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Targeted delivery of an *Mecp2* transgene to forebrain neurons improves the behavior of female *Mecp2*-deficient mice

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Rett syndrome is an X-linked neurological condition affecting almost exclusively girls that is caused by mutations of the *MECP2* gene. Recent studies have shown that transgenic delivery of MeCP2 function to *Mecp2*-deficient male mice can improve their Rett-like behavior. However, as the brain of a Rett girl contains a mosaic of MeCP2 expressing and non-expressing neurons, and the over-expression of MeCP2 in neurons can induce a severe progressive neurological phenotype, testing whether functional rescue can be achieved by gene re-introduction strategies in a female model of Rett syndrome is warranted. To address this, we generated transgenic mice expressing an epitope-tagged *Mecp2* transgene in forebrain neurons. These mice over-express MeCP2 protein at about 1.6 times normal levels in cortex and develop impaired motor behavior by 9–12 months of age. To test whether forebrain-targeted MeCP2 restoration would improve behavior in female *Mecp2*$^{-/+}$ mice, we crossed these transgenics with *Mecp2*$^{-/+}$ mice and examined the behavioral properties of the female rescue mice for 1 year. These assessments revealed that the diminished rearing activity, impaired mobility and the diminished locomotive activity of female *Mecp2*$^{-/+}$ mice were restored to wild-type levels in the rescue mice. These results show that improvement of Rett-like behavior can be achieved in *Mecp2*$^{-/+}$ females by targeted gene re-introduction without inducing deficits relating to MeCP2 over-expression.

INTRODUCTION

Rett syndrome is a pediatric neurological disorder first described in 1966 that affects primarily young girls (1). Typical symptoms appear gradually over time and include stereotypical hand wringing, loss of acquired speech, cognitive impairment, loss of locomotive ability, breathing irregularities, difficulty in eating and intractable seizures (2–4). Physically, Rett girls tend to have significantly shorter life spans, are growth retarded and, of particular note, the brain of a Rett child is both undersized and under-weighted. In fact, the average size of a mature Rett brain is approximately the same as that of a 12-month infant (5–7). Despite the reduced brain size, however, decreases in neuronal number have not been detected. Rather, a decrease in the degree of dendritic branching has been reported, particularly in cortical brain regions (8). These observations have led to the hypothesis that Rett syndrome is a condition in which the brain either fails to mature or is incapable of maintaining a mature phenotype (6,9,10).

Loss-of-function mutations within the gene encoding methyl CpG-binding protein type 2 (*MECP2*) are responsible for the majority of Rett syndrome cases (11). Mutant mice have been developed that completely or partially lack MeCP2, and each of these lines develops a collection of behavioral impairments reminiscent of clinical Rett syndrome (12–15). These models have recently been utilized for proof-of-principle studies showing that global (16) and neuronally targeted (17–19) re-introduction of functional MeCP2 improves at least some of the behavioral abnormalities

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of male Mecp2-deficient mice. Furthermore, phenotypic improvement can also be achieved in symptomatic male and female MeCP2-deficient mice by reactivating endogenous *Mecp2* expression (20). Collectively, these studies show that the Rett-like phenotype of these mutant mice is not irremediable and raises the possibility that gene re-introduction strategies may have clinical potential.

Because of the X-linked nature of *MECP2* and random X chromosome inactivation, however, the brain of a Rett girl contains a mosaic of *MECP2* wild-type and *MECP2* mutant-expressing neurons (3,7,21). As moderate overexpression of MeCP2 is sufficient to induce neurological impairment (16,17), it remains unknown whether a similar targeted transgenic complementation strategy that was beneficial in *Mecp2*-null males would also be beneficial in female subjects, where the re-introduction would elevate the expression of MeCP2 in the neurons expressing the wild-type allele. In this study, we address this issue by testing whether the behavioral deficits of female *Mecp2*$^{-/+}$ mice can be improved by the ectopic re-introduction of an epitope-tagged *Mecp2* transgene specifically in forebrain neurons.

RESULTS

Transgenic mice over-expressing MeCP2 in forebrain neurons develop delayed behavioral impairments

Mutant mice were generated in which a tetracycline response element (TRE)-containing promoter cassette was fused to a hemagglutinin (HA)-tagged mouse *Mecp2-e2* cDNA (Fig. 1). Four lines of transgene-positive founder mice were identified by Southern blot and PCR. Each line was expanded by backcrossing with C57B/6 wild-type mice (see Materials and Methods). None of these single-transgenic lines expressed the transgene at detectable levels in brain, indicating that the random insertion of the transgene did not promote its activation (not shown). Each transgenic line was viable, fertile and their adult body weight did not differ from wild-type mice of the same litter (data not shown). Mice from each founder line were crossed with calcium calmodulin kinase IIα promoter-driven reverse tetracycline transactivator (CamKII-rtTA) transgenic mice to generate the inducible transgenic mice. For simplicity and consistency with a recent report (19), these double transgenic mice will be referred to as 'inducible transgenics'. In contrast to expectation, however, immunoblot analysis of cortical tissue from inducible transgenic line B mice revealed that the transgene was expressed basally in the absence of tetracycline or analog (Fig. 1). None of the other founder lines expressed the transgene under basal conditions. The relative prevalence of total MeCP2 immunoreactivity in cortical tissue from these inducible mice was 160.4 ± 19.8 times that of wild-type cortex (Fig. 1). In comparison, the level of MeCP2 expression in cortical tissue from Mecp2$^{-/+}$ and female rescue transgenic mice was 48.7 ± 5.8 and 112.1 ± 6.4%, respectively (Fig. 1).

Immunohistochemical assessments of the brain from adult inducible transgenic line B mice revealed that the HA-MeCP2 transgene was expressed in cortex and striatum, whereas little or no transgene immunoreactivity was detected in thalamus or brain stem (Fig. 2). High-resolution

