Mild overexpression of MeCP2 causes a progressive neurological disorder in mice

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Mutations in the X-linked methyl-CpG-binding protein 2 (MECP2), encoding a transcriptional repressor, cause Rett syndrome and a variety of related neurodevelopmental disorders. The vast majority of mutations associated with human disease are loss-of-function mutations, but precisely what aspect of MeCP2 function is responsible for these phenotypes remains unknown. We overexpressed wild-type human protein in transgenic mice using a large genomic clone containing the entire human MECP2 locus. Detailed neurobehavioral and electrophysiological studies in transgenic line MeCP2Tg1, which expresses MeCP2 at ~2-fold wild-type levels, demonstrated onset of phenotypes around 10 weeks of age. Surprisingly, these mice displayed enhanced motor and contextual learning and enhanced synaptic plasticity in the hippocampus. After 20 weeks of age, however, these mice developed seizures, became hypoactive and ~30% of them died by 1 year of age. These data demonstrate that MeCP2 levels must be tightly regulated in vivo, and that even mild overexpression of this protein is detrimental. Furthermore, these results support the possibility that duplications or gain-of-function mutations in MECP2 might underlie some cases of X-linked delayed-onset neurobehavioral disorders.

INTRODUCTION

Rett syndrome is an X-linked progressive neurological disease that primarily affects females. After achieving some early developmental milestones, affected children lose acquired skills such as purposeful hand use and speech. Social behavior becomes impaired, many girls develop seizures and anxiety, and the vast majority display a continual, stereotypical hand-wringing motion (1).

Mutations in the methyl-CpG-binding protein 2 (MECP2) gene have been identified in up to 90% of Rett syndrome cases (2–7) and various other neurological disorders of differing severity (8). Disease-causing mutations vary from substitutions of conserved residues to deletions and insertions that cause frameshift mutations (2,4,7,9–11). Most of these mutations are predicted to cause either total or partial loss of function (12,13). In females, favorable patterns of X-chromosome inactivation (XCI) can mitigate the effects of a particular mutation; the spectrum of phenotypes can thus vary from classic Rett syndrome to milder forms of mental retardation (14) or autism (15), and in rare instances, carrier females can be completely asymptomatic owing to heavily skewed XCI (16). In males, the type and location of mutation dramatically affect the phenotype: severe inactivating mutations cause neonatal encephalopathy (16), whereas hypomorphic alleles yield X-linked mental retardation with or without psychosis (17–19). It is interesting that, despite the many different mutations and the broad expression of MECP2, dysfunction of this protein consistently results in a primarily neurological phenotype.

MeCP2, a protein that binds to methyl-CpG dinucleotides (20), is thought to recruit co-repressor and histone deacetylase complexes to repress gene expression through chromatin modification (21,22). Mice null for MeCP2 (MeCP2⁰⁰⁰) (23) and mice that contain only a C-terminal fragment of MeCP2 (24) display a severe neurological phenotype that results in death in early adulthood in males. Mice that bear a truncating mutation after amino acid 308 (MeCP2³⁰⁸) develop

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a progressive neurological syndrome reminiscent of Rett syndrome, with loss of motor skills, behavioral abnormalities, seizures, kyphosis and stereotypical forepaw motions (25).

While in vitro and some in vivo studies have shown MeCP2 to be a transcriptional repressor (21,26–30), its functions in the central nervous system (CNS) remain unclear. Microarray studies using mRNAs from MeCP2 loss-of-function mice have detected only minor changes in gene expression (31). Recently, analysis of neuronal cultures from MeCP2 null mice revealed that MeCP2 binds to regulatory elements of the brain-derived neurotrophic factor promoter and affects its expression (28,32). At this time, however, there is no evidence that any gene directly regulated by MeCP2 is responsible for any particular aspect of the phenotypes seen in patients or mouse models.

Other functions attributed to MeCP2 include histone deacetylase-independent repression (33), maintenance of DNA methylation (34), histone methylation (35), assembly of secondary chromatin structure independent of its methyl-CpG DNA binding capability (36) and interactions with RNA splicing factors (37). Regardless of the exact cellular functions of MeCP2, if Rett syndrome and other related diseases are caused by a loss of some function of MeCP2, then enhancing endogenous MeCP2 levels or introducing a wild-type MeCP2 using gene therapy could present a therapeutic option.

