

Environmental enrichment ameliorates a motor coordination deficit in a mouse model of Rett syndrome – *Mecp2* gene dosage effects and BDNF expression

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Abstract

Rett syndrome, commonly associated with mutations of the methyl CpG-binding protein 2 (*MECP2*) gene, is characterised by an apparently normal early postnatal development followed by deterioration of acquired cognitive and motor coordination skills in early childhood. To evaluate whether environmental factors may influence the disease outcome of Rett syndrome, we tested the effect of environmental enrichment from 4 weeks of age on the behavioural competence of mutant mice harboring a *Mecp2*^{tm1Tam}-null allele. Our findings show that enrichment improves motor coordination in heterozygous *Mecp2*^{+/-} females but not *Mecp2*^{-/-} males. Standard-housed *Mecp2*^{+/-} mice had an initial motor coordination deficit on the accelerating rotarod, which improved with training then deteriorated in subsequent weeks. Enrichment resulted in a significant reduction in this coordination deficit in *Mecp2*^{+/-} mice, returning the performance to wild-type levels. Brain-derived neurotrophic factor (BDNF) protein levels were 75 and 85% of wild-type controls in standard-housed and environmentally enriched *Mecp2*^{+/-} cerebellum, respectively. *Mecp2*^{-/-} mice showed identical deficits of cerebellar BDNF (67% of wild-type controls) irrespective of their housing environment. Our findings demonstrate a positive impact of environmental enrichment in a Rett syndrome model; this impact may be dependent on the existence of one functional copy of *Mecp2*.

Introduction

Classic Rett syndrome (RTT) is largely caused by mutations in the X-linked gene for methyl CpG-binding protein 2 (MeCP2) and is characterised by an apparently normal early postnatal development followed by progressive loss of acquired motor and cognitive skills (Amir *et al.*, 1999; reviewed by Williamson & Christodoulou, 2006). MeCP2 is primarily expressed in mature neurons and acts as a transcriptional repressor by interacting with DNA sequences containing methylated CpGs (Nan *et al.*, 1998; Kishi & Macklis, 2004) to regulate the activity of specific targets, including brain-derived neurotrophic factor (BDNF; Chen *et al.*, 2003; Martinowich *et al.*, 2003).

The heterogeneity in the phenotypic manifestation of RTT is likely to arise from a combination of genetic and environmental factors. The severity of the phenotype is broadly correlated with the location and the nature of the mutations in the gene (Christodoulou & Weaving, 2003; Bienvenu & Chelly, 2006; Scala *et al.*, 2007). However, variation in severity and progression of the disease was observed between patients with similar mutations (Scala *et al.*, 2007) and in

monozygotic twins (Migeon *et al.*, 1995; Ogawa *et al.*, 1997; also reviewed in Clarke *et al.*, 2001), highlighting the potential involvement of environmental factors.

Compelling evidence suggests that neurons are not irrevocably damaged by the absence of MeCP2 during development, as the re-expression of *Mecp2* in mutant mice is capable of suppressing the mutant phenotype (Giacometti *et al.*, 2007; Guy *et al.*, 2007). This ability to prevent the development of RTT symptoms raises the important therapeutic implication that symptomatic amelioration may be achieved by restoring normal neuronal functionality in postnatal life. Environmental enrichment (EE) in wild-type (WT) and mutant mice has shown beneficial effects on behavioural phenotype as well as cellular and molecular factors, including BDNF expression (e.g. Kempermann *et al.*, 1997; Rampon *et al.*, 2000; Spires *et al.*, 2004; also reviewed in van Praag *et al.*, 2000; Nithianantharajah & Hannan, 2006). In several models of neurological disorders, mice housed in enriched environments with objects of varying textures, sizes, shapes and colours to stimulate enhanced sensory, cognitive and motor activity have shown delayed onset and progression of disease symptoms (van Dellen *et al.*, 2000; Hockly *et al.*, 2002; Martinez-Cue *et al.*, 2002; Bezard *et al.*, 2003; Arendash *et al.*, 2004; Spires *et al.*, 2004; Jankowsky *et al.*, 2005; Lazarov *et al.*, 2005; Restivo *et al.*, 2005; Wolf *et al.*, 2006; McOmish *et al.*, 2007).

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Here we investigate the effects of EE on hemizygous (Hemi) male and heterozygous (Het) female *Mecp2*^{tm1Tam}-null mutant mice (Pelka *et al.*, 2006), and assess whether some degree of MeCP2 expression is required to mediate amelioration of the disease phenotype. Using age-matched WT and mutant littermates, we conducted behavioural tests for motor deficits of clinical relevance to RTT. BDNF protein levels in specific brain regions were also assessed based on the proposed role of this neurotrophin in the pathogenesis of RTT and experience-dependent neuronal plasticity.

Materials and methods

Mice

The *Mecp2*^{tm1Tam} mice (Pelka *et al.*, 2006) were originally bred on a 129 background then crossed to C57BL6 for two generations, followed by breeding amongst offspring of the same generation (F2 intercross) for 3–5 generations with breeder changes. The mixed background was necessary to delay onset of symptoms in males to enable behavioural testing (see Pelka *et al.*, 2006; for details). However, age-matched littermates were used in all experiments to control for possible effects of genetic background unrelated to the *Mecp2* mutation (reviewed by Wolfer *et al.*, 2002). After weaning, mice were sent from the Children's Medical Research Institute in Sydney to the Howard Florey Institute in Melbourne. Animals were maintained in a 12-h light–dark cycle. All behavioural tests were conducted between 08:00 and 18:00 h during the light phase, blind to the genotype. Mice were killed by cervical dislocation on completion of behavioural testing for collection of brain tissue samples. All experiments were approved by the Howard Florey Institute Animal Ethics Committee and carried out in accordance with the requirements of the National Health and Medical Research Council (Australia).

Age and spacing of behavioural tests

The schedule of behavioural tests was designed with allowance for rest periods to minimise stress to the mice (Fig. 1). However, the more rapid onset and progression of symptoms in the males necessitated a shorter interval between tests. The age at which each test was conducted was ± 4 days of the mean group-age for Hemi *Mecp2*^{+/y}-mutant males and ± 7 days for Het *Mecp2*^{+/-}-mutant females.

