, \( n = 3 \) mice) were not significantly different, indicating normal viability of the neurons in Mecp2-null slices (data not shown).

In the lower panels of each figure, the fEPSP slope is presented as a function of time (minutes). The data are expressed as the mean ± SEM. The statistical significance of the differences between the groups was evaluated using repeated measure ANOVA. The differences were considered significant at \( P < 0.05 \) for all points later than 60 min postinduction. Differences in posttetanic potentiation were not significant (\( P > 0.5 \)). Scale bars, 0.2 mV, 5 ms.
13% of baseline, 8 slices/6 mice; presymptomatic Mecp2-null animals exhibited potentiation of 135 ± 7.9% of baseline, 9 slices/3 mice).

The preparation of slices and the examination of LTP in Mecp2-null and wild-type control animals were performed with the experimenter blind to the genotype of the animals. Despite the observation that on average, LTP was impaired in symptomatic Mecp2-null compared to age-matched wild-type animals, we noted that in each of the groups there was some proportion of slices that failed to exhibit greater than 20% potentiation at 50–60 min posttetanus (no LTP) while others expressed potentiation greater than 20% at 50–60 min posttetanus (LTP). Basal neurotransmission in those slices that failed to express LTP was indistinguishable from those with LTP (data not shown). The average LTP reported above includes all slices tested; thus, we used a Chi-square test for a two-way contingency table analysis to evaluate whether there was a statistically significant difference in the proportions of LTP versus no LTP depending on genotype (Mecp2-null versus wild-type). In presymptomatic animals, there was no difference between Mecp2-null (4 out of 17 slices had no LTP) and wild-type (5 out of 14 slices had no LTP) animals; Pearson χ² (1, n = 31) = 0.553, P = 0.457. However, in symptomatic mice, the proportion of no LTP to LTP and genotype was found to be significantly related, Pearson χ² (1, n = 38) = 5.983, P = 0.014; 11 out of 20 Mecp2-null failed to exhibit >20% potentiation at 50–60 min posttetanus, while only 3 out of 18 wild-type slices had no LTP. Since some slices, albeit a significantly smaller proportion, of symptomatic Mecp2-null animals did express some LTP, it is likely that the loss of Mecp2 does not disable the cellular mechanisms underlying LTP, but rather affects the threshold for induction or the expression mechanism(s) of LTP. To test for this possibility, we then examined LTP induction by theta-burst stimulation.

**Theta-burst stimulation fails to induce LTP in symptomatic Mecp2-null mice**

LTP, while typically induced by brief high-frequency stimulation, can also be induced using a more physiological pattern of activity with short trains of theta frequency (5 Hz) stimulation. The signaling mechanisms involved in the induction of LTP by theta-burst stimulation differ in several ways from conventional tetanus-induced LTP, including the activation of the mitogen-activated protein kinase (MAPK) pathway (Watabe et al., 2000) and an involvement of endogenous BDNF (Kang et al., 1997; Chen et al., 1999; Kossel et al., 2001). In light of the very recent demonstrations that the promoter for BDNF is a target of normal Mecp2 regulation (Chen et al., 2003; Martinovich et al., 2003), we sought to determine the effects of Mecp2 loss on theta-burst-stimulation-induced LTP. Following at least 15 min of baseline test stimulation (1 stimulus every 20 s), the Schaffer collateral–commissural projections of acute hippocampal slices were stimulated by 3 trains of 10 theta-bursts (4 pulses at 100 Hz) at 200 ms intervals, and those trains were separated by 60 s. As with the tetanus-induced LTP, there was no significant difference in LTP exhibited by presymptomatic Mecp2-null mice compared to their age-matched wild-type littermate controls (see Fig. 2C; repeated measure ANOVA at 60–80 min postinduction, P = 0.31; fEPSP slopes of wild-type control were 148 ± 8.0% of baseline, 8 slices/6 mice; Mecp2-null were 133 ± 9.3% of baseline, 8 slices/4 mice). The magnitude of potentiation in the first minute following the tetanic stimulation was not significantly different between the wild-type and Mecp2-null slices (repeated measure ANOVA at 0–1 min, P = 0.167; at 1 min posttetanus, wild-type control animals exhibited potentiation of 167 ± 11% of baseline; Mecp2-null animals exhibited potentiation of 161 ± 7.6% of baseline). Strikingly, in slices prepared from symptomatic animals, LTP induced by theta stimulation was not maintained, decaying to baseline values within an hour of induction. At 60–80 min postinduction, the difference between symptomatic Mecp2-null and age-matched wild-type control slices in the magnitude of LTP was statistically significant (see Fig. 2D; repeated measure ANOVA at 60–80 min postinduction, P = 0.04; fEPSP slopes of wild-type control were 137 ± 8.1% of baseline, 10 slices/5 mice; Mecp2-null was 111 ± 9.0% of baseline, 11 slices/8 mice).