We therefore sought to investigate the ramifications of artificially elevating MeCP2 levels as well as to develop a tool for further analysis of the function of MeCP2. We developed a mouse model that transgenically expresses MeCP2 under the endogenous human promoter by using a large insert genomic clone from a P1-derived artificial chromosome (PAC) that contains the MECP2 locus. Luikenhuis et al. (38) also recently developed MeCP2 transgenic mice in which they targeted the MeCP2 cDNA into the Tau locus, creating a fusion protein with the first exon of Tau. Although they reported some neurologic dysfunction in their homozygous mice, additional studies are required to determine whether the reported dysfunction is due to the excess MeCP2, lack of Tau in the background of excess MeCP2 or some combination thereof.

Development and characterization of our PAC transgenic mice, which lack the confounding effects of loss of function of other neuronal proteins, yielded surprising information about the importance of MeCP2 levels for neuronal plasticity and function.

RESULTS

Expression pattern of PAC-derived MeCP2 is similar to that of the endogenous protein

Transgenic mice were generated though microinjection of the linearized insert from PAC671D9 (AF031078) into fertilized oocytes of FVB mice. This 99 kb human PAC clone contains all of the exons of MECP2, including the regions that would be responsible for both the short and long isoforms (Supplementary Material, Fig. S1A) (39,40). Careful sequence analysis of this PAC clone revealed that MECP2 is the only gene within the clone, and that it does not include any transcriptional units for neighboring genes.

We chose a genomic clone to study overexpression of MECP2 for several reasons. First, large insert clones such as PACs and BACs tend to give more physiologic expression patterns than cDNA inserts expressed from a heterologous promoter (41–43). Second, the gene’s first intron is large and conserved, suggesting that it may contain regulatory elements (44). Third, there are multiple evolutionarily conserved polyadenylation sites, which may indicate that the 3′-untranslated region (3′-UTR) has physiological importance (45,46). Fourth, endogenous MeCP2 protein levels do not directly correlate with RNA levels (47), suggesting post-transcriptional regulation that may be due to non-coding regions. Fifth, it was recently discovered that there are two protein isoforms of MeCP2 derived from alternative splicing that vary in abundance between brain and non-brain tissue (39,40). Lastly, mutations in exon 1, which are predicted to cause loss of the long isoform, are associated with classic Rett syndrome (40).

Six potential founders were generated and all appeared to contain the entire PAC (Supplementary Material, Fig. S1B and C). The MeCP2Tg2 founder was sub-fertile and therefore unable to transmit the transgene; so no further analysis was performed on this line. The MeCP2Tg3 showed sex-specific differences: males have a more severe phenotype, whereas females have a variable and milder phenotype.

To evaluate which lines expressed MECP2 RNA from the PAC transgene, northern analysis was performed on the five lines in which the transgene was transmitted. A human-specific 3′-UTR probe allowed us to detect the 10 kb human transcript. Four of the five transgenic lines expressed MECP2 RNA (Fig. 1A). To assess the protein levels, western analysis was performed on whole brain extracts (Fig. 1B). The antibody used cross-reacts with both the human and mouse proteins, which are approximately the same size. Therefore, to quantify protein levels, we determined total MeCP2 levels, which include both the mouse and human proteins. We found that the various transgenic lines have differing levels of protein, estimated at 2×, 7×, 1× and <2× endogenous levels for lines 1, 3, 11 and 22, respectively. The level of protein in MeCP2Tg1 is similar to wild-type, making it difficult to determine whether transgenic protein is present or not. The extracts analyzed from the MeCP2Tg3 line in Figure 1B are made from a male mouse. Female mice from the MeCP2Tg6 line were shown to have lower levels of protein than the male mice (data not shown), which is consistent with their milder phenotype. This supports a possible integration of the PAC into the X-chromosome in this particular line and suggests XCI as an explanation for lower expression and a milder phenotype in females.