Housing conditions

At 4 weeks of age, WT and mutant littermates (female Het and male Hemi) were randomly assigned to single-sex standard housing (SH) or EE. EE consisted of larger-sized home cages with nesting material and a variety of objects with differing textures, shapes and sizes, including running wheels (van Dellen *et al.*, 2000; Spires *et al.*, 2004). The objects in the box were changed every 2 days to maintain novelty. Standard mouse boxes contained nesting material only. Animals in both environments were housed communally in groups of five or six. EE commenced at 4 weeks of age and continued for the duration of the study.

Diet supplementation and health monitoring

Food and water were available *ad libitum* in all boxes. Due to the rapid phenotype onset and consequent decline in health in the males, each box was additionally provided with mashed rodent pellets in containers on the cage floor (Jugloff *et al.*, 2006). Male mice were checked twice weekly for symptoms of disease and animals showing signs of ill health were monitored daily.

Behavioural tests

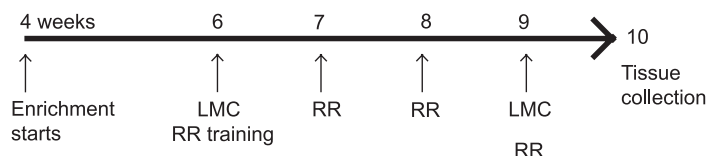
Accelerating rotarod

Mice were placed on an accelerating rotarod (7650; Ugo Basile, Comerio, VA, Italy) that accelerates from 4 to 40 rpm over 300 s. The latency to fall onto a platform below was recorded. Motor learning was assessed by conducting one session per day for five consecutive days (Crawley, 2007), then comparing the performance on the fifth day to that on the first. Motor coordination was assessed from single rotarod sessions at intervals throughout the study. All rotarod trials were concluded after 300 s. Parameters examined were performance on initial exposure to the rotarod (day 1 of training), change in performance after 5 days of training, and maintenance of performance level.

Female mice. Motor learning was assessed at 12 weeks and coordination at 20, 23, 26 and 29 weeks of age. Motor learning was also assessed in a second female cohort at 6 weeks of age.

Male mice. Motor learning was assessed at 6 weeks and coordination at 7, 8 and 9 weeks of age.

Male Timeline



Female Timeline

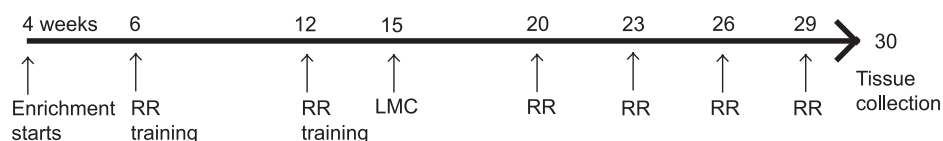


FIG. 1. Diagram of the experimental timeline in male and female cohorts. LMC, locomotor cell; RR, rotarod.

TABLE 1. *P*-values for Fig. 2a female rotarod Mann–Whitney tests

	Training		Testing			
	Day 1	Day 5	Week 20	Week 23	Week 26	Week 29
SH WT vs. EE WT	0.1903	0.393	0.7959	0.5787	0.7394	0.6842
SH WT vs. SH Het	0.006*	0.004*	0.0006*	< 0.0001*	< 0.0001*	0.001*
SH WT vs. EE Het	0.829	0.2743	0.572	0.203	0.122	0.122
EE WT vs. EE Het	0.315	0.8968	0.408	0.083	0.237	0.0545
SH Het vs. EE Het	0.006*	0.059	0.008*	0.0006*	0.0055*	0.015

*Significant differences; $\alpha = 0.01$ for all comparisons. SH WT is the control group. EE, environmental enrichment; Het, heterozygous; SH, standard housing; WT, wild-type.

Locomotor activity cell

Mice were placed in a 27×27 cm Tru Scan Photobeam Arena (E63–10; Coulbourn Instruments, Allentown, PA, USA) for 30 min to monitor the level of activity in a novel environment. Room lighting was maintained at a dim 8–10 lux to minimize the effects of anxiety on locomotion and exploratory behaviour. Data were recorded using Tru Scan 2.0 software provided by Coulbourn Instruments. Parameters measured were total distance moved, time spent in margins of arena and rearing activity. Locomotor activity was assessed at 15 weeks of age in females and at 6 and 9 weeks of age in males.

Total protein quantitation and BDNF ELISA

Upon completion of behavioural testing, brains from female mice killed at 30 weeks and males at 10 weeks were used to assess BDNF protein levels. Brain tissue was rapidly dissected and frozen at -80°C . Whole cortex, hippocampus, striatum and cerebellum were sonicated in lysis buffer (Pang *et al.*, 2006) and the DC Protein Assay (BioRad, Hercules, CA, USA) was used to quantify total protein in supernatant. The E-max BDNF ELISA kit (Promega, Madison, WI, USA) was used to calculate total BDNF levels, which were expressed as a proportion of the SH WT control group. The ELISA was validated using recombinant BDNF (Regeneron, Tarrytown, NY, USA).

Statistical analyses

The statistical packages SigmaStat and Prism were used for data analysis. All data was assessed for normal distribution using the D'Agostino and Pearson normality test. Comparisons were made with the SH WT control group and between groups with common environment or genotype factors. Groups were compared using ANOVA with the appropriate number of factors, followed by *post hoc* pairwise Bonferroni comparisons, or pairwise Mann–Whitney tests in cases of non-normally-distributed data. One-way ANOVA, Wilcoxon or *t*-tests were used to compare data within groups. Results were considered significant at an α level of 0.05. The rotarod results were analysed using nonparametric statistical tests as the test cut-off point of 300 s generated non-normally-distributed data. In this instance, α of 0.01 was applied for multiple comparisons.

Results

Behaviour: Het female mice

This is the first time that behavioural testing of *Mecp2*^{tm1Tam} Het female mice has been reported. EE resulted in a dramatic improvement in the performance baseline and maintenance of motor coordination of

female Het mice in the rotarod test for cerebellar motor learning and coordination. Briefly, the mice in this study were randomly allocated to either EE, in which a novel environment was maintained throughout the study duration by the inclusion and regular changing of toys, or SH, in which mice were housed with bedding material only (see Materials and methods for details).

