Postnatal Loss of Methyl-CpG Binding Protein 2 in the Forebrain is Sufficient to Mediate Behavioral Aspects of Rett Syndrome in Mice

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Background: Mutations in the methyl-CpG binding protein 2 (MeCP2) gene cause Rett syndrome (RTT), a neurodevelopmental disorder that is accompanied by a broad array of behavioral phenotypes, mainly affecting females. Methyl-CpG binding protein 2 is a transcriptional repressor that is widely expressed in all tissues.

Methods: To investigate whether the postnatal loss of MeCP2 in the forebrain is sufficient to produce the behavioral phenotypes observed in RTT, we have generated conditional MeCP2 knockout mice.

Results: These mice display behavioral abnormalities similar to RTT phenotypes, including hindlimb clasping, impaired motor coordination, increased anxiety, and abnormal social behavior with other mice. These mice, however, have normal locomotor activity and unimpaired context-dependent fear conditioning, suggesting that the behavioral deficits observed are the result of loss of MeCP2 function in postnatal forebrain and not the result of generalized global deficits.

Conclusions: These data highlight the important role of MeCP2 in the forebrain and suggest that even partial loss of MeCP2 expression in these brain regions is sufficient to recapitulate features of RTT.

Key Words: MeCP2, Rett syndrome, knockout, behavior, animal model

Rett syndrome (RTT) is a neurodevelopmental disorder that is one of the leading causes of mental retardation and autistic-like behavior in females, with an estimated prevalence of 1 in 10,000–15,000 live female births. In general, children affected with RTT experience normal development up to the age of 6–18 months, at which time they then enter a period of regression (Hagberg et al. 1983). Most RTT defects predominantly originate from the central nervous system, including mental retardation, autistic-like behavior, seizures, disturbances of sleep, problems with gait, and stereotypical hand movements. In addition, most children afflicted with RTT show a loss of social and cognitive abilities.

Recent work has demonstrated that RTT is an X-linked dominant disorder that in most patients (>90%) results from mutations in the methyl-CpG binding protein 2 (MeCP2) gene (Amir et al. 1999, 2000; Bienvenu et al. 2000; Huppke et al. 2000; Wan et al. 1999). These disease-causing mutations result in loss of MeCP2 function (Ballestar et al. 2000; Yusufzai and Wolffe 2000). The MeCP2 gene encodes a DNA-binding protein that binds to methylated cytosines in mammals. Normally, MeCP2 is believed to act as a transcriptional repressor by binding to target gene promoters and silencing their transcription in many different tissues. Although MeCP2 is expressed in many tissues, it is interesting that most of the RTT defects are pronounced in the central nervous system.

Attempts to examine whether the loss of MeCP2 contributes to an altered behavioral phenotype have focused on a genetic model (MeCP2<sup>308/+</sup>), in which a truncating mutation was inserted into the mouse MeCP2 gene (Shahbazian et al. 2002). These MeCP2<sup>308/+</sup> mice show many of the classic features of RTT patients; however, because the expression of the truncating mutation is widespread, these mice do not address whether the selective loss of MeCP2 function in the brain can account for the behavioral phenotypes observed in RTT patients. The early postnatal lethality of MeCP2<sup>+/−</sup> mice prevents their use in behavioral characterization (Chen et al. 2001; Guy et al. 2001; Tate et al. 1996). To circumvent these potential problems, conditional knockout (KO) mice, in which floxed MeCP2 mice were crossed with calcium-calmodulin-dependent protein kinase II (CaMKII)-Cre transgenic mice to selectively delete MeCP2 in the forebrain, have been generated (Chen et al. 2001). This CaMKII-Cre<sup>93</sup> mouse line expresses Cre recombinase in forebrain regions during early postnatal development. This approach allowed us to determine whether the loss of forebrain MeCP2 during early postnatal development contributes to RTT behavioral abnormalities.

In this study, we examined the role of forebrain MeCP2 in behavior, including motor coordination, locomotor activity, anxiety, associative learning and memory, and social behavior. We found that conditional MeCP2 KO mice display impaired motor coordination, increased anxiety, and abnormal social interactions with other mice but show normal responses in the other behavioral measures. These data suggest that the postnatal loss of MeCP2 in the forebrain is sufficient for the development of many of the behavioral phenotypes exhibited by RTT patients and highlights the utility of these mice as animal models for RTT.