In order to identify the transgenic human MeCP2 protein specifically, we crossed mice from one of the MeCP2Tg1 lines (MeCP2Tg+) to the MeCP2308 mice, which carry a truncated protein. This allows us to separate the endogenous protein from the transgenically expressed human protein. We found that the level of human MeCP2 in the MeCP2Tg1 line is comparable to that of the endogenous mouse protein in the wild-type mouse and that both isoforms are translated (Fig. 1C). It is of note that the short isoform is more readily detectable in wild-type human tissues when compared with wild-type mouse tissues, a pattern conserved in the transgenic animals. This demonstrates that the human protein is in fact being made, that its levels are similar to endogenous levels.
of the mouse protein in this line and that both the long and short isoforms of MeCP2 are encoded by the transgene.

Multi-tissue western analysis was performed to verify that the distribution of the protein product from the PAC mirrors that of the endogenous MeCP2. We found that, as in the wild-type mouse, MeCP2 is high in brain, lung and spleen, low in heart and kidney and undetectable in liver and skeletal muscle (Fig. 1D). To evaluate the cellular and sub-cellular distribution of MeCP2 in the transgenic mice, we performed immunohistochemical analysis. Throughout the brain, the expression pattern in the transgenic mice mirrored that of the wild-type animals and localized to heterochromatic foci as expected (Fig. 1E).

**MeCP2 transgenic mice develop a progressive neurological disorder and die prematurely**

Transgenic lines that expressed human MeCP2 developed a progressive neurological phenotype that varied in onset and severity according to the level of protein expressed. All transgenic mice appeared grossly normal at birth, but the mice from high-expressing lines manifested severe abnormalities within days to weeks, whereas mice from lower-expressing lines developed neurological phenotypes only at later ages (Supplementary Material, Table S1). The progressive neuro-behavioral abnormalities detected by clinical observation consisted of forepaw claspimg when hung by the tail,
aggressiveness (an increased propensity to bite), kyphosis and hypoactivity characterized by a freezing-like behavior. Premature death was seen to some degree in all the lines. The majority of the males of the MeCP2Tg3 line died by 3 weeks and 30% of the MeCP2Tg1 line died between 20 weeks and 1 year of age. Interestingly, the mice that survive past 1 year tend to live a normal lifespan.

**MeCP2 transgenic mice do not manifest anxiety or hypoactivity up to 20 weeks of age**

In order to further elucidate the neurological defects in MeCP2Tg mice, we conducted a battery of behavioral tests. The MeCP2Tg1 line of mice was chosen for these tests because they had a phenotype intermediate to that of male MeCP2Tg3 mice, which mostly died by 3 weeks, and mice from the MeCP2Tg22 line, which had a mild onset and late progression. Because of the progressive nature of the phenotype, behavioral tests were performed at different ages between 6 and 20 weeks. (Beyond 20 weeks the freezing-like behavior and severe hypoactivity observed at older ages would confound the interpretation of behavioral assays.)

We evaluated the level of activity and anxiety-like behavior in the mice using the light/dark box and the open field. Animals were tested at both 10 and 20 weeks of age. There were no differences in performance in the light/dark test at either age (Supplementary Material, Fig. S2).

No differences were discerned between wild-type and transgenic mice in the open field assay, when comparing the distance traveled in the center of the field to the total distance traveled. Transgenic mice did, however, show increased vertical activity, i.e. rearing, at both 10 and 20 weeks, which is taken to indicate less anxiety (Fig. 2A). Despite the observed hypoactive behavior at older ages, there were no differences observed in activity at either timepoint tested (data not shown).

**MeCP2Tg1 mice display abnormal behavior on the dowel test**

The dowel test was utilized as a test of gross motor coordination. This test measures the length of time it takes a mouse to walk off an elevated wooden dowel and the number of times it walks off the dowel successfully without falling off, using 120 s as the maximum test duration. Mice were tested at 6, 10 and 20 weeks of age. At all time points, both the wild-type and transgenic mice remained on the dowel for the maximum time (data not shown), but transgenic mice moved much less than wild-type mice while on the rod.

We measured the latency to take the first step and the latency and frequency of walking off the rod. At all time points, the latency of the MeCP2Tg1 mice to take the first step was increased (Fig. 2B), and therefore they walked off the rod fewer times (data not shown). Exactly why the transgenic mice hesitated to walk off the rod is not clear. There was no evidence of impaired coordination at this age; after the transgenic mice walked off the rod once, they were comfortable walking off again, although this was admittedly rare because most of the mice spent the entire trial inactive on the rod.