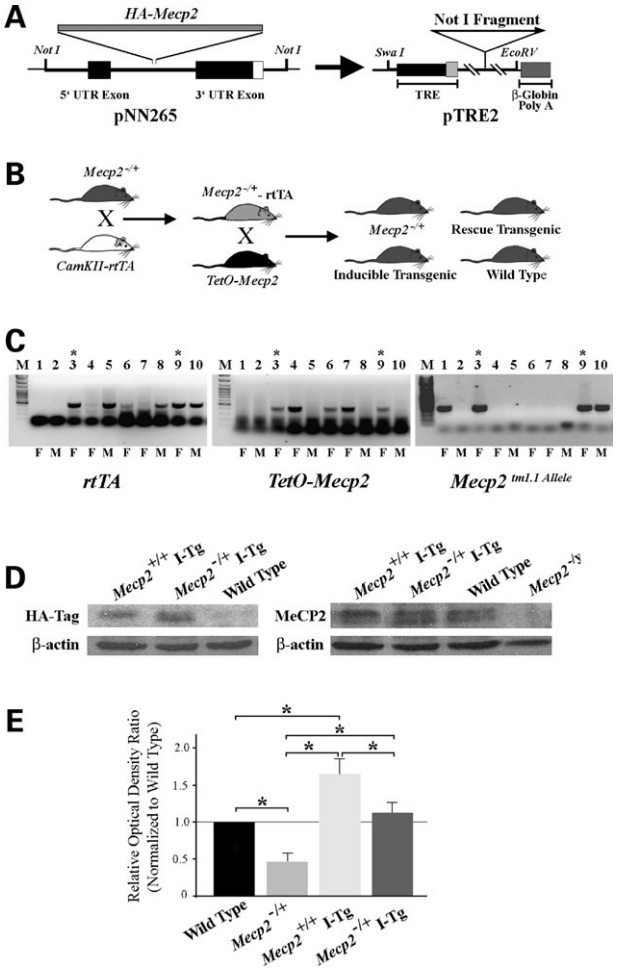

Figure 1. Generation of inducible transgenic mice. (**A**) Illustration of the transgene cassettes employed. The *HA-Mecp2* cDNA was initially introduced into the pNN265 shuttle vector that contains a hybrid 5′ exon to facilitate nuclear export of the transcribed mRNA, and a 3′ untranslated exon with an SV40 polyadenylation signal (30). This expression carriage was excised from pNN265 with *Not*1 and placed downstream of the TRE of the pTRE2 vector. The mini CMV promoter within the TRE is denoted in gray. The transgene for micro-injection was excised from this plasmid by restriction digestion with *Swa*1 and *Eco*RV. (**B**) Schematic illustration of the breeding scheme used to generate the different cohorts of transgenic mice. Female *Mecp2*$^{-/+}$ mice were crossed with male CamKII-rtTA mice to introduce the rtTA factor into the *Mecp2*$^{-/+}$ background. The female double mutants were then crossed with transgenic line B mice. The mice born from this cross were evaluated for a 1 year period and then genotyped and assigned to groups. (**C**) Example of PCR-based genotyping. The different panels show the PCR detection of the rtTA factor (left panel), the *HA-Mecp2* transgene (middle panel) or the mutant Mecp2 allele (right panel) in mice from two litters. In this group, number 6 is an over-expressing inducible transgenic, whereas numbers 3 and 9 are rescue mice (denoted by asterisks). (**D**) Detection of expressed transgene in cortical tissue. Shown are representative immunoblots of cortical tissue derived from an inducible transgenic mouse, a transgenic rescue mouse and a wild-type mouse. The left panel shows HA immunoreactivity, whereas the right panel shows total MeCP2 immunoreactivity in samples from the same mice. An MeCP2$^{-/y}$ mouse cortical sample (right panel) illustrates specificity. (**E**) Histogram depicting the normalized expression level (mean ± standard error, $n = 3$ for each) of MeCP2 protein in cortical tissue from inducible transgenic line B mice, female rescue mice and female *Mecp2*$^{-/+}$ mice. The values represent the MeCP2 densitometric immunoreactive intensity of the inducible transgenic, female *Mecp2*$^{-/+}$ and female rescue mice, normalized to the intensity of the wild-type mouse sample on the same blot. Asterisks denote statistically significant differences between the indicated groups (one-way ANOVA, $P < 0.05$ with Bonferroni *post hoc* correction for multiple comparisons).

