Selective preservation of MeCP2 in catecholaminergic cells is sufficient to improve the behavioral phenotype of male and female Mecp2-deficient mice

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Rett syndrome (RTT) is a neurodevelopmental disorder caused primarily by mutations of the X-linked MECP2 gene. Although the loss of MeCP2 function affects many neural systems, impairments of catecholaminergic function have been hypothesized to underlie several of the cardinal behavioral deficits of RTT patients and Mecp2-deficient mice. Although recent Mecp2 reactivation studies indicate that RTT may be a reversible condition, it remains unclear whether specifically preserving MeCP2 function within a specific system will be sufficient to convey beneficial effects. Here, we test whether the selective preservation of MeCP2 within catecholaminergic cells will improve the phenotype of Mecp2-deficient mice. Our results show that this targeted preservation of MeCP2 significantly improves the lifespan, phenotypic severity and cortical epileptiform discharge activity of both male and female Mecp2-deficient mice. Further, we found that the catecholaminergic preservation of MeCP2 also improves the ambulatory rate, rearing activity, motor coordination, anxiety and nest-building performances of Mecp2-deficient mice of each gender. Interestingly, our results also revealed a gender-specific improvement, as specific cortical and hippocampal electroencephalographic abnormalities were significantly improved in male, but not female, rescue mice. Collectively, these results support the role of the catecholaminergic system in the pathogenesis of RTT and provide proof-of-principle that restoring MeCP2 function within this specific system could represent a treatment strategy for RTT.

INTRODUCTION

Rett syndrome (RTT) is an X-linked pediatric neurodevelopmental disorder and a leading genetic cause of mental retardation in females (1,2). Affected individuals are typically born without any obvious deficit and commonly meet most early developmental milestones. However, typically between 6 and 18 months of age, girls affected with RTT typically lose acquired skills and undergo a period of regression in which additional symptoms begin to manifest (1,3,4). Commonly, RTT patients are affected with symptoms such as cognitive impairment, loss of coordinated locomotive ability, stereotypic hand movements during wakefulness, intractable seizures, breathing irregularities, gastrointestinal abnormalities and bone density deficits, among others (3). Physically, the brain of a RTT patient tends to be undersized (5), and neurons in...
the RTT brain show increased neuronal packing density (6,7) and display diminished levels of dendritic complexity (6,8–10). Collectively, these observations have spawned the hypothesis that RTT is a condition in which brain development stalls and synapses fail to mature properly (2,11,12).

Mutations within the X-linked gene encoding methyl-CpG-binding protein 2 (MeCP2) have been identified as the predominant causes of RTT (13). Mouse models have been generated that lack MeCP2 function, and both male and female MeCP2-deficient mice recapitulate several cardinal features of clinical RTT (14,15). These models have been utilized for ‘proof-of-concept’ studies showing that global reintroduction of MeCP2 (16,17), or targeted reintroduction of MeCP2 into specific large populations of neurons or glia (18–22), improved at least some of the behavioral reductions of MeCP2 into specific large populations of neurons or glia (18–22), improved at least some of the behavioral deficits of MeCP2-deficient mice. Collectively, these studies show that the Rett-like phenotype of MeCP2-deficient mice is not irreversible and raises the possibility that gene reintroduction strategies may have clinical potential. Although encouraging, repopulating large regions of the brain with MeCP2 remains a challenging prospect clinically. Given this, a key question that arises is whether specifically maintaining MeCP2 function within small populations of defined neurons would be sufficient to make a meaningful impact on RTT deficits.