**Enhanced cerebellar motor learning and hippocampal learning in the MeCP2Tg1 mice**

Because the MeCP2Tg1 mice failed to move on the dowel, we could not get an accurate measure of their coordination; we therefore decided to use the rotating rod analysis to evaluate motor coordination and cerebellar learning, as indicated by how long a mouse can stay on a rod whose rotational motion is accelerated throughout the test.

At 6 weeks of age no significant difference was detected between wild-type and MeCP2Tg1 mice (Fig. 3A). At 10 weeks of age a naive set of MeCP2Tg1 mice were tested and were found to perform similarly on the first day of the test, indicating no defect in coordination. By the second day, however, the transgenic mice performed better than wild-type animals. Performance of the transgenic mice was significantly different from the wild-type littermates over the trial at 10 weeks of age (Fig. 3B). The improved performance in days 2–4 suggested that enhanced cerebellar motor learning might contribute to this phenotype. We therefore tested those mice that were tested at 10 weeks again at 20 weeks of age. The difference in performance upon retesting was also significant. The most striking difference was noted on the first day of trials (Fig. 3C), suggesting that the MeCP2Tg1 mice have an enhanced motor memory and possibly improved cerebellar dependent motor learning. The performance of the transgenic mice plateaued near the end of the 20 week test (Fig. 3C) because the animals remained on the rod for the duration of the test; this should not be construed to indicate that they did not continue to improve.

We used conditioned fear analysis to assess both hippocampal- and amygdala-dependent learning. In this task, the mice are trained in a novel environment with a neutral stimulus (a tone) paired with a foot shock. The mice are placed in a different context with the same tone (cued test) or back in the same environment without a tone (contextual test) 24 h later. Freezing is measured to determine whether they associate either the context or the tone with the previously received foot shock. Contextual learning is dependent on both the amygdala and the hippocampus, while associating the tone or cue with the foot shock is dependent on the amygdala (48,49). We performed this test on 20 week old mice. There was no statistical difference in the amount of freezing in the absence of a paired stimulus either during training (data not shown) or before the tone during cued test (Fig. 3D), indicating that the background amount of freezing is comparable at 20 weeks. No difference was detected between the wild-type and transgenic mice when the tone was given in the cued test (Fig. 3D), suggesting that overexpression of MeCP2 does not affect amygdala function. The contextual test, however, showed a higher percentage of freezing in the MeCP2Tg1 mice when compared with wild-type littermates (Fig. 3E), which indicates MeCP2 overexpression augments hippocampal learning.

**Synaptic plasticity is enhanced in MeCP2Tg1 mice**

Changes in synaptic plasticity are thought to be associated with changes in the ability to learn. Therefore, the possible ramifications of MeCP2 overexpression on synaptic transmission and plasticity were explored. We investigated neurotransmission
at the Schaffer-collateral synapses in hippocampal area CA1. First, we assessed basal synaptic transmission by examining the correlation between the slopes of evoked field excitatory postsynaptic potentials (fEPSPs) and fiber volleys, known as the input/output relationship (I/O). The fiber volley represents depolarization of presynaptic terminals and the fEPSP represents depolarization of dendrites.

MeCP2Tg1 and littermate controls had comparable I/Os (Supplementary Material, Fig. S3A), so overall Schaffer-collateral synaptic connectivity is apparently unaffected by MeCP2 overexpression. Another measure of synaptic function utilizes a short-term form of synaptic plasticity known as paired-pulse facilitation (PPF). PPF is measured by evoking two synaptic stimuli in rapid succession (within 300 ms). PPF is a presynaptic phenomenon whereby the second synaptic response is enhanced relative to the first (50). PPF was greatly enhanced when measured from MeCP2Tg1 animals relative to littermates (Fig. 4A). Given that the MeCP2Tg1 animals have comparable synaptic connectivity to wild-type animals, the enhancement in PPF observed in MeCP2Tg1 animals suggests that changes in the presynaptic terminal induced by MeCP2 overexpression have enhanced the ability of MeCP2 synapses to exhibit PPF.