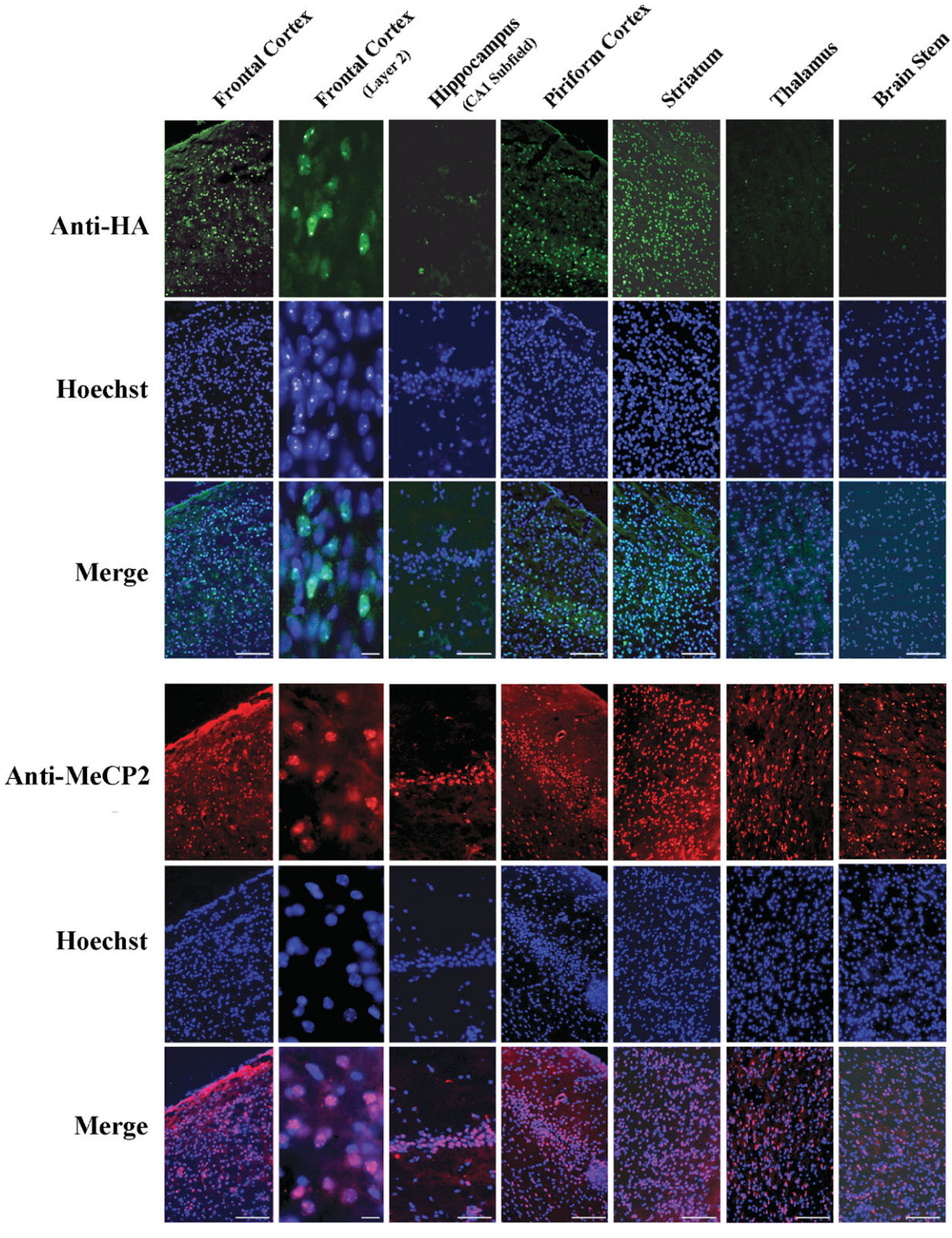

Figure 2. Immunohistochemical localization of the HA-MeCP2 protein in brain regions of adult inducible transgenic mice. The two sets of panels show individual fluorescence channels and a merged image of the same field to compare the expression profile of the inducible transgene with endogenous MeCP2. In the top panels, transgene immunoreactivity is detected with anti-HA antibodies (green) in specific brain regions of an adult line B inducible transgenic mouse. In the bottom panels, endogenous MeCP2 immunoreactivity (red) is detected in the same brain regions of an adult wild-type mouse. In each set, Hoechst 33215 is included to label all cell nuclei in the field. Strong transgene immunoreactivity is observed in frontal cortex, piriform cortex and striatum, whereas little or no transgene immunoreactivity is detected in hippocampus, thalamus and brain stem. The higher magnification image of frontal cortex shows the expressed transgene to localize within the nucleus, where it displays a staining pattern similar to endogenous MeCP2. Scale bars for low and high magnification panels are 100 and 10 μm, respectively.

fluorescence microscopy revealed that transgene immunoreactivity localized largely within the nucleus, where it displayed a predominantly punctate staining pattern that overlapped with major satellite DNA regions (Fig. 2). Occasional neurons were identified in cortex and striatum, in which the transgene displayed perinuclear or diffuse cytoplasmic staining, but these neurons constituted <10% of the total transgene-positive population. It is worth noting that in this inducible transgenic line, little or no transgene expression was detected within the hippocampus (Fig. 2), despite strong transgene expression being previously observed in the hippocampus of other inducible transgenic mice generated with the same CamKII-rtTA transgenic mouse (22). Although the reason for this atypical expression profile is unclear, it may result from position effects relating to the site of transgene integration in the genome.

MeCP2 over-expression in forebrain neurons is sufficient to promote delayed behavioral impairments

Previous studies have demonstrated that mice over-expressing MeCP2 at two-fold or greater levels throughout the brain develop severe behavioral impairments (16,17). To test whether the modest elevation in MeCP2 selectively in forebrain neurons would also induce phenotypic deficits, the behavior of the inducible transgenic mice was assessed using the open-field behavioral apparatus (see Materials and Methods). These assessments were done first between 3 and 6 months of age, and then again on the same cohort of subjects between 9 and 12 months of age. No significant differences in total activity, rearing activity, time spent motionless or average activity speed were observed between wild-type and the inducible transgenic mice at 3–6 months of age (Fig. 3). By 9–12 months of age, however, several significant impairments were detected in the inducible transgenic mice. Compared with age-matched female wild-types, female inducible transgenics at this age displayed 26% less total active time, 46% fewer total rearing events and spent twice the amount of time immobile (Fig. 3). Although less active overall, when these mice were active their average activity speed did not differ from wild-type mice. This suggests that the observed behavioral impairments do not arise from neuromuscular axis deficits, but rather from neuronal impairment. Thus, consistent with what has been observed previously for higher levels of MeCP2 over-expression (16,17), the modest over-expression of MeCP2 selectively in forebrain neuronal populations is sufficient to induce behavioral impairments in mice, but with a more delayed onset time and an overall diminished level of severity (16,17).

To examine whether the over-expression of MeCP2 in forebrain neurons had any effect on motor co-ordination, we examined the balance performance of the inducible transgenics on the rotorod apparatus. No significant differences in the average latency time before falling off the rotating rod, or the rotational speed that was tolerated before falling, were detected between the inducible transgenic and wild-type mice (Fig. 3). In addition, the rotorod paradigm that we employed allowed task improvement rate to be assessed. Consistent with this, no significant differences in improvement rate were observed between wild-type or line B inducible transgenic mice (Fig. 3).

The impaired open-field performance of female Mecp2$^{-/+}$ mice is partially corrected by expressing the Ha-MeCP2 transgene in forebrain neurons

We then assessed whether introducing HA-MeCP2 transgene expression into forebrain neurons of *Mecp2*$^{-/+}$ mice would improve any of their Rett-like behavioral phenotypes. Heterozygous female *Mecp2*$^{-/+}$ mice develop a mild and temporally delayed behavioral phenotype compared with complete male *Mecp2*$^{-/y}$ mice (23), with notable behavioral impairments becoming evident only by about 9 months of age (20). Consistent with these previous observations, our cohort of 3–6-month-old female *Mecp2*$^{-/+}$ mice did not display any significant differences from age-matched female wild-types in total active time, total number of rearing events, time spent motionless or in their average activity speed in the open-field tests (Fig. 4). The behavior of our rescue mice at this stage of development also failed to show any differences from wild-type in the open field (Fig. 4).

At 9–12 months of age, however, behavioral deficits in the open-field parameters were readily evident in the female *Mecp2*$^{-/+}$ mice. Compared with age-matched female wild-type mice, this cohort of mutants displayed 43% less total active time, 64% fewer total rearing events, spent more than twice as much time immobile and made 47% fewer complete exploratory trips in the open-field test (Fig. 4). These behavioral impairments are similar to what has been reported previously for female *Mecp2*$^{-/+}$ mice at this age (20,23), and intriguingly, their impaired behavior was similar in magnitude to what was observed for our inducible transgenic mice (Fig. 3). In contrast to the impaired performances observed for both the female *Mecp2*$^{-/+}$ mice (Fig. 4) and the inducible transgenics at 9–12 months of age (Fig. 3), the behavior of our rescue mice in several open-field parameters was significantly improved and restored to wild-type levels (Fig. 4). For example, the total time spent mobile, the average rate of mobility, the total number of rearing events and the number of complete cage explorations for the rescue group were equivalent to wild-type levels and significantly improved above the values of the female *Mecp2*$^{-/+}$ mice. These results therefore indicate that the ectopic re-introduction of MeCP2 into female *Mecp2*$^{-/+}$ mice can rescue at least some of their behavioral deficits without introducing the deficits caused by MeCP2 over-expression. Similarly, as our inducible transgenic mice also displayed deficits in these behaviors, these results indicate that a mosaic diminution of MeCP2 can also rescue the impaired behavior resulting from its over-expression.