Methods and Materials

Generation of MeCP2 Conditional Knockout Mice

The CaMKII-Cre<sup>93</sup> line was on a mixed 129/BALBC background that was back-crossed to a C57BL/6 line. The floxed MeCP2 was also on a similar mixed background and was back-crossed to C57BL/6 mice. Male CaMKII-Cre<sup>93</sup> mice were crossed with female floxed MeCP2 mice, and the resulting bigenic mice exhibited no obvious initial phenotypic difference (Chen et al. 2001). All experiments were performed on littermates derived from this mating paradigm to ensure analysis by matched...
control (CTL) mice and to minimize the genetic differences. Genomic DNA was isolated from tails for genotyping by polymerase chain reaction analysis, as previously described (Chen et al 2001).

Fluorescent Immunohistochemistry
Mice were perfused, and brains were treated as previously described (Monteggia et al 2004). Briefly, sections were incubated with rabbit anti-MeCP2 (1:1000; Affinity Bioreagents, Golden, Colorado) or in 3% normal goat serum, 3% Tween 20 in phosphate-buffered saline (PBS), overnight. The sections were rinsed with PBS and then incubated with goat antirabbit fluorescent secondary antibody (1:200; Jackson ImmunoResearch, Westgrove, Pennsylvania) for 2 hours. Sections were rinsed in PBS, dehydrated, and coverslipped. For the cerebellum sections, immunofluorescence was performed with cells double-labeled for MeCP2 and calbindin (1:500; Chemicon, Temecula, California), and proteins were visualized with CY2 and CY3 fluorophore-labeled secondary antibodies (Perrotti et al 2004).

Western Blot Analysis
Western blot analysis was performed as previously described (Perrotti et al 2004). Briefly, brain regions were dissected, homogenized, and protein concentration determined by the modified Lowry method (Biorad, Hercules, California). Samples of total protein (30 μg) were electrophoresed on an 8% sodium dodecylsulfate polyacrylamide gel. Proteins were transferred to nitrocellulose membrane, then blocked in 1× Tris-buffered saline (TBS) with 1% Tween 20 and 5% nonfat dry milk for 1 hour at room temperature. The blots were then incubated in the presence of the rabbit anti-MeCP2 (1:2000; Affinity Bioreagents), or mouse anti-actin (1:10,000; ICN, Irvine, California) in blocking solution overnight at 4°C. The blots were washed in 1× TBS with 1% Tween 20, incubated with a peroxidase-labeled goat anti-rabbit immunoglobulin (IgG; 1:2500; Vector Laboratories, Burlingame, California) or goat antimouse IgG (1:20,000; Vector Laboratories), then washed in 1× TBS. Bands were visualized with enhanced chemiluminescence (Amersham, Piscataway, New Jersey). Densitometry of the immunoreactivity was quantitated with the NIH Image analysis software.

Behavioral Overview
All experiments were approved by the University of Texas Southwestern Medical Center Animal Care and Use Committee. All behavioral testing was done on male mice 4–6 months of age, unless otherwise specified. Control mice were littermates of the MeCP2 conditional KO mice. For all experiments, data were presented as mean ± SE, and significance was set at \( p < .05 \).

Locomotor Activity and Rotarod
Locomotor activity was assessed as previously described (Monteggia et al 2004). For the rotarod test, each mouse was placed on the rotarod (ITC Life Science, Woodland Hills, California), which was then activated and its speed ramped up from 0 to 45 rpm in 60 sec. The time to fall off the rotarod or turn one full revolution was measured. The mouse was returned to its original cage for 1 hour. The test was repeated for a total of 5 runs. Data were analyzed by a two-way analysis of variance. Elevated Plus Maze
Mice were placed in the center of a cross-shaped maze (each arm 33 cm × 5 cm) that was elevated 2 ft above the floor. The maze had two open arms and two closed arms (25-cm-tall walls on the closed arms). The behavior of the mice was monitored for 5 min. with a video camera, and then an observer blind to the genotype of the animals analyzed the tapes. The time spent in the closed and open arms and the number of explorations of open arms was determined. Student’s \( t \) test was used to analyze the data.

Open Field
Mice were tested for their activity during 6 min in a 72-cm-diameter open field under dim lighting. A video tracking system (Ethovision 3.0; Noldus, Leesburg, Virginia) was used to measure the time spent in the center and borders of the open field. Data were analyzed by Student’s \( t \) test.