Motor learning

Motor learning was assessed in female mice at 12 weeks of age (for clarity of the text, the results of Mann–Whitney comparisons, $\alpha = 0.01$, have been placed in Table 1). On initial exposure to the rotarod (day 1; Fig. 2a), SH Het mice had a coordination deficit relative to SH WT and EE Het mice. EE rescued baseline coordination in Het mice, with the time spent on the rotarod by the EE Het matching that of SH and EE WTs. Despite an initial deficit, motor learning ability was preserved in the SH Het mice as their performance improved after the 5-day training period (day 1 vs. 5, Wilcoxon signed-rank test, $\alpha = 0.01$, $P = 0.004$). The SH WT group also improved marginally with training (day 1 vs. 5, Wilcoxon signed-rank test, $\alpha = 0.01$, $P = 0.016$), and achieved maximal performance by day 5, whilst the EE Het group showed high levels of performance at both day 1 and day 5 ($P = 0.156$). In both the 12-week and the 6-week test, described below, the EE WT group reached a performance ceiling (300 s) on their first exposure to the rotarod. The EE WT group was therefore unable to improve further and their performance remained constant over the five training days ($P = 0.742$). After the training period (day 5, Fig. 2a), the SH Het demonstrated a deficit in performance relative only to the SH WT group.

As a coordination deficit was already present in SH Het mice at 12 weeks of age, a second cohort was tested for motor learning only at 6 weeks of age (Fig. 2b). On day 1, the SH Het mice performed as well as the EE Het and SH WT mice (Mann–Whitney test, $\alpha = 0.01$, SH Het vs. EE Het $P = 0.147$, SH Het vs. SH WT $P = 0.483$). After 5 days of training, the four groups performed at a similar level ($P > 0.44$). The SH Het mice improved their performance over the training period (Wilcoxon signed-rank test, day 1 vs. 5, $P = 0.002$), whilst the other groups achieved maximal performance (SH WT $P = 0.156$, EE WT $P = 0.383$, EE Het $P = 0.084$).

Motor coordination

Motor coordination was monitored at weeks 20, 23, 26 and 29 (Fig. 2a and Table 1). Although the SH Het mice improved with training, the level of coordination acquired on day 5 was lost in subsequent weeks, with these mice performing significantly worse than the SH WT group at each week of testing. Importantly, EE prevented the progressive coordination deficit from developing in EE Het mice. Coordination in the EE Het mice remained similar to both WT groups for the study

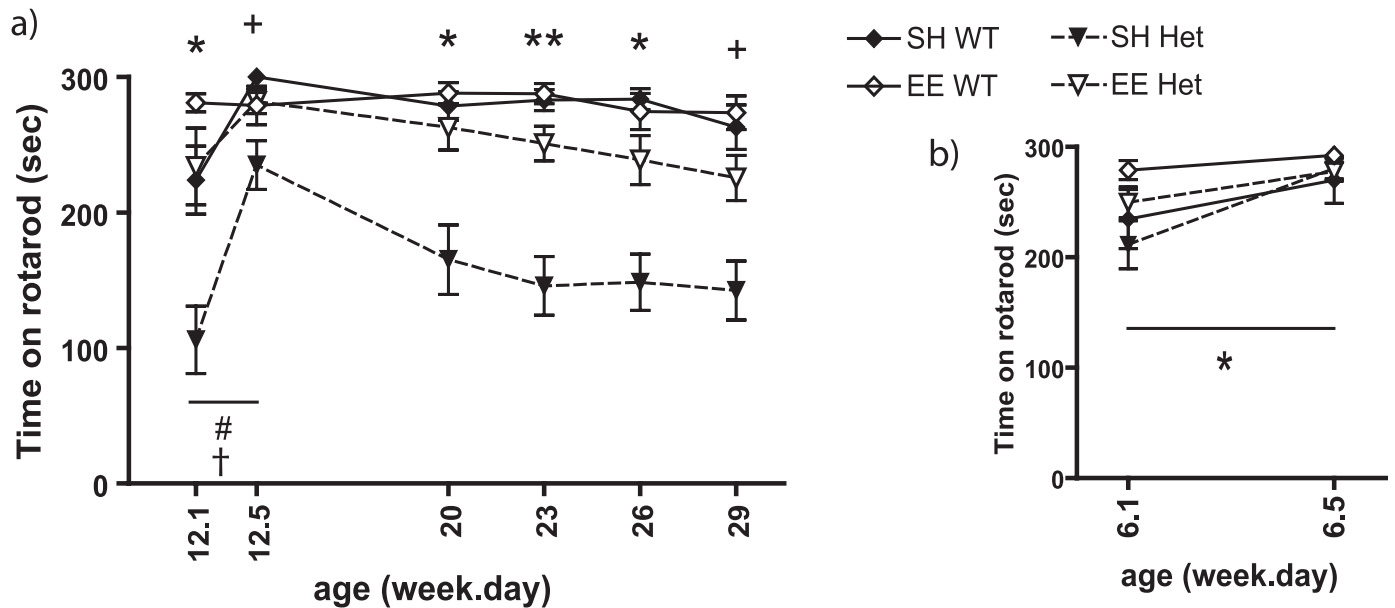


FIG. 2. Female rotarod performance. Values are mean \pm SEM. (a) First cohort, 12–29 weeks of age. Motor learning day 1 (week 12.1) vs. day 5 (week 12.5): performance improved in SH Het ($^{*}P = 0.004$) and SH WT ($^{*}P = 0.016$; Wilcoxon test). EE WT and EE Het groups had already achieved high performance on day 1. Motor coordination: EE Het performance remained similar to both WT groups for the study duration and was superior to SH Het mice until 26 weeks of age. Week 12.1: $^{*}P < 0.01$ SH Het vs. SH WT and EE Het. Week 12.5: $^{*}P < 0.01$ SH Het vs. SH WT. Week 20: $^{*}P < 0.01$ SH Het vs. SH WT and EE Het. Week 23: $^{**}P < 0.001$ SH Het vs. SH WT and EE Het. Week 26: $^{*}P < 0.01$ SH Het vs. SH WT and EE Het. Week 29: $^{+}P < 0.01$ SH Het vs. SH WT; (all Mann–Whitney tests, $\alpha = 0.01$). Numbers included: SH WT, 10; EE WT, 10; SH Het, 9; EE Het, 8. (b) Second cohort, 6 weeks of age. Motor learning day 1 (week 6.1) vs. day 5 (week 6.5): improved performance in SH Het ($^{*}P = 0.002$; Wilcoxon test). There were no differences between groups on either day 1 or day 5. Numbers included: SH WT, 8; EE WT, 12; SH Het, 11; EE Het, 13.

duration and was superior to SH Het mice until 26 weeks of age. There was no significant difference between the EE and SH Het groups at 29 weeks of age.