The impaired balance and center-field rearing activity of female Mecp2$^{-/+}$ mice is not improved by forebrain expression of the HA-MeCP2 transgene

In addition to the impairments in open-field behavior presented earlier, female *Mecp2*$^{-/+}$ mice of 9–12 months age also demonstrated behavioral impairment on the rotorod task and showed significantly diminished rearing activity in the center region of the open-field apparatus (Fig. 5). In contrast

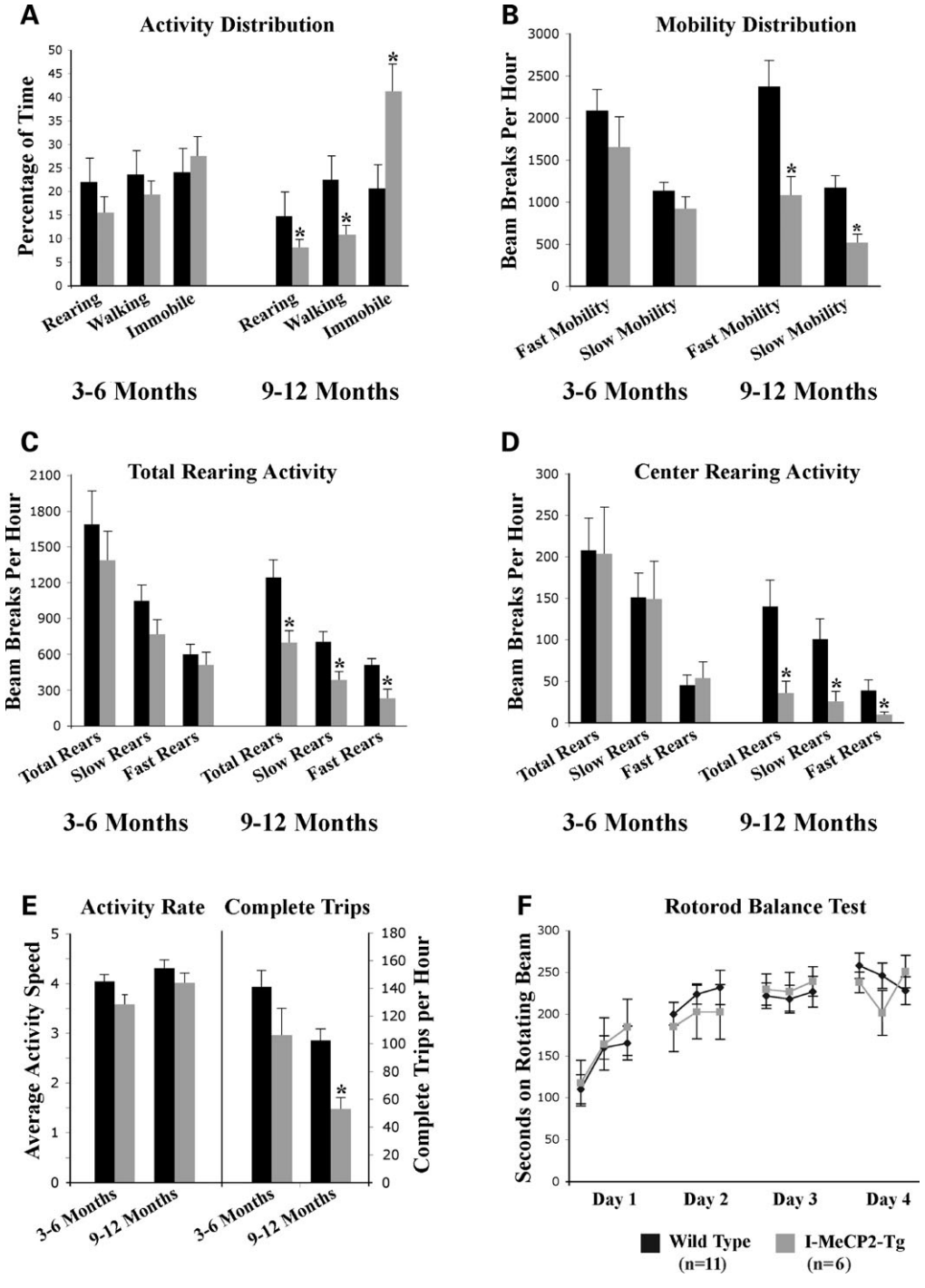

Figure 3. The open-field behavior of the inducible transgenic mice is initially preserved, but impairments develop later in life. (**A**)–(**E**) Histograms showing the behavioral performances of 3–6-month-old and 9–12-month-old wild-type mice (black bars) and inducible transgenic mice (gray bars) in different open-field tests. (**F**) Illustration of the performance of 9–12-month-old wild-type and inducible transgenic mice on the rotorod test. (A) The percentage of time during the 1 h observation window that the mice spent rearing, walking or immobile. (B) The number of beam breaks associated with fast or slow movement for the younger and older mice. Fast movement is defined as consecutive beam breaks occurring <200 ms apart, whereas slow movement denotes consecutive beam breaks occurring with a time interval of >200 ms. (C) The total number of beam breaks corresponding total rearing activity, and the number of fast and slow rears during the observation period. The same fast and slow criteria as mentioned earlier are used. (D) The total number of beam breaks in the center field of the open-field box, and the number of breaks corresponding to fast and slow rearing activities in this region. The center field is defined as beam breaks that do not involve the detection lasers along the periphery of the open-field apparatus. (E) The average rate at which the subject performs an activity is shown in the left panels, whereas the average number of complete front-to-rear exploration trips taken by a subject during the observation period is presented in the right panels. Asterisks denote statistically significant differences between wild-type and inducible transgenics at the specific task and age (one-way ANOVA, $P < 0.05$). (F) The time subjects balanced themselves on an accelerating rotating rod (presented in seconds). The test was performed three times a day for four successive days. The time spent on the rod was recorded when the subject fell. Mice that held onto the rod and circled during a trial were excluded from analysis. No significant differences were observed in the rotorod performance between the wild-type and inducible transgenic mice, and no differences were observed in the rate at which the different groups improved their performance on the task (one-way ANOVA, $P < 0.05$).