Fear Conditioning
The fear conditioning paradigm was assessed as previously described (Powell et al 2004). Briefly, mice were placed in individual chambers for 2 min followed by a loud tone (90 dB) for 30 sec, then immediately followed by a .8-mA footshock for 2 sec. Mice remained in the box for 1 min, at which time they again received a loud tone (90 dB) for 30 sec and then an immediate .8-mA footshock for 2 sec. The mice were immediately removed and placed back into their home cages. Each chamber was cleaned with 70% ethanol between animals. To test for context-dependent fear conditioning, 24 hours later the mice were placed back in the same boxes without a tone or shock and their behavior videotaped. The amount of time the animal spent freezing was assessed by the FreezeFrame program (Actimetrics, Wilmette, Illinois). Freezing behavior was defined as no movement except for respiration. Four hours later, the cue test was performed. To test for cue-dependent fear conditioning, mice were placed in a novel environment with no tone or shock for 3 min, followed by 3 min of the tone. The amount of time the mice spent freezing was assessed as described above. Data were analyzed by Student’s \( t \) test.

Pain Sensitivity
Responses to footshock were assessed 2 weeks after the fear-conditioning paradigm to allow the mice time to extinguish from the context. Mice were placed back in the individual chambers (MedAssociates, St. Albans, Vermont) for 2 min to habituate. The intensity of shock required to elicit flinching, jumping, or vocalization was determined by an observer blind to their genotype by delivering a 1-sec shock every 30 sec starting at 0 mA and increasing the shock by .05 mA each time until the animal vocalized its response. The maximum intensity was .6 mA.

Startle Testing
Startle testing was performed in SR-Lab System startle chambers (San Diego Instruments, San Diego, California). Each chamber contained a Plexiglas cylinder resting on a platform and a loudspeaker that produced the acoustic stimuli. Underneath the platform, a piezoelectric transducer converted the animal’s vibrations to a signal that was recorded on a computer. Individual mice were placed into the Plexiglas cylinder. The background noise (70 dB) was presented alone for 5 min, after which the animal was presented with 36 trials (six trial blocks repeated six times in a random order). The trial blocks were 70, 80, 90, 100, 110, or 120 dB, with an average intertrial time of 9 sec. The startle values were recorded for all trials.

Social Interaction with Other Mice
To assess whether the conditional MeCP2 KO mice would interact normally with other mice, we used two social interaction

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paradigms aimed at measuring social interest and social recognition, respectively. In the first paradigm, social interest was defined as the approach that the conditional MeCP2 KO or CTL animal has to an unknown social target. Conditional MeCP2 KO and CTL mice were individually housed for 1 week before testing. The testing room was maintained in complete darkness, and the video recording was performed with a camera equipped with an infrared filter. An experimental mouse was placed in an open-field environment (42 × 42 cm) containing an empty wire mesh enclosure (10 × 6.5 cm) located against one of the walls (no target), and its movements recorded with a video tracking system (Ethovision) for 2.5 min. Immediately thereafter, a C57BL/6 male mouse (Target) was placed behind the wire mesh, and the movements of the experimental animal was recorded for an additional 2.5 min. The wire mesh allowed visual and olfactory interactions between the mice but prevented direct physical contact. During the No Target and Target sessions, everything was identical except for the absence or presence of the C57BL/6 mouse. The video tracking system measured the duration of time spent in the interaction zone (8-cm-wide area surrounding the wire mesh enclosure), as well as the latency to enter into the interaction zone. The experimental animal was then returned to its home cage.

The second social interaction paradigm was used to evaluate social recognition of the experimental mice (Podhorna and Didriksen 2004). In this paradigm, the test mice were placed in individual cages for 10 min in the dark before testing. A 4-week-old juvenile C57BL/6 mouse was then placed in the cage of a test animal for 2 min, and the amount of time the experimental animal interacted with the juvenile mouse was recorded with the Observer program (Noldus). The animals were left together for an additional 8 min to further stimulate social interactions. After a total of 10 min, both animals were returned to their home cages. The same juvenile mouse was then placed back in the cage with the same experimental animal for the next 3 days, and the amount of time the experimental animal interacted with the juvenile was calculated. On the fifth day, a novel C57BL/6 juvenile mouse was placed into the experimental animal’s cage, and the amount of time the experimental animal interacted with this juvenile was recorded.