We next investigated the capacity for Schaffer-collateral synapses to express a form of plasticity known as long-term potentiation (LTP). LTP refers to a phenomenon whereby synaptic responses are elevated in response to specific patterns of synaptic stimulation. MeCP2 overexpression led to a robust enhancement of LTP immediately after high-frequency stimulation (HFS; 2 × 1 s, 100 Hz stimulation) that persisted for at least 90 min (Fig. 4B). These results suggest that overexpression of MeCP2 enhances synaptic plasticity.

Behavioral seizures and EEG abnormalities are seen in older transgenic mice

Clonic seizures were visually observed in the MeCP2Tg1 mice as early as 20 weeks. Electroencephalographic (EEG) traces were taken while the mice were in the cage. Although no clonic seizures occurred during the trace recordings, at 20 weeks of age abnormal spikes and waves were occasionally seen (Fig. 5). No obvious behavioral abnormalities were associated with these spikes and waves at this age. By 50 weeks, visually observed seizures were more frequent. Recordings at this age revealed frequent abnormal spikes and waves, at times being sustained for many seconds (Fig. 5). The spike and brief spike–wave discharges at this age were always associated with behavioral immobility (akinetic seizures), which reverted to normal behavior upon cessation of the EEG discharge.
Double mutant MeCP2\textsuperscript{Tg1};MeCP2\textsuperscript{Bird} null mice are phenotypically normal

To be certain that the human transgene was functioning normally in the mouse background, we crossed the MeCP2\textsuperscript{Tg1} mice to MeCP2\textsuperscript{Bird} mice, which are null for the MeCP2 protein. We obtained four genotypes from each mating: wild-type, MeCP2\textsuperscript{Bird/y}, MeCP2\textsuperscript{Tg1} and double mutants (MeCP2\textsuperscript{Bird/y};MeCP2\textsuperscript{Tg1}). The MeCP2\textsuperscript{Bird/y};MeCP2\textsuperscript{Tg1} mice have reached 33 weeks of age and remain indistinguishable.
from the wild-type littersmates. Clearly, the premature death of the male null mice, which typically occurs around 10–11 weeks on this genetic background, has been rescued by the transgene. Interestingly, the loss of the endogenous protein also rescues the neurological abnormalities seen in the MeCP2Tg1 mice up to 33 weeks thus far, as assessed by clinical observation.

**DISCUSSION**

We have developed a mouse model that transgenically expresses MeCP2 under the endogenous human promoter. The MeCP2Tg1 mice appear clinically normal until ∼10–12 weeks of age, when they develop forepaw clasping. Intriguingly, early in life (up to 20 weeks) the animals display enhanced cerebellar and hippocampal learning along with increased synaptic plasticity. Nevertheless, with age the animals develop seizures, hypoactivity and spasticity. Between 30 weeks and 1 year, kyphosis develops, hypoactivity becomes more severe and seizures become more frequent. Abnormal EEG patterns are accompanied by frequent akinetic episodes. Eventually many animals stop grooming themselves, become ataxic and die prematurely (Fig. 6). These phenotypes are clearly modulated by the levels of MeCP2. The multiple transgenic lines provide strong evidence that higher protein levels correlate with more severe phenotypes. Summing up, even a modest excess of endogenous MeCP2 levels can cause a progressive neurological phenotype.

We also crossed MeCP2 null mice with our overexpressing transgenics. Analysis of double-mutant MeCP2null/Tg1 animals demonstrated two important features of the transgene. First, expression of a human MeCP2 transcript rescues the loss of the mouse MeCP2. This argues that the human protein functions properly in a mouse background and that the transgene expresses properly in at least the cells where MeCP2 function is necessary. Second, reduction of MeCP2 levels by removing the endogenous allele rescues the overexpression phenotype found in the transgenic line. This argues that the effects seen in the transgenic mice are truly because of overexpression of MeCP2 rather than some other unspecified effect of the transgene.