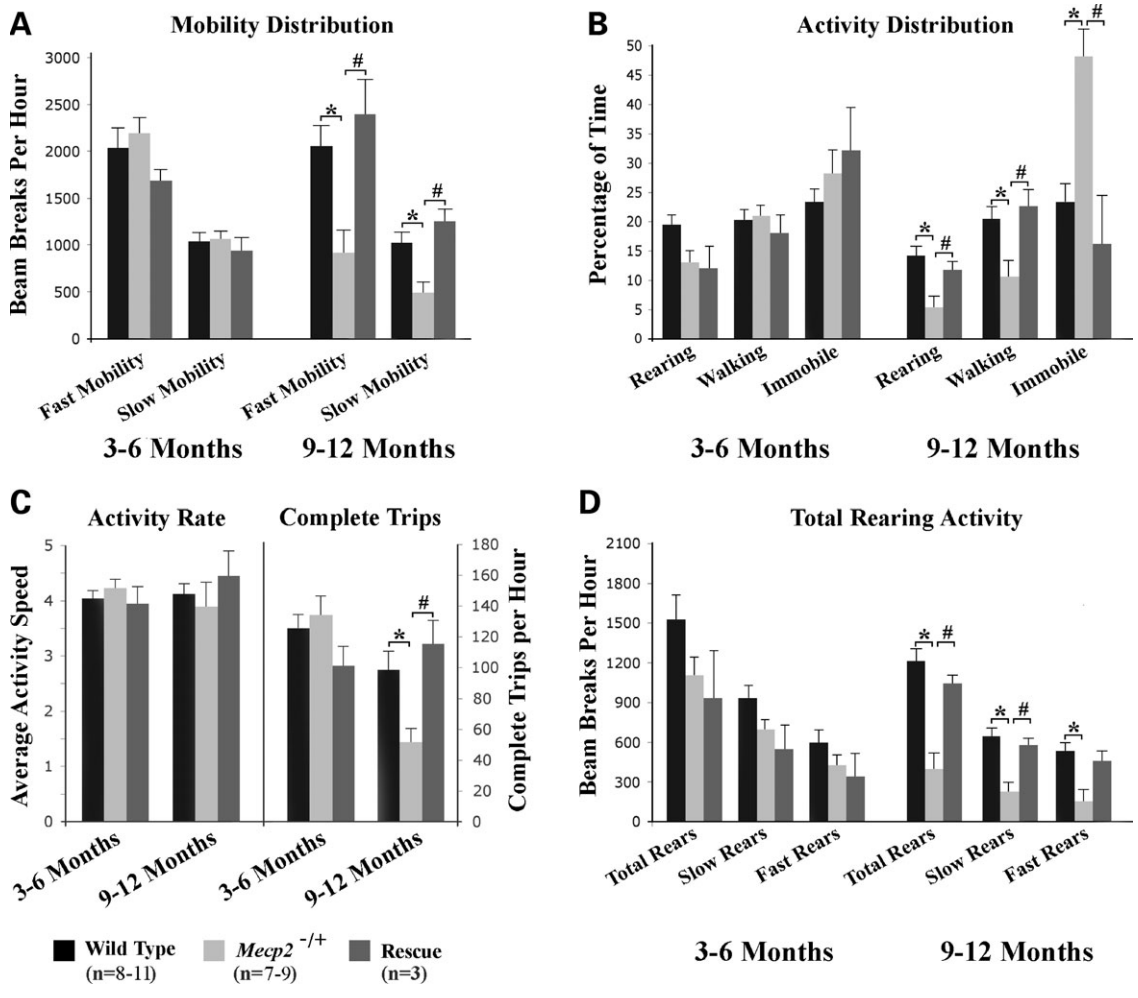

Figure 4. The open-field behavioral impairments of female *Mecp2*$^{-/+}$ mice are improved by re-introducing HA-MeCP2 into forebrain neurons. (**A**)–(**D**) Histograms showing the open-field behavioral performance properties (mean and standard error) of female wild-type mice (black, $n = 8–11$), female *Mecp2*$^{-/+}$ mice (lighter gray, $n = 7–9$) and female *Mecp2*$^{-/+}$ mice expressing the HA-MeCP2 transgene in forebrain neurons (darker gray, $n = 3$) at 3–6 months and 9–12 months of age. Fast and slow movements are as defined in Figure 3. No significant differences in any of these open-field parameters were observed in the cohorts at 3–6 months of age. At 9–12 months of age, however, the cohort of female *Mecp2*$^{-/+}$ mice displayed significantly less fast and slow mobility counts (A), spent significantly less time rearing and walking, but more time immobile (B), made significantly fewer complete exploration trips in the test cage (C) and made significantly fewer fast and slow rearing events (D) compared with wild-type controls. Each of these behavioral deficiencies (except fast rearing, where the *P*-value was 0.078) was significantly improved and restored to wild-type levels in the female rescue mice expressing the HA-MeCP2 transgene in forebrain neurons. $^{*}P < 0.05$ compared with wild-type mice; $\#P < 0.05$ compared with *Mecp2*$^{-/+}$ mice (one-way ANOVA with Tukey's *post hoc* correction for multiple comparisons).

to the restoration of behavioral performances presented earlier, however, neither the impaired rotorod balance performance nor the deficit in center field rearing of the heterozygous *Mecp2*$^{-/+}$ mice was significantly improved in the rescue mice. In the open-field test, 9–12-month-old female *Mecp2*$^{-/+}$ mice displayed <3% the number of center field rears in the open-field apparatus compared with age-matched female wild-types. Both their fast and slow center rearing activities were similarly affected (Fig. 5). Although there was a trend towards modest improvement in the average center field rearing activity of the rescue mice (18% of wild-type levels), this increase was not statistically different from the *Mecp2*$^{-/+}$ mice ($P = 0.084$, Fig. 5). A similar modest trend but overall lack of significant improvement compared with *Mecp2*$^{-/+}$ mice was observed for the fast and slow center rearing activities ($P = 0.068$ and 0.089, respectively) of the rescue mice. On the rotorod apparatus, the lack of behavioral improvement in the rescue mice was clearly apparent. The performance of 9–12-month-old female *Mecp2*$^{-/+}$ mice on the rotorod test was significantly impaired compared with wild-type for each trial after the first on day 1 (Fig. 5). The performance of the rescue mice on the rotorod was not significantly different on any trial from female *Mecp2*$^{-/+}$ mice and remained well below the performance levels of the wild-types. Thus, although functional improvement was achieved in some behavioral tasks by targeted re-introduction of MeCP2, these data also show that the forebrain expression of the *HA-Mecp2* transgene was not able to correct the balance deficiency or the diminished center field rearing activity of older female *Mecp2*$^{-/+}$ mice.