**Results**

**Conditional MeCP2 KO Mice Show a Selective Deletion of MeCP2 in Forebrain Regions**

To determine the regional distribution of the MeCP2 deletion in the conditional KO mice compared with CTL littermates, we used fluorescent immunohistochemistry. In all animals, the MeCP2 staining was localized to the neuronal nuclei. The conditional MeCP2 KO mice showed a clear reduction in MeCP2 staining in the prefrontal cortex, striatum, nucleus accumbens, hippocampus, and amygdala compared with the CTL littermates (Figure 1A); however, the level of MeCP2 in the habenula was unchanged between KO and CTL mice (Figure 1A). To examine MeCP2 levels in the cerebellum, specifically the Purkinje cells and the cells in the molecular layer, which normally express MeCP2, we performed dual fluorescent immunocytochemistry with MeCP2 and calbindin. The level of MeCP2 expression in the Purkinje cells, as well as the cells within the molecular layer, was indistinguishable between the conditional MeCP2 KO mice and CTL littermates (Figure 1A). To confirm our immunohistochemistry findings, we performed Western blot analysis of the MeCP2 protein in these specific brain regions, with the exception that the striatum and nucleus accumbens were combined owing to our inability to reliably separate these brain regions by gross dissection in mice. The amount of MeCP2 protein was analyzed as a ratio between MeCP2 and actin, the control protein, to ensure that an equal amount of protein was loaded onto the gel and transferred to the membrane. The Western blot data showed a significant reduction of MeCP2 protein in the cortex (reduction to 59% of control animals), striatum/nucleus accumbens (74%), hippocampus (64%), and amygdala (78%) of the conditional KO mice compared with CTL littermates (Figure 1B). The level of MeCP2 in the cerebellum of the KO mice, however, was unchanged (96%) compared with CTL mice.

**Conditional MeCP2 KO Mice Have Abnormal Motor Coordination**

The conditional MeCP2 KO mice were indistinguishable from CTL littermates by gross examination until approximately 16 weeks of age, at which time the KO mice were significantly heavier than the CTL mice. A weight phenotype has previously been reported for these conditional MeCP2 KO mice at this age (Chen et al. 2001). To more closely examine this phenotype, we weighed the conditional MeCP2 KO and CTL mice weekly from 7 to 24 weeks of age. The KO mice exhibited a significant increase in weight starting at 13 weeks of age (p < .05) that became more pronounced in older animals (Figure 2).

Because RTT patients suffer from motor impairments, we examined our mice for motor coordination deficits. Hindlimb and forelimb clasping has been observed in several mouse models of motor dysfunction (Auerbach et al. 2001; Guidetti et al. 2001; van Dellen et al. 2001). In conditional KO mice at 16 weeks of age, we observed a sustained clasping, a retraction of the hindlimbs toward the midline, when briefly suspending the mouse by its tail. To assess whether the conditional KO mice displayed motor coordination deficits, we used the rotarod test. The KO mice performed significantly worse than the wild-type CTL littermates on the rotarod (Figure 3A, trials 4 and 5, p < .05; interaction genotype/trial). These data are not that surprising, given the hindlimb clasping that was observed in the KO animals at approximately 16 weeks of age; however, at 16 weeks of age the KO mice also showed a significant weight phenotype compared with CTL littermates (Figure 2). To ensure that the motor coordination deficit was not the result of the weight phenotype, we examined 12-week-old KO mice and wild-type CTL littermates, using the rotarod paradigm. Conditional MeCP2 KO mice performed significantly worse on the rotarod test than wild-type mice at 12 weeks of age, even though this time point preceded any observed hindlimb clasping or weight phenotype (Figure 3B, trials 4 and 5, p < .05; interaction genotype/trial).

**Conditional MeCP2 KO Mice Exhibit Normal Locomotor Activity**

To assess locomotor activity in the conditional MeCP2 KO mice, we placed the animals individually into a novel cage for 2 hours and recorded the number of consecutive beam breaks, an indication of horizontal locomotor activity. Conditional MeCP2 KO mice exhibited a significant decrease in locomotor activity within the first 5 min of testing compared with CTL littermates (Figure 4, p < .05); however, this initial level of hypoactivity was followed by a normal level of activity for the remainder of the test period. The total amount of activity over the 2-hour period was indistinguishable between the two groups (see Figure 4, inset). Testing an animal in a novel environment has been shown to produce a mild stress effect that can initially affect locomotor activity.
Conditional MeCP2 KO Mice Have Increased Anxiety-like Behaviors

To test whether the initial hypoactivity of the conditional MeCP2 KO mice in the locomotor activity paradigm is the result of altered anxiety-like behavior, we next examined these mice in two common anxiety tests. In the elevated plus maze, conditional MeCP2 KO mice showed a significant decrease in the amount of time spent in the open arms compared with CTL littermates (Figure 5A, p < .05). The KO mice also showed a significant increase in the latency of their first entrance to the open arm (Figure 5B, p < .05).