General locomotor activity

Locomotor activity was assessed in female mice at 15 weeks of age. A two-way ANOVA of distance moved (Fig. 3a) indicated that EE decreased locomotion ($F_{1,43} = 7.35$, $P < 0.01$) but that genotype had no effect ($F_{1,43} = 1.48$, $P = 0.231$). *Post hoc* Bonferroni tests showed that although there was no difference in distance travelled between the two EE groups ($t = 2.07$, $P = 0.267$), the EE Het mice moved less than SH WT littermates ($t = 2.8$, $P = 0.045$) and also less than SH Het mice ($t = 3.1$, $P = 0.021$). This may be an EE-induced change in exploratory behaviour enhanced in mice with the *Mecp2* Het mutation. Enrichment was recently shown to alter exploration in WT and mutant mice in other behavioural tests (Nithianantharajah *et al.*, 2008). Time spent in the arena margin, which can be a measure of anxiety-like behaviour, did not differ between groups (Fig. 3b; two-way ANOVA, $F_{1,43} < 1.9$, $P > 0.18$). Rearing behaviour, as assessed by vertical plane entries, was analysed with Mann–Whitney pairwise comparisons due to its non-normal distribution (Fig. 3c). The two Het groups reared significantly less than the WT groups (SH Het vs. SH WT $P = 0.045$, EE Het vs. SH WT $P = 0.008$, EE Het vs. EE WT $P = 0.018$). There was no effect of EE on this behaviour.

Behaviour: Hemi male mice

In initial studies with the *Mecp2*^{tm1Tam} model of RTT, Hemi mutant males were shown to have a motor coordination deficit and a cerebellar motor learning deficit (Pelka *et al.*, 2006) corresponding to the ataxia and apraxia observed in RTT patients. The coordination

deficits were confirmed and extended in the current study, with Hemi mice showing poor coordination and inability to improve performance on the rotarod without displaying overt differences in floor plane movement in a novel environment. Housing the male mice in EE did not ameliorate the motor phenotype.

Motor learning and coordination

On first exposure of the male mice to the rotarod at 6 weeks of age (day 1; Fig. 4), the EE WT animals showed better performance than the SH WT group (Mann–Whitney test, $\alpha = 0.01$, $P = 0.0006$). The EE Hemi mice displayed reduced performance relative to the EE WT mice on day 1 (Mann–Whitney tests, $\alpha = 0.01$, $P = 0.001$) but not to SH WT ($P = 0.689$). The SH Hemi were also similar to SH WT on day 1 (Mann–Whitney test, $\alpha = 0.01$, $P = 0.034$). On day 5 (Fig. 4), upon completion of training, there was no difference in performance between the EE and SH WT, with both groups remaining for the maximum 300 s on the rotarod (Mann–Whitney test, $\alpha = 0.01$, $P = 0.243$). SH WT males showed significant improvement between days 1 and 5 as a result of training (Wilcoxon signed-rank test, $P = 0.004$), whereas the EE WT had already reached the performance ceiling on their first exposure to the test and therefore maintained their performance without further improvement (Wilcoxon test, $P = 0.688$). Both Hemi groups, however, remained inferior in performance on day 5 compared to the relevant WT groups (Mann–Whitney tests, $\alpha = 0.01$, SH WT vs. SH Hemi $P = 0.003$, SH WT vs. EE Hemi $P = 0.0004$, EE WT vs. EE Hemi $P = 0.001$). Neither of the Hemi groups improved performance with training, indicating impaired motor learning, and EE had no effect (Wilcoxon test day 1 vs. day 5: SH Hemi $P = 0.625$, EE Hemi $P = 1.00$). Continued assessment of motor coordination from 7 to 9 weeks of age showed persistent impairment in the Hemi groups (data not shown due to low numbers).