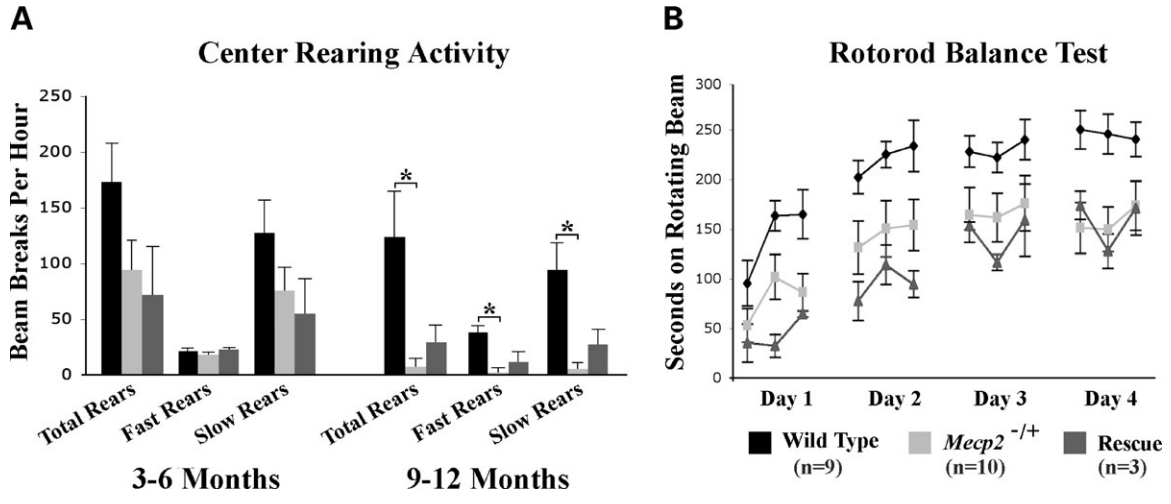

Figure 5. The ectopic re-introduction of the MeCP2 transgene protein in forebrain neurons does not improve the impaired center field rearing behavior or the rotorod performance of female *Mecp2*$^{-/+}$ mice. (**A**) The cumulative data (mean and standard error) of the center field rearing activity of the three cohorts of mice. At 9–12 months of age, the female *Mecp2*$^{-/+}$ mice showed significantly fewer total rears in the center field, and both their fast and slow rearing activities in the center field were diminished from wild-type levels. The behavior of the rescue cohort of mice ($n = 3$) in center field rearing was not improved from the female *Mecp2*$^{-/+}$ mice ($n = 7$–9). $^{*}P < 0.05$ compared with wild-type mice; $\#P < 0.05$ compared with *Mecp2*$^{-/+}$ mice (one-way ANOVA with Tukey's *post hoc* correction for multiple comparisons). (**B**) The cumulative data (mean and standard error) of the rotorod performance of the three cohorts of mice at 9–12 months of age. Both the female *Mecp2*$^{-/+}$ mice ($n = 10$) and the rescue mice ($n = 3$) performed significantly below wild-type levels ($n = 9$) for each trial day excluding the first. No differences were observed between the balance performance of the female *Mecp2*$^{-/+}$ mice and the rescue mice. $^{*}P < 0.05$ compared with wild-type, two-way ANOVA (genotype and trial) with Tukey's *post hoc* correction.

DISCUSSION

In this study, we tested whether specific aspects of the Rett-like behavior of heterozygous *Mecp2*$^{-/+}$ female mice could be improved by forebrain-targeted MeCP2 transgene delivery. Our strategy was to employ an inducible transgene system that in principle should allow the transgene to be induced at specific times by pharmacological activation of the transactivator protein. Such a system would allow one to activate the transgene both prior to and after the onset of Rett-like symptoms in the inducible transgenic mice. While the conditional inducibility of the tetracycline responsive system has been demonstrated previously (22), our mice expressed the transgene at a basal level, and this expression was sufficient to induce behavioral impairments at later stages of development. This result is not necessarily unexpected, as the ability of the rtTA factor to bind weakly to TREs in the absence of tetracycline has been shown previously (24,25). Although this basal expression precluded an induction study, the impaired behavioral performance of our mice comports well with previous studies, demonstrating global MeCP2 over-expression induces behavioral deficits (16,17). Collectively, these results highlight that even the modest over-expression of MeCP2 in specific neuronal populations is sufficient to promote delayed behavioral impairments in mice.

Our study also complements recent rescue reports on male *Mecp2*-null mice by Giacometti *et al*. (18) and Alvarez-Saavedra *et al*. (19). While each of these studies employed the CamKII promoter to directly or indirectly activate the expression of an *MeCP2* transgene in forebrain neurons, our study focused on female mice and included a longitudinal comparison of specific behavioral properties within cohorts of over-expressing, heterozygous *Mecp2*$^{-/+}$, and rescue mouse lines. We chose to assess female mice, as Rett syndrome is predominantly a female condition that culminates from a mosaic pattern of neurons expressing the wild-type or the dysfunctional *MECP2* allele. In addition, *Mecp2*$^{-/+}$ female mice remain reproductively fertile, display an apparent plateau phase in symptomatic progression and have a lifespan more consistent with what is seen clinically than *Mecp2*-null male mice (20,23). Importantly, the use of a female model as the rescue group also begins to test whether changing the mosaic expression pattern of MeCP2 across a population of neurons will be tolerated or induce behavioral impairments such as those seen in mice over-expressing MeCP2 at moderate levels (16,17). Our results indicate that for at least low levels of transgene expression, altering the mosaic expression pattern of MeCP2 across a population of forebrain neurons in *Mecp2*$^{-/+}$ female mice is tolerated and can be used to improve at least some of their Rett-like behavioral impairments.