To confirm this increased anxiety-like behavior, we also examined the mice in the open field paradigm. The conditional MeCP2 KO mice showed a significant decrease in the total amount of time they spent in the center of the open field environment compared with CTL littermates (Figure 5C, p < .05).
The conditional KO mice also showed a significant increase in latency to enter the center of the open field compared with the CTL mice (Figure 5D, \( p < .05 \)).

**Conditional MeCP2 Knockout Mice Display Impaired Fear Conditioning**

Conditional MeCP2 KO mice were assessed for emotional learning and memory with the fear conditioning paradigm. Mice were tested in a one-trial context and cue-dependent paradigm. Context-dependent fear conditioning is a task that requires an intact hippocampus and amygdala, whereas cue-dependent fear conditioning is dependent on the amygdala (LeDoux 2000; Maren 2001). In this paradigm, a mouse was habituated to a new environment and then received a loud auditory tone (90 dB, 2.8 kHz, 30 sec), followed by a footshock (0.8 mA, 2 sec). The mouse was then removed and returned to its home cage. Twenty-four hours later the mouse was placed back into the same environment, and the amount of time the animals spent freezing was recorded (context-dependent fear conditioning). Four hours later the mouse was placed in a completely novel environment, the tone was played, and the amount of time the animal spent freezing was recorded (cue-dependent fear conditioning).

The baseline level of freezing assessed before training was indistinguishable in the conditional MeCP2 KO mice and wild-type CTL littermates (Fig. 6A). In the context-dependent fear conditioning, conditional MeCP2 KO mice showed no difference in time spent freezing compared with CTL littermates (Figure 6A, \( p = .99 \)). In contrast, conditional MeCP2 KO mice showed a significant reduction in freezing in cue-dependent fear conditioning compared with their CTL littermates (Figure 6A, \( p < .05 \)). To test whether this deficit was due to a difference in pain sensitivity, we tested the response of KO and CTL mice to footshock over a range of shock intensities (.05 mA to a maximum of .6 mA). No differences between the ability of conditional MeCP2 KO mice and CTL wild-type littermates to respond (flinch, jump, or vocalize) to footshock were observed (Figure 6B). To rule out that the deficit in cue-dependent fear conditioning was due to hearing deficits in the mutants, we measured startle responses...
elicted by various acoustic intensities. The conditional MeCP2 KO and littermate CTL mice exhibited comparable startle responses at all acoustic stimuli (70–120 dB), indicating no difference of genotype (Figure 6C).

Conditional MeCP2 Knockout Mice Are Impaired in Social Interactions

To examine the social interactions of the conditional MeCP2 KO mice compared with CTL littermates, we used a two-trial social interaction test. In this paradigm, an experimental mouse was placed in an open-field target with a wire mesh at one end of the field (No Target). The amount of time each experimental mouse entered a specific interaction zone, defined as an area near the wire mesh, was calculated with the Ethovision video tracking system. The conditional MeCP2 KO mice showed slightly less time in the interaction zone than CTL mice (Figure 7A), which might be the result of the initial hypoactivity observed in these mice (Figure 4). After 2.5 min, a 10-week-old C57BL/6 mouse was placed behind the wire mesh (Target), and the experimental animal’s movement was recorded for an additional 2.5 min. The CTL mice showed a significant increase in the amount of time in the interaction zone when another mouse (Target) was present compared with the No Target baseline (Figure 7A) ($p < .05$). In contrast, the conditional MeCP2 KO mice showed no difference in time spent in the interaction zone, whether another mouse was present or not (Figure 7A). In this paradigm, the conditional MeCP2 KO mice also showed a significant increase in latency to enter the interaction zone when the Target mouse was present compared with the No Target session (Figure 7B) ($p < .01$), whereas the CTL mice showed no difference between the two sessions (Figure 7B).