It is informative to compare the phenotypes of mice that overexpress MeCP2 with those that lack normal MeCP2 function. MeCP2Tg1 mice, which reproduce many of the features of Rett syndrome, carry a truncated hypomorphic MeCP2 allele. Both MeCP2Tg1 and MeCP2Tg1/C3/C3 develop progressive neurological syndromes, but with different and frequently inverse phenotypes. The transgenic mice show enhanced coordination and cerebellar motor learning, whereas the MeCP2Tg1/C3/C3 mice suffer impaired coordination and cerebellar learning (25). MeCP2Tg1 mice display anxiety as measured by decreased center-to-total-distance ratio and decreased vertical activity in the open field (25). The transgenic mice, on the other hand, display an increase in vertical activity, which is associated with less anxiety. Furthermore, the MeCP2Tg1 mice display enhanced synaptic plasticity as measured by increased PPF and increased LTP, whereas the loss-of-function mutants show decreased PPF and decreased LTP (Moretti and Zoghbi, unpublished data). Although both overexpression and loss-of-function mice develop seizures, the types of seizures are different clinically and electrophysiologically (25). The abnormal forepaw movements differ in kind as well: the MeCP2Tg1 mice have stereotypies reminiscent of the handwringing movement seen in Rett syndrome patients, whereas the MeCP2Tg1/C3 mice display forelimb claspig, a feature of many different neurologically impaired mutant mice.

The two mouse models, however, share three striking similarities: the phenotypes appear after a period of overtly normal...
development, affect CNS function and are progressive. This is particularly interesting in light of the recent finding that a patient with double the normal levels of MeCP2 RNA has a preserved speech variant form of Rett syndrome (51). The finding that MeCP2 is expressed in mature neurons and that its abundance increases postnatally in these cells throughout childhood in humans (47) argues that its function is critical in mature neurons, possibly after these neurons have engaged in synaptogenesis. The enhanced LTP and PPF in MeCP2Tg mice are consistent with an important role for this protein in regulating synaptic function and plasticity. The molecular mechanism by which overexpression of MeCP2 causes the neuronal dysfunction and altered plasticity and behavior is not clear at this time, but there are several possibilities. The extra MeCP2 could be titrating or sequestering interacting proteins. Alternatively, overexpressed MeCP2 may not be as tightly regulated if levels of modifying enzymes are limiting. Lastly, overexpression of MeCP2 could alter gene expression, perhaps indiscriminately repressing genes or repressing its normal targets in an unregulated manner. Future molecular profiling studies and comparisons with ongoing studies in MeCP2Tg mice may provide a mechanistic clue.

Luikenhuis et al. (38) recently developed transgenic mice in which the MeCP2 cDNA was targeted into the Tau locus, creating a fusion protein with the first exon of Tau. Those mice that are homozygous for the Tau–MeCP2 allele develop a progressive neurological phenotype. Some of the features observed in the Tau–MeCP2 mice overlap with those we observe in the MeCP2Tg animals: the mice were small at weaning, displayed progressive neurological problems and had reduced fertility (Supplementary Material, Table S1). There are notable differences, however, between the PAC transgenic mice and the Tau–MeCP2 mice. Tremors and swaying, reported features of the Tau–MeCP2 mice, were not observed in the MeCP2Tg mice, and we observed ataxia only at the very terminal stage in transgenic mice that died prematurely. Moreover, the Tau–MeCP2 mice do not show premature death, whereas a modest increase of MeCP2 levels in the correct spatio–temporal distribution is lethal in MeCP2Tg mice. Other phenotypes such as seizures, enhanced learning and enhanced synaptic plasticity were not evaluated or reported in the Tau–MeCP2 mice (38). It is possible that some of the phenotypes in the Tau–MeCP2 mice are due to the additive effects of MeCP2 overexpression and Tau loss-of-function in this double mutant. In fact, differences between the MeCP2Tg and the Tau–MeCP2 mice could be attributable to a variety of factors, such as patterns of MeCP2 expression (Tau versus MECP2 promoters), presence or absence of the 3′-UTR or alternative splicing to include exon 1. Furthermore, the function of MeCP2 may be altered due to its fusion with 31 amino acids from Tau.