It is becoming increasingly appreciated that MeCP2 expression must be maintained within a fairly narrow window in neurons, and perhaps within subsets of neurons within specific populations, to avoid behavioral consequences. Using a method of estimating MeCP2 transgene expression previously employed (17,19), immunoblot analysis of cortical tissue from our inducible transgenic mice revealed that they express MeCP2 at $\sim 160 \pm 19.8\%$ wild-type levels, and our rescue mice express MeCP2 at $112 \pm 6.4\%$ wild-type (Fig. 1). The heterozygous *Mecp2*$^{-/+}$ mice expressed MeCP2 at $48.8 \pm 6.4\%$. If the transgene is expressed in the same number of cells overall as endogenous MeCP2, this would indicate that the average transgene expression level would be approximately 0.6 times that of normal MeCP2. We attempted to identify the number of neurons co-expressing endogenous MeCP2 and the ectopically expressed transgene using dual-label immunohistochemistry, but to date our attempts have not been successful (the HA tag appears to be

blocked in double-label procedures). As an alternative strategy to estimate expression, we counted the number of MeCP2 or HA-positive neurons in random fields from position-matched cortical and striatal sections of female wild-type and transgene-expressing inducible transgenic mice. No significant differences in the number of transgene-expressing and MeCP2-expressing neurons were detected in either region of these respective mice (data not shown). Although caution is warranted in interpreting such results, as MeCP2 expression levels vary significantly in magnitude between different neurons (26), these data are consistent with the level of transgene expression in our over-expressing mouse being slightly below, but not greater than, that of endogenous MeCP2 in cortical neurons. The relatively modest behavioral impairments seen in these mice, compared with the more severe phenotypes observed in transgenic mice over-expressing MeCP2 at greater levels (16,17), would seem consistent with this estimate. Importantly, though, the behavioral improvement in the open-field tasks seen in the female rescue mice demonstrates that modest changes in mosaic MeCP2 expression levels can be tolerated, at least in forebrain neurons, without causing over-expression related behavioral impairments. As the expression of wild-type MeCP2 in neurons of *Mecp2*$^{-/+}$ mice is diminished from wild-type levels and influenced by mosaic pattern (27), a low level of MeCP2 re-introduction into a subset of MeCP2-deficient neurons could also affect the expression of adjacent wild-type-expressing neurons, and thereby provide benefit. Additional experiments will be required to test this possibility. Taken together, however, these results do provide the first evidence that at least some of the Rett-like deficiencies of *Mecp2*-deficient female mice can be corrected by gene re-introduction. It will be of interest to determine whether a similar phenotypic improvement will be observed in female *Mecp2*$^{-/+}$ mice over-expressing an MeCP2 transgene at higher levels. In addition, it is also worth noting that the improved behavior of our rescue group indicates that at least some of the behavioral deficits caused by the over-expression of MeCP2 (Fig. 3) can be improved by a mosaic diminution of MeCP2 expression.

Our observation that some of the behavioral deficiencies of female *Mecp2*$^{-/+}$ mice can be corrected by transgenic re-introduction adds to the growing evidence that Rett syndrome is not an irremediable condition. Recently, however, Alvarez-Saavedra *et al*. (19) reported that targeted restoration of MeCP2 into forebrain neurons using a similar CamKII promoter-driven transactivating system to what we employed failed to rescue the primary phenotypic abnormalities of *Mecp2*$^{-/y}$ and *Mecp2*$^{308/y}$ male mice. Although the results of our study may appear to contrast with theirs, they focused on *Mecp2*-deficient male mice, which have a more severe phenotype than *Mecp2*$^{-/+}$ female mice, and employed a more robust transactivator system (tTA versus rtTA). Despite these differences, and the lack of significant improvement of lifespan, tremor onset or breathing irregularities observed in their inducible male rescue mice, it should be noted that two common outcomes were seen in our respective studies: both found that targeted re-introduction improved the mobility times of *Mecp2*-deficient mice in the open field, and neither found an improvement in the rescue mice on the rotorod test (19). A similar restoration of mobility was observed during the symptomatic phase of *Mecp2*$^{-/y}$ mice in the rescue study of Giacometti *et al*. (18), although this effect dissipated as the male mice acquired a more severe phenotype.

Although the reason for the lack of pronounced behavioral improvement in the rotorod and center field exploration in our rescue mice remains unclear, there are several possible explanations. In addition to involving motor cortex and striatum, which are targeted by the CamKII promoter and express the MeCP2 transgene in our rescue mice, the cerebellum and spinal cord also contribute to rotorod performance (28). Few neurons within either of these structures expressed the MeCP2 transgene in our inducible transgenic mice (not shown). Thus, if the deficiency in rotorod performance arises, at least in part, from impaired function in neurons of the cerebellum and spinal cord, the lack of significant improvement in our rescue animals would not be surprising. Similarly, anxiety levels negatively influence center field exploration and rearing, and a recent study by McGill *et al*. (29) demonstrated elevated levels of corticotrophin-releasing hormone expression in the paraventricular nucleus of the hypothalamus in *Mecp2*-deficient mice. As transgene expression was not detected in this brain region in our inducible transgenics, this imbalance in stress axis regulation would likely be retained in our rescue mice. Thus, although it is possible that the inability to rescue these behavioral deficits relates to improper expression levels of the transgene in specific neurons, or simply that these behaviors are not amenable to being rescued, an alternative explanation is that the transgene was not expressed in the correct population of neurons to address these deficits. Although additional experiments will be required to delineate the reason, the lack of pronounced improvement in these tasks by our rescue mice strengthens our conclusion that the rescue observed in the open-field tasks arises directly from the re-introduction of MeCP2 into the targeted forebrain neurons. Taken together, these results support the possibility that targeted gene re-introduction strategies—if maintained within a tolerable window of expression—could be used to improve some of the behavioral deficiencies caused by the mosaic loss of MeCP2.

MATERIALS AND METHODS

Generation of HA-MeCP2 transgenic mice

Transgenic mice were generated through a facility maintained by the University of Toronto Faculty of Medicine at the Ontario Cancer Institute (Toronto, Ontario, Canada). The transgene cassette consisted of an HA-tagged Mecp2-e2 cDNA that was fused to an SV40 3′ untranslated sequence placed into the PNN265 transgene shuttle vector (30). This carriage was excised from the vector by restriction digestion with *Not*I and subcloned into a modified pTRE2 vector (Clontech, Palo Alto, CA, USA) to generate a construct in which the *HA-MeCP2* transgene is downstream of an inducible promoter under the control of TREs. This transgene carriage was excised from the vector by restriction digestion with *Swa*1 and *Eco*RV, resolved by gel electrophoresis, purified and micro-injected into pronuclei of fertilized oocytes derived from superovulated FVB mice using standard procedures (31).

At 24 h following injection, the oocytes were transplanted into pseudopregnant mice, and offspring containing the transgene were identified by Southern blot or PCR analysis of DNA extracted from tail biopsy. The primers used to detect the Mecp2 transgene were forward 5′-CCATATGACG TCCCAGACTATGCT-3′ and reverse 5′-AAGGAGGTGTCT CCCACCTTTTCA-3′. Individual founders were then transferred to the Toronto Western Research Institute Animal Facility for housing and line expansion. Four founder mice carrying the HA-tagged MeCP2 transgene cassette were crossed and expanded on C57Bl/6 backgrounds for at least seven generations before the study was initiated.