In the second social interaction paradigm, we assessed the ability of the conditional MeCP2 KO mice to directly interact with another mouse. We placed either the conditional MeCP2 KO mice or the wild-type littermate CTL mice into individual cages in the dark for 10 minutes to habituate. We then placed a 4-week-old juvenile male C57BL/6 mouse into the cage of each experimental mouse. We chose to use a juvenile mouse instead of an adult mouse because of its smaller size and less-threatening appearance to the experimental animal. The amount of time the experimental animal interacted with the juvenile mouse over a 2-min period was determined with the Observer program (Noldus). On the initial interaction, the conditional MeCP2 KO mice showed a decreased trend in the amount of interaction with the juvenile mouse compared with the CTL mice (Figure 7C). We then tested the same experimental animal with the same juvenile mouse repeatedly for 4 consecutive days. At each day of testing, the conditional MeCP2 KO mice interacted less with the juveniles compared with the CTL mice. On day 5, a novel juvenile C57BL/6 mouse was placed into each experimental cage, and the amount of time the experimental mouse interacted with this juvenile was determined. The CTL mice showed a significant increase in the amount of time they interacted with the novel juvenile compared with the previous juvenile after 4 consecutive days of interaction (Figure 7C). In contrast, the conditional MeCP2 KO mice showed only a slight increase in the amount of time they interacted with the novel mouse compared with the previous juvenile animal (Figure 7C). Although the conditional MeCP2 mice interacted less with the juvenile mice on all days of testing compared with the CTL mice, the only significant difference between the groups was on day 5 ($p < .05$).
Discussion

The results presented in this study show that conditional MeCP2 KO mice display behavioral deficits that are characteristic of many features of RTT. These conditional MeCP2 KO mice have motor coordination deficits, increased anxiety, and impaired social interactions with other mice. These mice, however, have normal locomotor activity and unimpaired context-dependent fear conditioning, suggesting that the behavioral deficits observed are the result of loss of MeCP2 function in postnatal brain and not the result of generalized global deficits.

Findings from the present study differ in several important respects from previously published data characterizing mice with a truncated MeCP2 (MeCP2<sup>308</sup>/y) mutation (Shahbazian et al 2002). First, the previous study used a knock-in approach to express the truncating MeCP2 mutation into the <i>Mecp2</i> gene. We used a strategy in which we selectively deleted MeCP2 from postnatal brain to investigate the role of this gene in brain in mediating behavior. Second, we found that the conditional MeCP2 KO mice have a different behavioral profile than the MeCP2<sup>308</sup>/y mutant mice. Although both lines initially exhibit no obvious phenotypes, the MeCP2<sup>308</sup>/y line develops hindlimb clasping at an earlier age than the conditional MeCP2 KO mice. The MeCP2<sup>308</sup>/y mice also exhibit additional phenotypes (kyphosis and spontaneous myoclonic seizures) that were not observed in the conditional KO mice. The conditional KO mice, however, had a more pronounced impairment in motor coordination, as assessed by the rotarod, than was observed in the MeCP2<sup>308</sup>/y mice. This impairment in motor coordination in the conditional KO mice preceded the observed hindlimb clasping and suggests that subtle changes in motor coordination might be present even before the emergence of the more overt phenotype. We found that the conditional KO mice had an initial level of hypoactivity that was followed by normal locomotor activity, whereas the MeCP2<sup>308</sup>/y mice showed initial normal locomotor activity followed by a period of reduced activity. One observation that was noted in both animal mutant models was abnormal social interactions; however, we found that the conditional KO mice displayed an impaired ability to interact with wild-type mice, even juvenile mice that would not be perceived as a threat, suggesting that the lack of MeCP2 impairs social interaction. In contrast, wild-type mice were found to interact significantly less with the mutant MeCP2<sup>308</sup>/y mice, suggesting that the wild-type mice were actively avoiding the mutant mice. There were some consistent findings between the conditional MeCP2 KO mice and the MeCP2<sup>308</sup>/y mice, including an increased anxiety-like behavior compared with wild-type littermates, as well as normal context-dependent fear conditioning.