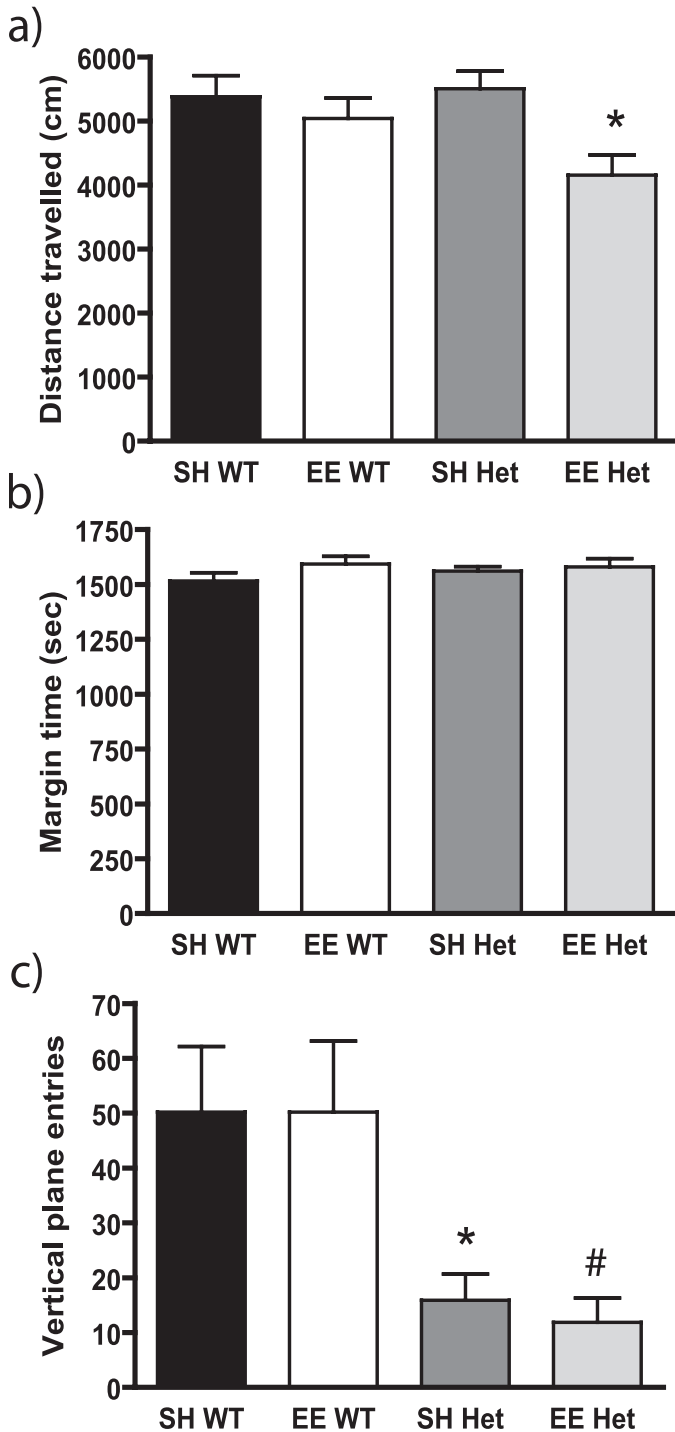


FIG. 3. Female locomotor behaviour at 15 weeks of age. (a) Distance traveled: main effect of environment ($P < 0.01$, two-way ANOVA). *Post hoc* Bonferroni tests showed that EE Het traveled less than SH WT and SH Het groups ($*P < 0.05$) but were similar to EE WT ($P = 0.27$). (b) Time spent in margin zone, no significant difference between groups (two-way ANOVA). (c) Vertical plane entries. Both Het groups showed significantly fewer vertical entries than did relevant WT groups ($*P < 0.05$ SH Het vs. SH WT; $*P < 0.05$ EE Het vs. SH WT and EE WT; Mann–Whitney tests). Numbers included: SH WT, 11; EE WT, 12; SH Het, 11; EE Het, 13. Values are mean \pm SEM.

General locomotor activity

Locomotor activity was measured at 6 and 9 weeks of age as an indicator of general activity levels. Impaired locomotion resulting

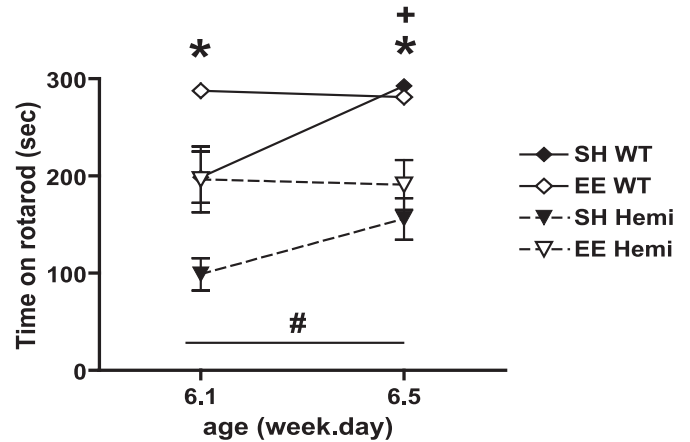


FIG. 4. Male rotarod performance at 6 weeks of age. Motor learning day 1 (week 6.1) vs. day 5 (week 6.5): performance improved in SH WT ($*P = 0.004$; Wilcoxon test). No improvement in performance was observed for either Hemi group. EE WT achieved maximal performance on day 1. Week 6.1: EE WT performance was superior to all other groups ($*P < 0.01$; Mann–Whitney tests). Week 6.5: WT groups had performance which was superior to that of Hemi groups ($*P < 0.01$ SH Hemi vs. SH WT, $*P < 0.01$ EE Hemi vs. SH WT and EE WT; Mann–Whitney tests). Numbers included: SH WT, 9; EE WT, 10; SH Hemi, 5; EE Hemi, 5. Values are mean \pm SEM.

from progression of disease symptoms may alter the outcome of behavioural tests; hence locomotor activity was assessed before and after the remainder of the behavioural testing series. Figure 5a shows that at 6 weeks of age there was no effect of genotype or environment on the total distance travelled, indicating that the Hemi mice were capable of adequate movement for exploration (two-way ANOVA, $F_{1,25} < 3.8$, $P > 0.06$). There was also no difference in time spent in the margins of the activity cell (Fig. 5b, two-way ANOVA, $F_{1,25} < 4.45$, $P > 0.508$). Rearing activity (Fig. 5c), however, varied between genotypes. The SH Hemi mice reared significantly less than their control WT littermates, (nonparametric distribution; pairwise Mann–Whitney comparisons, SH Hemi vs. SH WT $P = 0.029$). No rearing was recorded for the EE Hemi group, which precluded statistical analyses. There was no effect of environment on rearing in the WT mice (Mann–Whitney test, $P = 0.968$). A similar pattern was observed for all three movement parameters for animals surviving at 9 weeks of age (data not shown).

BDNF protein levels in brain tissues

Changes in BDNF protein levels in cortex, striatum, cerebellum and hippocampus of the female mice were determined at 30 weeks of age (Fig. 6a–d). BDNF expression was also assessed in the cerebellum of male mice at 10 weeks of age (Fig. 7). Briefly, brain regions were sonicated and the supernatant collected, then total BDNF protein was assayed using a BDNF ELISA kit. BDNF protein content of the SH Het/Hemi, EE Het/Hemi and EE WT groups were expressed as a percentage of that of the SH WT control group (Chang *et al.*, 2006).

For the female mice, the BDNF concentration in the cerebellum showed a significant effect of genotype (Fig. 6a; two-way ANOVA, genotype: $F_{1,26} = 24.52$, $P < 0.001$) but not environment ($F_{1,26} = 2.52$, $P = 0.124$) or interaction ($F_{1,26} = 0.36$, $P = 0.55$). Bonferroni *post hoc* tests indicated that BDNF was significantly decreased in the cerebellum of SH Het mice relative to SH WT ($t = 3.93$, $P = 0.003$). The SH Het and EE Het groups did not