MeCP2-deficient and rtTA mice

The *Mecp2*-deficient mouse model generated by Guy *et al*. (12) in which exons 3 and 4 of the *Mecp2* gene were ablated by Cre recombinase-mediated excision (obtained from Jackson Laboratories, Bar Harbor, ME, USA) was used for this study. Genotyping was performed via the polymerase chain reaction using the primers and conditions described previously (32). The wild-type *Mecp2* allele was detected with the common primer 5′-GGTAAAGACCCATGTGACCC-3′ and the wild-type allele-specific primer 5′-GGCTTGCCAC ATGACAA-3′, and in a separate reaction, the mutant *Mecp2* allele was detected by the common primer aforementioned, paired with the mutant allele-specific primer 5′-TCCA CCTAGCCTGCCTGTAC-3′. The 'Tet-On' transactivator mice we used to activate the Mecp2 transgene express an rtTA cDNA under the control of the calcium calmodulin kinase IIα promoter. These mice, which have been described previously [line 1237 (22)], were obtained from Dr J. Roder (Mt Sinai Hospital, Toronto, Ontario, Canada). The primers used for detecting the rtTA factor by PCR were forward 5′-ACTCAGCGCTGTGGGGCATTT-3′ and reverse 5′-AATCGTCTAGCGCGTCGGCAT-3′.

Tissue isolation

Tissue preparation for immunoblots was done as described previously (33). In brief, mice were sacrificed by decapitation and their brain removed from the skull and chilled on an ice-cold dissection disc. The cortex was isolated, frozen on dry ice and homogenized in immunoblot lysis buffer (150 mM NaCl, 1 mM 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). Following homogenization, genomic DNA was sheared by sequential passage through 18, 20 and 25 gauge needles, and the homogenate was then centrifuged at 12 000 *g* for 5 min to remove insoluble material. Aliquots of the soluble lysates were frozen and stored in liquid nitrogen until use. Protein concentrations were determined by the Folin method (Sigma-Aldrich).

Gel electrophoresis and immunoblotting

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 as described previously (33). The proteins were transferred electrophoretically to nitrocellulose in standard transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) and pre-hybridized overnight at 4°C in blocking solution [Tris-buffered saline containing 0.05% (v/v) Tween-20 (TBST) containing 5% (w/v) non-fat dried milk]. Blots were incubated with primary antibody diluted in blocking solution for 12–16 h at 4°C. The following antibodies were used: anti-HA (Covance, Cumberland, VA, USA; 1/1000 dilution), anti-MeCP2 (07–013, Upstate Biochemicals, Lake Placid, NY, USA; 1:1000 dilution) and anti-GAPDH (RGM2, Advanced Immunochemicals, Long Beach, CA, USA; 1/2000 dilution). After washing in TBST (3× 20 min/wash), goat anti-mouse HRP secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA; 1/5000 dilution) was applied and incubated for 60 min at room temperature. After extensive washing in TBST, specific immunoreactivity was visualized using Lumiglo-enhanced chemiluminescence (New England Biolabs, Mississauga, Ontario, Canada) with Biomax film (Eastman Kodak, Rochester, NY, USA).

Immunohistochemistry

Anti-MeCP2 and anti-HA immunohistochemistry was conducted as described previously (32,34). Mice were anesthetized in a halothane bell jar and transcardially perfused with a 2% paraformaldehyde/1 × PBS solution. The brain was extracted from the skull and postfixed overnight in fixative solution supplemented with 10% sucrose. Sections of 20 μm were obtained by cryosectioning, mounted onto slides and stored at −80°C until use. Sections were pre-hybridized in 4% bovine serum albumin, 3% goat serum and 0.1% Triton X-100 for 2 h at room temperature, and primary antibodies applied for 48 h at 4°C in a humidified chamber. The polyclonal anti-MeCP2 (Upstate Biochemicals) was used at a dilution of 1/200, and the monoclonal anti-HA (Covance) was used at a dilution of 1/100. After primary incubation and extensive washing in PBS, biotinylated anti-rabbit or biotinylated anti-mouse secondary antibody was applied (1/300 dilution, Vector Labs) for 30 min at room temperature. The sections were then washed extensively in PBS and incubated with FITC-conjugated or Texas Red-conjugated avidin (Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. Nuclei within the sections were stained with Hoechst 33258 (1/4000 dilution). Signal was then detected using a Zeiss Axioplan epifluorescence microscope. Controls for immunohistochemical examinations consisted of sections processed equivalently without primary antibody or sections evaluated for autofluorescence with primary antibody but without secondary antibody. Neither of the control conditions generated significant signal when compared with the test condition. Background was defined as the level of signal detected in the control conditions.

Behavioral assays

Animal behavior was assessed both in an open-field arena and on an accelerating rotarod (35,36). The open-field

performance of the mice was assessed at both 3–6 and 9–14 months of age, and the rotorod performance was assessed for the same mice at 9–12 months of age. An automated movement detection system (AM1053 activity monitors; Linton Instrumentation, UK) was used to measure motor behavior in the open-field arena. Briefly, this apparatus consists of a plexiglass box (20×30 cm^2) surrounded by a housing frame (45×25 cm^2) that houses an array of 24 infrared beams forming a grid across two levels. As the mouse moves, a beam is broken and an activity count, dependent on the type of movement, is registered. Slow activity counts are registered if successive beam breaks are separated by >200 ms. Several behavioral parameters are measured using this system depending on the location, level and succession of the activity counts. These include total activity, locomotion and exploratory behaviors such as total and center field rearing. In all, we assessed 14 parameters in the open-field arena. These parameters were the percentage of time spent walking, rearing or immobile; the number of fast and slow locomotive mobility counts; the number of total rearing events; the number of fast and slow rearing events in the entire field; the number of total rearing events in the center region of the open field; the number of fast and slow rears in the center region of the open field; the average speed at which an activity occurs; the number of times the animals moved from one end of the long axis of the cage to the other without changing direction. These behavioral assessments were conducted between 10:00 and 13:00 to minimize circadian effects, and each subject was examined in the open field only once at each age to prevent acclimation to the open-field apparatus. For the rotorod behavioral test, mice were examined three times per day, on four successive days, with a rest time of 60 min between each of the three daily trials. Mice were placed on the rotating rod, whose rotational speed increased from 3 to 35 rpm during the 5 min trial time. The time at which a mouse fell from the rotating rod was recorded automatically by laser beam break for each trial. Subjects were excluded if they did not actively balance on the rotating rod (e.g. held on and rotated in a circle). Statistical analysis for both the open-field and rotorod tests was performed using either one-way or two-way ANOVA, followed by a Tukey's *post hoc* correction for multiple comparisons where applicable.

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Conflict of Interest statement. The authors declare no conflict of interest.

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