In sum, our studies provide firm evidence that the CNS is exquisitely sensitive to MeCP2 expression levels and suggest that this protein’s levels and function are tightly regulated. Any therapies directed at increasing the levels of MeCP2 in patients must be considered carefully to avoid further neurological impairments. Furthermore, our findings are of diagnostic importance. Increased MeCP2 RNA levels were reported in a single case of autism and in a case of pervasive developmental disorder, in addition to the patient with preserved speech variant of Rett syndrome (51,52). Although the data in these three cases do not unequivocally prove that increased MeCP2 RNA levels are responsible for the phenotypes, the data from our study support this possibility by showing that MeCP2 overexpression can lead to a postnatal neurological phenotype. Therefore, gain-of-function mutations and duplications of MECP2 in humans might result in postnatal neurodevelopmental problems, including variants of Rett syndrome and autism.

**MATERIALS AND METHODS**

**Creation of transgenic mouse lines**

PAC671D9 DNA was digested with NotI. The ~99 kb insert was separated from vector on a pulse-field gel. The fragment was cut out and the gel was digested with GELase enzyme (Epicentre) and purified on a Centricon 100 column (Millipore Corporation). The DNA was then washed off the column with microinjection buffer (10 mM Tris pH 8.0; 0.1 mM EDTA), and was injected into FVB single cell zygotes at concentrations of 0.4 and 0.8 ng/μl using standard procedures.

**Northern blot analysis**

RNA was purified from fresh brain tissue homogenized in a dounce homogenizer using Trizol Reagent (Invitrogen). Into each lane of a standard formaldehyde gel, 20 ng were loaded. After running, the gel was transferred onto Zetaprobe membrane (Biorad) in 10× SSC (1× SSC is 3 mM NaCl; 0.3 M sodium citrate). The membrane was then hybridized using ExpressHyb hybridization solution (BD Biosciences) using a labeled probe from the last 700 bp of the 3′-UTR of human MECP2.
Western blot analysis

Protein was purified from fresh tissue into protein extraction buffer [2% SDS; 25 mM DTT; 100 mM Tris, pH 6.8; 1 x complete protease inhibitors (Roche)]. Brain and liver were homogenized with a dounce homogenizer while other tissues were broken up with a tissue grinder. Proteins were run on SDS–PAGE gels (53) at 25 µg/lane for most gels and 100 µg/lane for the multi-tissue blot. Proteins were then transferred to supported nitrocellulose membrane using 10 mM CAPS; 10% methanol. Membranes were blocked with 5% non-fat dry milk (Bio-Rad) in TBS-T (100 mM Tris–Cl, pH 7.5; 150 mM NaCl; 0.1% Tween 20). Anti-MeCP2 antibody (Upstate) was used at a dilution of 1:1000, anti-GAPDH (Advanced Immuno Chemical) at 1:10 000 and anti-β-tubulin (The Developmental Studies Hybridoma Bank at the University of Iowa) at 1:1000. Secondary antibodies were HRP conjugated and were developed using chemiluminescence.

Immunohistochemistry

Mouse tissues were fixed by transcardial perfusion with PBS-buffered 4% formaldehyde and were washed. Tissue was dehydrated and impregnated with paraffin. Sections 5 µm in thickness were cut and mounted onto plus slides. Antigen retrieval was performed with citric acid (pH 6.0) for 3 x 5 minutes in the microwave. Nuclear digestion was performed with proteinase K. MECP2N15 or MECP2C17 (47) was used at a dilution of 1:100 for three nights at 4°C. Biotinylated antirabbit secondary antibodies were used from the ABC-Elite kit (Vector Laboratories).

Behavioral studies

Light/dark box. A 44 x 21 x 21 cm light/dark box was used. This box is divided into two chambers: a dark chamber occupying approximately one-third of the box and a light chamber occupying approximately two-thirds of the box. A small opening, large enough to allow movement of the mice between the light and dark, connected the two chambers.

Each mouse was placed in the light chamber. The time the mouse spent on each side of the box was recorded, with a transition between light and dark only recorded when all four paws of the mouse changed sides. The test was conducted for 10 min. Data were analyzed using the Student's t-test.

Open field test. The open field test was performed using a computer-operated Digiscan optical animal activity system (RXYZCM, Acuscan). Mice were placed in the center of a 40 x 40 cm open field space. Data was collected in 2 min intervals for 30 min. Data was analyzed in 10 min increments using a two-way ANOVA with repeated measures.