**Long-term depression (LTD) is absent in symptomatic Mecp2-null mice**

LTD induced by low frequency stimulation (LFS) is a form of NMDA-receptor-dependent long-lasting synaptic plasticity believed to be of particular importance during early development. Indeed, the magnitude of LTD expressed in rodent hippocampus is age-dependent, declining with age. Given the NR2 subunit expression profile in the symptomatic mice consistent with a mechanism of developmental arrest, in combination with the delayed onset of behavioral symptoms, we hypothesized that the form of synaptic plasticity prevalent early in development, namely LTD, would be preserved in the presymptomatic Mecp2-null mice. Thus, we examined LTD in the presymptomatic (3–5 week old) Mecp2-null and wild-type mice. We found that LTD expression in Mecp2-null slices was indistinguishable from age-matched wild-type controls (see Fig. 3A; repeated measure ANOVA at 60–75 min postinduction, P = 0.25; fEPSP slopes of wild-type control were 89 ± 4.8% of baseline, 10 slices/4 mice; Mecp2-null was 81 ± 4.8% of baseline, 11 slices/3 mice).

While LTD in rodents has been shown to decline with age, the age at which Mecp2-null mice become symptomatic (>6 weeks of age) is relatively early. Indeed, we found adequate LTD expression in wild-type hippocampal slices prepared from 6–9 weeks old mice (see Fig. 3B; fEPSP slopes at 60–70 min postinduction 78 ± 1.2% of baseline, 5 slices/3 mice). Strikingly, there was no LTD expression in slices from the symptomatic Mecp2-null mice (fEPSP at 60–70 min postinduction 92 ± 1.2% of baseline, 5 slices/3 mice). Thus, both NMDA-receptor-dependent LTD and LTD are absent in the symptomatic Mecp2-null mice, while preserved prior to the onset of behavioral phenotype.

**The efficacy of basal Schaffer collateral–CA1 neurotransmission is unaltered in Mecp2-null mice**

While Mecp2 is expressed throughout the developing embryonic brain, its expression increases with synapse formation and cellular differentiation (Shahbazian et al., 2002; Jung et al., 2003; Mullaney et al., 2004). Thus, as indicated by our findings on LTP and LTD, the loss of Mecp2 function may not be relevant early in brain development, but rather becomes evident concurrent with the establishment and/or maintenance of neuronal function. This is consistent with our observation that, as previously reported (Chen et al., 2001; Guy et al., 2001), the onset of behavioral abnormalities of Mecp2-null mice (hypoactivity, hind-limb clasping, tremor) was
delayed until approximately 6 weeks of age. To examine the general properties of basal neurotransmission in presymptomatic and symptomatic MeCP2-null mice compared to wild-type, littermate, age-matched controls, we performed extracellular recordings of Schaffer collateral–CA1 synaptic responses to single electrical stimuli. Stimulating and recording electrodes were placed in the stratum radiatum in area CA1 of a hippocampal slice, and fEPSPs were evoked by stimuli of incrementally increasing strengths. Stimulus–response curves obtained from MeCP2-null slices were not significantly different from wild-type over a wide range of stimulus intensities (for presymptomatic (n = 9 slices/5 mice) and symptomatic (n = 10 slices/5 mice) MeCP2-null and age-matched 3–5 weeks old wild-type (n = 11 slices/6 mice) and >6 weeks old wild-type (n = 10 slices/4 mice) animals; repeated measures ANOVA, P = 0.68; Fig. 4A). Furthermore, Wilcoxon signed ranks test (non-parametric test) showed that the fEPSP slope corresponding to a given presynaptic fiber volley did not differ between MeCP2-null and wild-type slices (P = 0.16 for presymptomatic MeCP2-null and P = 0.64 for age-matched wild-type; P = 0.58 for symptomatic MeCP2-null and P = 0.65 for age-matched wild-type; Fig. 4B). Thus, the baseline properties of synaptic function in response to single stimuli appear to be unaltered by deletion of MeCP2.