The conditional MeCP2 KO mice generated in this study show that selective loss of MeCP2 in broad forebrain regions is sufficient to mediate many RTT features (increased anxiety, impaired motor coordination, and social deficits) but not other deficits observed in the truncated mouse line (tremors, seizures, and kyphosis). These data start to provide a framework to elucidate the neural circuits that are involved in the loss of MeCP2 in mediating these behaviors. These data also suggest

Figure 6. Conditional methyl-CpG binding protein 2 (MeCP2) knockout (KO) mice exhibit impaired cue-dependent fear conditioning. Conditional MeCP2 KO mice displayed normal baseline freezing before training. (A) After a one-trial fear conditioning paradigm, the conditional MeCP2 KO mice were indistinguishable compared with littermate control (CTL) mice in the average amount of time spent freezing when tested 24 hours after training for contextual memory. In contrast, conditional MeCP2 KO mice displayed a significant decrease when tested for cue-dependent memory (KO mice, n = 13; CTL mice, n = 16; p < .05). (B) The conditional MeCP2 KO mice have similar responses to footshock sensitivity as the wild-type littermate CTL mice (KO mice, n = 7; CTL mice, n = 8; p > .05). (C) The conditional MeCP2 KO mice exhibited a similar startle response at multiple pulse intensities, ranging from 70 to 120 dB (KO mice, n = 8; CTL mice, n = 8; p > .05). Values are means ± SEM.
that the two mouse models for RTT, the mutant MeCP2<sup>308/Y</sup> mice and the conditional KO mice, are strikingly different. Expression of a disease causing MeCP2 mutation on a null MeCP2 background might reveal a more severe phenotype compared with the absence of MeCP2. This might be due to mutant MeCP2 interacting with other proteins and preventing the substitution of MeCP2 by a related protein, such as Mbd1 and Mbd2. Therefore, mutant MeCP2 might exert a dominant negative effect, whereas the loss of MeCP2 might allow a partial substitution by a related protein to alleviate some of the phenotype (Zhao et al 2003).

One of the most surprising findings of this study is that loss of MeCP2 in broad forebrain regions produces impaired motor coordination. The CaMKII-Cre driver line used in these studies does not mediate recombination in the neuromuscular axis or in the cerebellum, a brain region where MeCP2 is highly expressed (Jung et al 2003). Our immunocytochemistry data show that MeCP2 levels in the cerebellum are indistinguishable in conditional KO and CTL littermate mice (Figure 1A); however, the loss of MeCP2 was pronounced in the motor cortex of the conditional KO mice (data not shown). Within the basal ganglia circuitry, we observed loss of MeCP2 in the striatum, ventral pallidum, ventral tegmental area, and motor thalamic nuclei of the conditional KO mice but normal “wild-type” levels of expression in the globus pallidus and substantia nigra (data not shown). Our data suggest that the loss of MeCP2 in broad forebrain regions, including some regions of the basal ganglia, is sufficient to mediate motor impairments and that alterations in MeCP2 expression in the cerebellum and neuromuscular axis of these conditional KO mice can be excluded as the cause of the motor deficits. More targeted deletion of MeCP2 within these specific brain regions is necessary to address the role of MECP2 in these specific brain regions in mediating RTT-based motor impairments.

Our findings indicate that a partial reduction of MeCP2 in broad forebrain regions is sufficient to recapitulate symptoms of RTT. Conversely, previous studies have demonstrated that overexpression of wild-type MeCP2 (approximately two- to threefold higher levels of expression) in mice produces symptoms of RTT as well as behavioral and electrophysiological abnormalities (Collins et al 2004; Luikenhuis et al 2004). Taken together, these data suggest that slight alterations in MeCP2 levels can produce profound behavioral effects and further highlight the importance of tightly regulating MeCP2 expression.

In our characterization of the conditional MeCP2 KO mice, we found a weight phenotype that might be the result of the genetic background of the mice rather than the loss of MeCP2 per se. Previous work demonstrated that this floxed MeCP2 line, when crossed to either CaMKII-Cre or Nestin-Cre, produces a significant gain of body weight (Chen et al 2001); however, another
study has shown that a Nestin-Cre driven conditional MeCP2 KO line has low body weight compared with CTL littermates (Guy et al 2001). Given that this latest study found that MeCP2 null KO mice had varying weight phenotypes depending on the genetic background (Guy et al 2001), it is quite possible that the same phenotypic effect is observed with the conditional KO line.

Our results show that postnatal loss of MeCP2 in the forebrain is sufficient to mediate behavioral aspects of RTT in mice. This information is important for understanding the neurobiological basis of RTT, begins to provide a framework of the neural circuitry that is involved in mediating aspects of the disease, and may contribute to a better understanding of other disorders, such as autism, that share similar features with RTT.

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