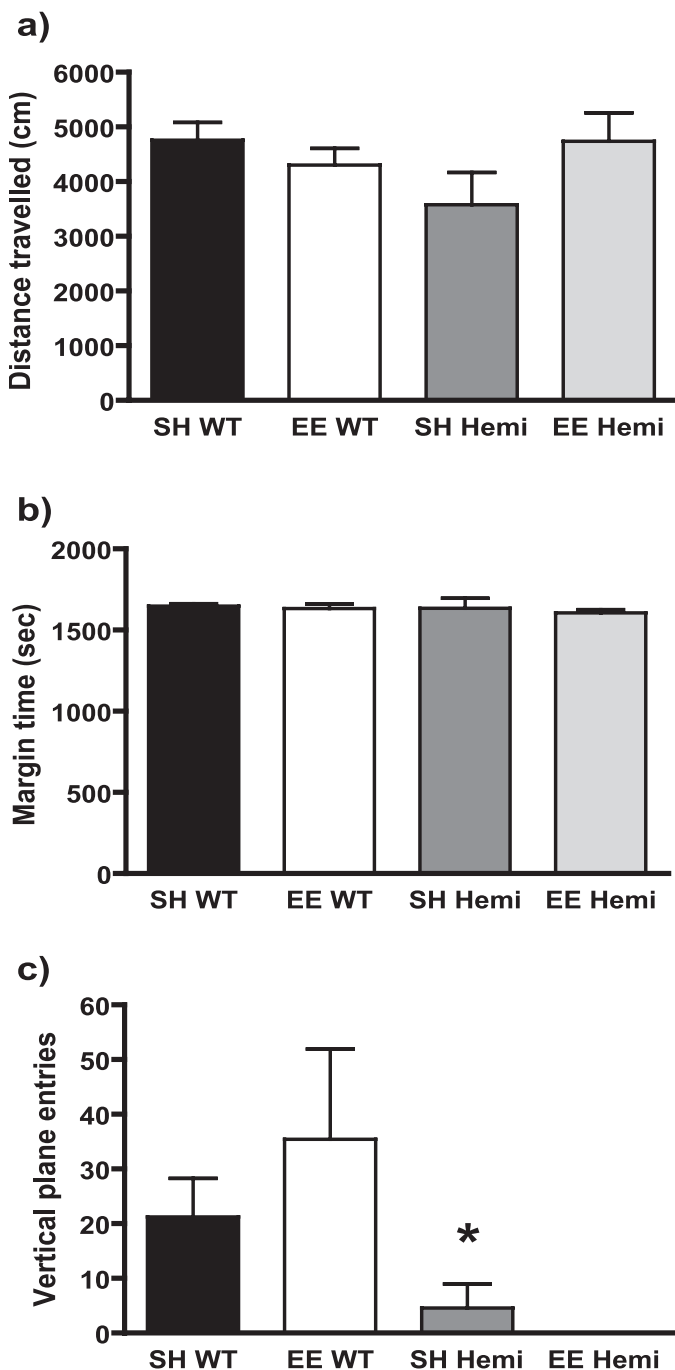


FIG. 5. Male locomotor behaviour at 6 weeks of age. (a) Distance traveled, no significant difference between groups (two-way ANOVA). (b) Time spent in margin zone, no significant difference between groups (two-way ANOVA). (c) Vertical plane entries. EE Hemi group showed no vertical plane entries. SH Hemi showed significantly fewer vertical entries than did WT groups (* $P < 0.05$, SH Hemi vs. SH WT; Mann–Whitney tests). Numbers included: SH WT, 9; EE WT, 10; SH Hemi, 5; EE Hemi, 5. Values are mean \pm SEM.

differ significantly; however, the EE Het group showed levels of cerebellar BDNF similar to those of SH WT mice ($t = 2.38$, $P = 0.15$), albeit lower than the EE WT ($t = 3.08$, $P = 0.029$). In the hippocampus, there was a significant positive effect of environment on BDNF (Fig. 6b; two-way ANOVA: $F_{1,26} = 6.04$, $P = 0.021$) but none of the *post hoc* pairwise comparisons showed any significant difference. No statistically significant differences

were found in the cortex (Fig. 6c; $F_{1,26} < 1.4$, $P > 0.25$) or striatum (Fig. 6d; $F_{1,26} < 1.8$, $P > 0.57$).

In the male cerebellum there was a significant effect of genotype, with Hemi mice having a significantly lower BDNF concentration (Fig. 7; two-way ANOVA: $F_{1,19} = 69.32$, $P < 0.001$). Bonferroni *post hoc* tests indicated that both SH and EE Hemi groups displayed a deficit in cerebellar BDNF relative to WT groups (SH WT vs. SH Hemi: $t = 5.45$, $P < 0.001$; SH WT vs. EE Hemi: $t = 6.16$, $P < 0.001$; EE WT vs. EE Hemi: $t = 6.35$, $P < 0.001$).

Discussion

This study documents a marked rescue in motor coordination and a partial normalization in cerebellar BDNF expression of Het female *Mecp2*^{tm1Tam} mice, a model of RTT, resulting from exposure to EE.

There are several mouse models of RTT (Chen *et al.*, 2001; Guy *et al.*, 2001; Shahbazian *et al.*, 2002; Collins *et al.*, 2004; Pelka *et al.*, 2006) which recapitulate aspects of the clinical phenotype found principally in human females Het for *MECP2* mutations. The majority of mouse studies have been conducted on male Hemis which display a more severe phenotype than that of Het female mice, which harbour both normal and *Mecp2*-deficient cells due to X-chromosome inactivation (Young & Zoghbi, 2004; Watson *et al.*, 2005). Male mice have a phenotype progression reminiscent of, although more severe than, RTT patients and provide insight into the outcome of total loss of MeCP2 function. However, the existence of one *Mecp2* copy, and therefore some level of MeCP2 function, in the female *Mecp2*^{+/-} mice is closer to human RTT at the molecular level (reviewed by LaSalle, 2004; Stearns *et al.*, 2007). We opted to investigate both Hemi and Het models of RTT for this reason.

In the current study *Mecp2*^{+/-} mice exhibited deficits in motor coordination which worsened progressively, developing an apparent motor coordination deficit at 12 weeks of age but not at 6 weeks. Housing these mice in an environment that stimulates sensory, motor and cognitive activity reversed the deficit in motor coordination. The EE Hets maintained their performance near WT levels up to 29 weeks of age, whilst the SH *Mecp2*^{+/-} mice lost the motor skills acquired through training. This contrasts with Hemi male mice which show a more severe phenotype, with early onset of symptoms and progressive decline (Pelka *et al.*, 2006) which EE was unable to ameliorate.