**Paired-pulse facilitation and posttetanic potentiation**

To examine whether presynaptic function is affected by MeCP2 loss, we examined paired-pulse facilitation (PPF) in MeCP2-null and age-matched, wild-type, littermate animals. PPF is examined by measuring fEPSP responses to two stimuli delivered at short interstimulus intervals to the Schaffer collateral inputs. PPF is a transient form of plasticity commonly used as a measure of presynaptic function, where the response to the second stimulus is enhanced due to residual calcium in the presynaptic terminal following the first stimulus. Shown in Fig. 5A, we examined PPF in response to five different interstimulus intervals ranging from 50 to 250 ms. In presymptomatic animals, MeCP2-null and wild-type animals were indistinguishable in the amount of PPF observed at all intervals (repeated measures ANOVA, P = 0.154; Fig. 5A). In contrast, in symptomatic animals, MeCP2-null slices exhibited significantly less PPF at 50 ms and 100 ms interstimulus intervals than the age-matched wild-type slices (repeated measures ANOVA, P = 0.015; post-hoc Student’s t test of 50 and 100 ms interstimulus intervals, P = 0.003 and P = 0.009, respectively; Fig. 5B), supporting the hypothesis that the loss of MeCP2 function may become evident only later in development as neuronal function and mechanisms of synaptic plasticity mature.

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**Fig. 3.** Long-term depression is absent in MeCP2-null hippocampal slices of symptomatic mice. (A) No significant difference was observed between LTD induced by 900 stimuli delivered at 1 Hz in presymptomatic MeCP2-null (open circles) and wild-type control (closed circles) slices (repeated measure ANOVA at 40–60 min postinduction, P = 0.25; fEPSP slopes of wild-type control were 89 ± 4.8% of baseline, 10 slices; MeCP2-null were 81 ± 4.8% of baseline, 11 slices). Data points represent the normalized mean response ± SEM. Insets show representative traces of field recordings before and 60 min after LTD induction stimulation. Lower panels of each figure show the stability of the amplitudes of presynaptic fiber volleys (PSFV) before and after LTP induction. (B) LTD is expressed in wild-type hippocampal slices prepared from 6–9 week old mice (fEPSP slopes at 60–70 min postinduction 78 ± 1.2% of baseline, 5 slices), yet strikingly, there was no LTD expression in slices from the symptomatic MeCP2-null mice (repeated measure ANOVA at 60–70 min postinduction, P = 0.015; fEPSP at 60–70 min postinduction 92 ± 1.2% of baseline, 5 slices). Scale bars, 0.2 mV, 5 ms.

**Fig. 4.** The strength of basal neurotransmission does not differ between MeCP2-null and wild-type mice. (A) Input–output curves of field excitatory postsynaptic potential (fEPSP) slope (mV/ms) versus stimulus intensity (mA) at the Schaffer collateral–CA1 synapses in hippocampal slices from presymptomatic (triangles) and symptomatic (circles), MeCP2-null (open symbols) and wild-type (closed symbols) mice. Data are presented as mean response ± SEM. (B) No differences are apparent in plots of fEPSP slope (mV/ms) versus presynaptic fiber volley amplitude (mV) of slices from presymptomatic (triangles) and symptomatic (circles), MeCP2-null (open symbols) and wild-type (closed symbols) mice. Data points represent the normalized mean response ± SEM.
As a second measure of presynaptic plasticity, we examined the posttetanic potentiation (PTP) of fEPSPs in Mecp2-null and wild-type slices in the presence of the NMDA receptor antagonist d,l-2-amino-5-phosphonovaleric acid (APV; 50 μM) to prevent induction of LTP. PTP was elicited by a high frequency tetanic stimulus train (1 s at 100 Hz), resulting in an elevation of presynaptic calcium and short-term enhancement of transmission due to mobilization of the reserve pool of synaptic vesicles (Zucker, 1989). We observed no significant differences in the peak PTP (see Fig. 5C; repeated measure ANOVA, P = 0.83; fEPSP slopes of wild-type control were 217 ± 1.5% of baseline, 9 slices/3 mice; Mecp2-null was 221 ± 2.2% of baseline, 6 slices/3 mice), or in the time course of decay to baseline levels in symptomatic animals, indicating that the ability of Schaffer collateral–CA1 synapses to express PTP is not affected in the Mecp2-null animals.