Dowel crossings. Mice were placed on the center of a 0.9 cm wooden dowel suspended between two platforms. If the mouse walked off of the dowel onto the platform, the mouse was placed back in the center. The test lasted 2 min beginning when the mouse was placed on the dowel. Data were analyzed using the Student’s t-test.

Rotating rod. The mice were placed on a rotating rod (Ugo Basile) that accelerated from 3 to 30 r.p.m. The duration of each trial was a maximum of 600 s. The time at which they either fell off of the rod or displayed turning with the rod two times was recorded as the end of the trial for that mouse. This was repeated four times per day for 4 days, with a minimum of 10 min between each trial. Analysis was performed using a two-way ANOVA with repeated measures.

Conditioned fear. Mice were placed in a Med Associates/Actimetrics chamber system where 30 s tone was followed by a 2 s foot shock at ~1.5 mA. The tone–foot shock stimuli were repeated after 2 min. After 24 h the mice were tested for freezing in the same chamber with no tone to evaluate contextual fear. The mice were then tested for freezing when the shape, color, scent, texture and lighting of the chamber had all been changed, but the tone was sounded for 3 min after the animals have acclimated to the chamber for three minutes. Scoring for freezing is automated in this system. Analysis was performed using ANOVA and t-test analysis.
Animals were sacrificed by cervical dislocation, followed by Hippocampus slice preparation. Transverse slices (400 μm) were prepared with a Vibratome (Vibratome, St Louis, MO, USA). During isolation, slices were stored in ice-cold CS. After isolation, cortical tissue was removed and hippocampal slices were equilibrated in a mixture of 50% CS and 50% artificial cerebrospinal fluid (ACSF: 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH2PO4, 25 mM NaHCO3, 2 mM CaCl2, 1 mM MgCl2, 25 mM glucose) at room temperature for 30 min prior to transfer to the recording chamber.

Slice electrophysiology
Electrophysiology was performed in an interface chamber (Fine Science Tools, Foster City, CA, USA). Oxygenated ACSF (95%/5% O2/CO2, 30°C) was perfused into the recording chamber at a rate of 1 mL/min. Electrophysiological traces were digitized and stored using a Digidata 1320A and Clampex software (Axon Instruments, Union City, CA, USA). Extracellular stimuli were administered on the border of area CA3 and CA1 along the Schaffer-collaterals using Teflon-coated, bipolar platinum electrodes. fEPSPs were recorded in stratum radiatum with an ACSF-filled glass recording electrode (1–3 MΩ). The relationship between fiber volley and fEPSP slopes over various stimulus intensities was used to assess baseline synaptic transmission. All subsequent experimental stimuli were set to an intensity that evoked an fEPSP of area CA3 and CA1 along the Schaffer-collaterals using Teflon-coated, bipolar platinum electrodes. fEPSPs were recorded in stratum radiatum with an ACSF-filled glass recording electrode (1–3 MΩ). The relationship between fiber volley and fEPSP slopes over various stimulus intensities was used to assess baseline synaptic transmission. All subsequent experimental stimuli were set to an intensity that evoked an fEPSP that had a slope of 50% of the maximum fEPSP slope. Paired-pulse facilitation was measured at various interstimulus intervals (20, 50, 100, 200 and 300 ms). HFS LTP was induced by administering two 100 Hz tetani (1 s), with an intertetanus interval of 20 s. Synaptic efficacy was monitored 20 min prior to and 90 min following induction by recording fEPSPs every 20 s (traces were averaged for every 2 min interval). Slices that did not exhibit stable fEPSP slopes during the first 20 min of recording were excluded from the study.

EEG recordings
Each mouse was implanted with an electrode (Omnetics Connector Corp.) for EEG recordings as described (54). Avertin (1.25% tribromoethanol/amyl alcohol solution) was used to anesthetize the mice using a dose of 0.02 ml/g via intraperitoneal injection. Wires were implanted into the subdural space at the left and right side of the brain. Digital EEG activity and video were recorded for 4 consecutive days on each animal for 2 h samples at random times using Stellate Systems, Harmonie software version 5.6b.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG Online.

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