The difference in performance between the 6-week and 12-week time points suggests that the deficit in motor coordination in Het mice is progressive. The ability to learn the task, however, seems to be unaffected by a partial *Mecp2* deficiency, and the deficit in motor coordination at 12 weeks of age in SH Het mice did not correspond to a decline in distance moved in the locomotor arena. This implies that the lateral movement of SH Het mice was not hindered. Our results differ from those from other models, where female Het mice displayed hypoactivity when monitored over 12 h in the dark cycle at 6 weeks of age (Stearns *et al.*, 2007), and symptomatic females displayed hypoactivity in the open field test (Guy *et al.*, 2001). Rearing behaviour, however, was reduced in the Het mice in our study, an aspect of the phenotype that was not altered by EE. This is a clear contrast to the reversal of the rotarod deficit by EE, and suggests that the reduced rearing of the *Mecp2*^{+/-} and *Mecp2*^{-/-} mice may be due to altered exploratory patterns rather than impaired motor coordination.

The hind-limb clasping behaviour in *Mecp2* mutant mice, observed previously (Guy *et al.*, 2001; Shahbazian *et al.*, 2002; Chang *et al.*, 2006; Pelka *et al.*, 2006) as well as in the current

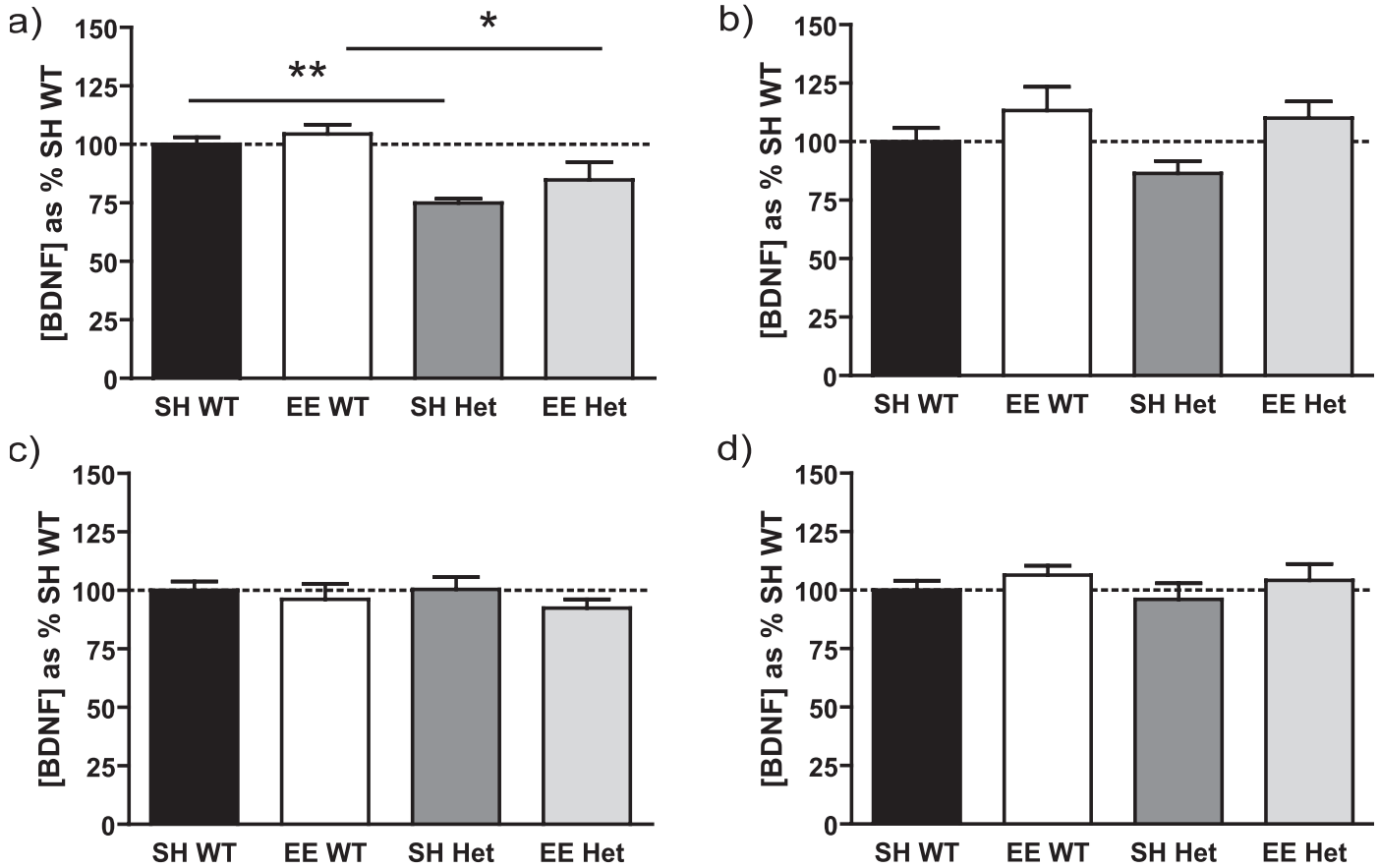


FIG. 6. BDNF expression in the female WT and Het mice, expressed as % of SH WT. (a) Cerebellum: significant effect of genotype (two-way ANOVA, $P < 0.001$). SH WT vs. SH Het, $***P < 0.01$; EE WT vs. EE Het, $*P < 0.05$ (*post hoc* Bonferroni tests). There were no differences between SH Het and EE Het or SH WT and EE Het. (b) Hippocampus: significant effect of environment (two-way ANOVA, $P < 0.05$); *post hoc* tests were not significant. (c) Cortex: no variation with genotype or environment. (d) Striatum: no variation with genotype or environment. Numbers included: SH WT, 8; SH Het, 7; EE WT, 8; EE Het, 7. Values are mean \pm SEM.