**Normal expression levels of hippocampal pre- and postsynaptic and cytoskeletal protein markers in symptomatic Mecp2-null mice**

The preservation of basal hippocampal neurotransmission in the symptomatic Mecp2-null mice suggests that the general mechanisms involved in synaptic morphogenesis and function must be preserved in the mutant animals. This further suggests that the representation of synaptic proteins would be preserved in the symptomatic mutant mice. Immunoblot analysis was used to test whether changes in presynaptic and postsynaptic proteins would be evident in the symptomatic hippocampal tissue. We first examined the representative presynaptic proteins synaptophysin-1, synaptotagmin-1 and synaptobrevin-2. As shown in Fig. 6A, no changes in the representation of these presynaptically relevant proteins were detected in the mutant tissue. We then examined the postsynaptic proteins PSD95 and nNOS. Consistent with the lack of change observed with the presynaptic markers, no differences in the immunoreactive prevalence of these postsynaptic proteins were detected in the symptomatic Mecp2-null hippocampus compared to wild-type age-matched controls (Fig. 6B). Taken together, these results indicate that Mecp2 deficiency does not lead to gross changes in proteins that are involved in basal neurotransmission.

Although losses in total neuronal number have not been observed, two cardinal features of the Rett brain and the Mecp2-null mouse brain are microcephaly and a decrease in the complexity of forebrain neurons (reviewed in Armstrong, 2002). If neurons are attenuated in size and complexity, then a decrease in the prevalence of cytoskeletal markers that are selectively expressed within neurons would be expected. However, comparisons of the immunoreactive protein levels of a set of cytoskeletal markers in the hippocampus of symptomatic and age-matched wild-type control mice reveal no significant difference in the prevalence of the neuronal somato-dendritic marker MAP2, the mature axonal marker neurofilament 200 or the cytoskeletal protein β-III tubulin (see 6C). Note that these findings are in contrast to previously published observations of reductions in dendritic cytoskeletal proteins in postmortem human tissues from Rett patients (Kaufmann et al., 1995; Kaufmann et al., 1997; Kaufmann et al., 2000).

**Discussion**

We have used the Mecp2-null mouse model to provide functional evidence in support of the hypothesis that Rett syndrome is a “clinical disorder of synaptic plasticity” (Johnston, 2004). Two principal findings on the functional consequences of MeCp2 loss emerge from our study. First, we demonstrate that NMDA-receptor-dependent LTP and LTD in the CA1 region of the symptomatic Mecp2-null mouse hippocampus are significantly attenuated compared to age-matched controls. Data presented here suggest that alterations in the developmental expression of NR2A and NR2B may be linked to the deficit in long-lasting activity-dependent synaptic plasticity. Second, consistent with our observation that there was no change in the representation of AMPA receptor subunits GluR1 and GluR2, or in pre- and postsynaptic proteins involved in synaptic function, basal properties of...
excitatory neurotransmission were unaltered in the Mecp2-null mice compared to wild-type controls.