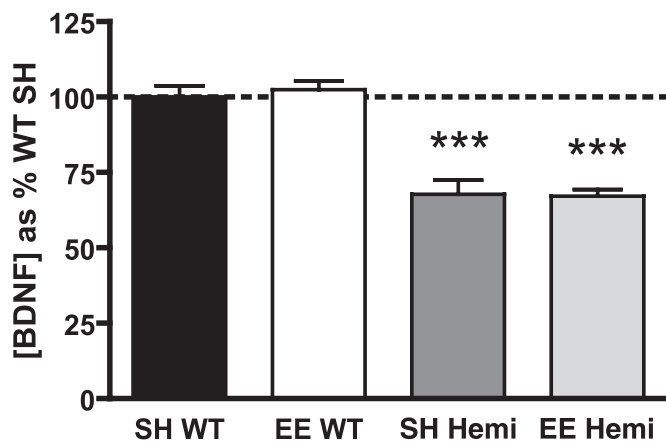


FIG. 7. BDNF expression in the cerebellum of male WT and Hemi mice, expressed as % of SH WT. Significant effect of genotype (two-way ANOVA, $P < 0.001$). $***P < 0.001$, SH Hemi vs. SH WT and EE Hemi vs. EE WT (*post hoc* Bonferroni tests). Numbers included: SH WT, 9; SH Hemi, 3; EE WT, 7; EE Hemi, 4. Values are mean \pm SEM.

study, is thought to mimic stereotypic hand-wringing in RTT patients. As noted by Chang *et al.* (2006), this phenotype is very similar in appearance to the hind-limb claspings displayed by *Bdnf* conditional mutant mice, and it has been speculated that a BDNF

deficit is involved in this behaviour in both mutants. At the final timepoint examined in the present study, the hind-limb claspings phenotype was apparent in 37.5% of EE Het and 55.5% of SH Het mice (data not shown).

Analysis of BDNF expression in the female mice indicated that BDNF protein levels were reduced to 75% of SH WT levels in the cerebellum of the SH Het (Fig. 6a), which is informative in the context of the impaired motor coordination observed in these animals. Enrichment raised mean cerebellar BDNF to 85% of SH WT levels. Although the EE Het group expressed levels of BDNF in the cerebellum that were statistically indistinguishable from the SH WT levels, the increase in BDNF in the Het mice with EE did not achieve statistical significance between EE Het and SH Het mice. Therefore, at the relatively old age of 30 weeks, there was only partial normalization of BDNF expression in the EE Het mice. This molecular result correlates with the rotarod performance of the Het mice at 29 weeks of age (Fig. 2a), just prior to tissue collection, when performance was starting to decline.

Analysis of BDNF in the male mice revealed a deficit in the cerebellum in the SH Hemi mice, at 67% of SH WT levels. Significantly, cerebellar BDNF levels were also 67% of SH WT controls in the EE Hemi mice, which were behaviourally unresponsive to EE for rotarod performance. A key role for BDNF in the pathogenesis of RTT is suggested, as deleting *Bdnf* in *Mecp2*-null mice results in the earlier onset of RTT-like phenotype whereas overexpression of BDNF in *Mecp2*-deficient mice extends their

lifespan and reduces the locomotor deficit (Chang *et al.*, 2006). Therefore, the beneficial effect of EE we observed in the *Mecp2*^{+/-} model of RTT may be due, at least partially, to upregulation of BDNF. It is noteworthy that this behavioural response was achieved by a relatively moderate form of home-cage EE (for review of published enrichment protocols used in rodent models of brain disorders, see Nithianantharajah & Hannan, 2006). A study in male *Mecp2*-knockout mice reported a significant decrease in BDNF levels at 6–8 weeks of age, with knockouts expressing only 79% of the BDNF protein level of WT mice in the cortex and 59% in the cerebellum (Chang *et al.*, 2006). Our results in the SH Hemi cerebellum are similar to these. Furthermore, the percentage change we observed in the Het cerebellum was milder than that in the Hemi null mice and correlates with the coordination differences observed between the models. The lack of BDNF deficit in the cortex of the *Mecp2*^{+/-} mice is not surprising either, as Wang *et al.* (2006) observed no difference in cortical or hippocampal BDNF protein levels in null male mice despite significant deficits in the brainstem and nodose ganglia. Here, a significant effect of EE on hippocampal BDNF was observed in the female mice irrespective of genotype. EE is known to induce neural plasticity, including enhancing adult hippocampal neurogenesis and synaptic plasticity (Kempermann *et al.*, 1997) and increasing neurotrophin expression (reviewed by van Praag *et al.*, 2000). As our data are consistent with actions on the cerebellum, molecular changes underlying experience-dependent synaptic plasticity will be of interest in future investigations. This could include other trophic factors and neurotransmitter receptors, as well as various presynaptic and postsynaptic signaling proteins.

Although it is difficult to make direct comparisons between the male and female cohorts used in this study due to differences in the duration of EE, age of tissue collection and stage of disease, the results are suggestive of the importance of *Mecp2* gene copy number in the response to EE, and not just phenotypic expression. The effect of EE on the *Mecp2*^{+/-} mice is therefore more likely to be mediated by cells expressing an active WT *Mecp2* allele. Further significance of *Mecp2* gene copy number in disease progression and potential for recovery of symptoms can be gleaned from recent studies documenting amelioration of RTT-like symptoms in animal models with restoration of *Mecp2* expression (Giacometti *et al.*, 2007; Guy *et al.*, 2007). Of note in these studies was a significant gene dosage effect, with some degree of phenotypic improvement in transgenic lines with only 10% of cells expressing *Mecp2* (Giacometti *et al.*, 2007). Collins *et al.* (2004) also showed the importance of tight regulation of MeCP2 levels *in vivo*, with mice expressing double the normal WT dose displaying disease symptoms with increasing age. Additionally, as MeCP2 levels influence excitatory synaptic strength via regulation of glutamatergic synaptic density (Chao *et al.*, 2007), the presence of some level of functional MeCP2 in the females (dependent on X-chromosome inactivation patterns) may afford the later onset and milder presentation, hence some capacity to respond to treatments including EE. As neuronal activity-dependent phosphorylation of MeCP2 appears to regulate dendritic patterning, spine morphogenesis and BDNF transcription (Zhou *et al.*, 2006; reviewed by Chahrour & Zoghbi, 2007) it is plausible that, in females, MeCP2 function may be upregulated by EE, reversing part of the phenotype.

The results of our study suggest that symptomatic alleviation of RTT may be achieved by enhanced sensory, cognitive and motor stimulation, underscoring the notion that EE should be actively incorporated into the treatment paradigm for RTT. Furthermore, molecular insights into these beneficial effects of EE may facilitate future development of new therapeutic approaches for RTT and other brain disorders.

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Abbreviations

BDNF, brain-derived neurotrophic factor; EE, environmental enrichment; Hemi, hemizygous; Het, heterozygous; MeCP2, methyl CpG-binding protein 2; RTT, Rett syndrome; SH, standard housing; WT, wild-type.

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