Previous studies have suggested that the Rett brain may be stalled in an immature stage of development. The mass of an adult Rett brain is approximately the same as that of a 1-year old infant, and the size and complexity of cortical layer II/III and layer V neurons are diminished from normal (Armstrong, 2002). There are also reports showing alterations in neuronal maturation markers in Rett children (Kaufmann et al., 1997), consistent with a model of developmental arrest. Importantly, by using a defined genetic mouse mutant that recapitulates many of the cardinal features of clinical Rett syndrome, it is possible to circumvent problems associated with co-morbidities and the small sample sizes of available human subjects, and allow a detailed study of the genetic, morphological, biochemical and physiological manifestations of the Mecp2 mutation. It is therefore interesting that our results using this mouse model differ substantially from what has been reported previously from patient studies. First, we do not observe significant differences in the prevalence of either β-III tubulin or MAP2 in the fully-symptomatic hippocampus. This isoform of tubulin is expressed predominantly on young neurons, while MAP2 is expressed predominantly on fully mature neurons. Thus, if the developmental state of the Mecp2-null hippocampus was stalled at an immature stage, one might expect to see an increase in β-III tubulin and a decrease in MAP2. This was not, however, the case in the symptomatic mice, as the representation of both markers did not differ from control. Furthermore, the expression of the axonal marker NF200 was also preserved, which suggests that the development and maturation of axons within the symptomatic hippocampus are not significantly affected by the lack of Mecp2. Consistent with this general observation that neuronal differentiation and maturation are not significantly affected, the presynaptic proteins synaptotagmin-1, synaptophysin-1 and synaptobrevin-2 were each present at normal levels in the hippocampi of these symptomatic mice. This further suggests that the number of synapses in the symptomatic hippocampus does not significantly differ from wild-type, and argues that synaptic release of neurotransmitter from these axonal terminals is also preserved—a prediction consistent with our observation that the basal properties of neurotransmission at CA1 synapses are not significantly different from age-matched wild-type controls. Taken together, these data suggest that the general development and gross function of the hippocampus are not dramatically affected by the lack of Mecp2. These results are somewhat surprising, given the pervading models of Rett syndrome which have suggested that significant impairment of synaptic efficacy in the Mecp2-deficient brain underlies the severe clinical condition.

LTP is the most widely studied form of synaptic plasticity in the mammalian nervous system, and can be related to many forms of synaptic modification occurring not only during learning and memory, but also throughout development, in seizure, with aging and in a host of psychiatric and neurologic disorders. Investigation of the ramifications of Mecp2 loss on hippocampal synaptic transmission and plasticity provides important functional insight to possible abnormalities in excitatory neurotransmission in neural circuits of the cerebral cortex, basal ganglia and brainstem that could underlie the cognitive, motor and respiratory...
dysfunctions of Rett syndrome patients. While at present we do not know the molecular mechanism that is responsible for the impaired LTP and LTD in the Mecp2-null mice, the altered representation of the NR2A and NR2B NMDA receptor subunits represents an intriguing possibility. On-going whole-cell patch clamp studies in these animals are underway to test the hypothesis that aberrant NMDA receptor expression and/or function underlie the observed changes. In the normal hippocampus, the expression of NR2A and NR2B is developmentally distinct, with the NR2B subunit being expressed predominantly in the immature tissue. Importantly, the NR2A subunit is more efficacious than NR2B in the generation of NMDA receptor-mediated, activity-dependent long-term potentiation at adult CA1 synapses (Liu et al., 2004). Therefore, our observations of a decreased prevalence of NR2A in the symptomatic Mecp2-null mouse hippocampus are consistent with the attenuated ability of these synapses to exhibit LTP. Interestingly, mice overexpressing Mecp2 exhibit enhanced cerebellar motor learning, and increased paired pulse facilitation and LTP (Collins et al., 2004), despite progressive neurological disorders, including seizure and impaired motor activity.

In sum, our studies provide the first electrophysiological evidence supported by biochemical findings that NMDA-receptor-mediated, activity-dependent synaptic plasticity of excitatory hippocampal neurons is abnormal in symptomatic Mecp2-null mice. Furthermore, we report the somewhat unexpected, and nonetheless very important finding that the properties of basal neurotransmission are preserved in these mice. Thus, any future therapies directed at ameliorating the synaptic consequences of Mecp2 loss must take into account the activity-dependence of the role of Mecp2 in regulating CNS function without overtly affecting the efficacy of neurotransmission under basal conditions. It remains an open question which changes in synaptic function are directly related to mutations in Mecp2 versus reflective of compensatory mechanisms aimed at overcoming the effects of Mecp2 loss. Nonetheless, because mechanisms underlying the regulation and modulation of excitatory neurotransmission in the hippocampus can be generalized to the function of a majority of glutamatergic synapses throughout the CNS, our findings provide the important functional insight to the fundamental deficits underlying many of the hallmark neurological features of Rett syndrome.

Acknowledgments

This work was supported by the Rett Syndrome Research Foundation and NIH (MH59800) grants to RMF, and grants to JHE from the Canadian Institutes of Health Research (MOP57765), and the Heart and Stroke Foundation of Canada (NA-5110). DGMJ is the recipient of an Epilepsy Canada postdoctoral fellowship. We thank members of the Fitzsimonds and Eubanks laboratories for their valuable comments on the manuscript, and Ms. M. Conraads for her insights and perseverance.

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