One candidate system that could potentially be targeted by gene therapy that has been strongly implicated in the pathophysiology of RTT is the catecholaminergic system (23–25). The majority of norepinephrine projections within the brain originate from neurons residing within the locus ceruleus and lateral tegmental area (26), although the majority of dopamine projections arise from neurons within the ventral tegmental area, arcuate nucleus or substantia nigra (27). These regions are well defined anatomically, and although comprised of relatively small numbers of neurons, their functions influence the activity of numerous cell types and neural systems throughout the brain. Further, deficits in catecholamine levels have been reproducibly seen in RTT patients and in MeCP2-deficient mice (28), and phenotypically, RTT patients frequently display dystonic movements, stereotypic hand movements, gate impairments, loss of ambulation and heightened levels of anxiety-related behavior (1,3,29). The importance of MeCP2 function in catecholaminergic cells has been recently demonstrated, as the selective ablation of MeCP2 from the catecholaminergic system induced RTT-like phenotypic impairments in mice (28). Collectively, these observations highlight the importance of the catecholaminergic system in RTT pathophysiology and demonstrate that abrogating MeCP2 function solely in catecholaminergic cells is sufficient to disturb neural homeostatic balance and cause behavioral deficits.

Given the results showing the targeted deletion of MeCP2 from only catecholaminergic cells can produce RTT-like deficits, we questioned in this study whether or not the selective preservation of MeCP2 function in catecholaminergic cells of MeCP2-deficient mice would improve aspects of their RTT-like phenotype. Our results show that as hypothesized, the preservation of MeCP2 function in these defined populations of cells is sufficient to improve phenotypic deficits in both male and female MeCP2-deficient mice.

RESULTS

Mecp2 is selectively preserved in tyrosine hydroxylase-expressing neurons in the ‘rescue’ mouse brain

To allow the selective preservation of functional MeCP2 expression within catecholaminergic cells, we crossed female MeCP2-deficient (Mecp2<sup>−/−</sup>) mice containing a ‘stop-flox’ MeCP2 allele (16) with transgenic mice expressing Cre recombinase from an exogenous rat tyrosine hydroxylase (TH) promoter (TH-cre) (30). For simplicity, we will refer to MeCP2-deficient mice as ‘non-rescue’ mice and MeCP2-deficient mice expressing Cre recombinase in TH-positive cells as rescue mice. To confirm the reactivation efficiency of MeCP2 expression in rescue mice, we employed dual-label immunohistochemistry and quantified MeCP2 expression within the catecholaminergic regions of the adult male rescue mouse brain (Fig. 1A–H). These results revealed that within the substantia nigra, 87.5 ± 5.0% of the MeCP2-positive neurons stained positively for TH, and conversely, 85.4 ± 3.4% of TH-positive neurons expressed MeCP2 (Fig. 1C and D). In the locus ceruleus, 81.3 ± 6.2% of MeCP2-positive neurons expressed TH, and 81.9 ± 5.7% of TH-positive neurons expressed MeCP2 (Fig. 1G and H). These co-expression percentages were comparable with those of adult wild-type littermate mice, where within the substantia nigra, 94.1 ± 3.1% of the MeCP2-positive neurons co-expressed TH, 96.9 ± 0.9% of TH-positive neurons co-expressed MeCP2, 92 ± 7.1% of MeCP2-positive neurons in the locus ceruleus co-expressed TH and 95 ± 4.5% of TH-positive neurons co-expressed MeCP2 (Fig. 1C, D, G and H). Few, if any, MeCP2-positive cells were observed within the cortex, hippocampus or cerebellum of rescue mice (Supplementary Material, Fig. S1A–C). However, although the majority of MeCP2 expression was restricted to TH-positive cells throughout the rescue mouse brain, reactivated MeCP2 expression was seen infrequently in some TH-negative cells within the periventricular and paraventricular nuclei of the hypothalamus (not shown) and in some neurons in the midbrain and brainstem of adult male rescue mice (Supplementary Material, Fig. S1D and E). The ectopic expression of the rescue MeCP2 protein in a small cohort of TH-negative cells in the adult brain was not unexpected, however, as the TH-Cre transgenic mouse we employed has been shown previously to activate a stop-flox reporter gene in scattered cells within these same areas (30). Further, it should be noted that there is evidence for transient expression of TH in small populations of cells during rodent brain development (31,32), which in our rescue mice could allow for transient Cre expression in these cells, and in sympathetic ganglia and certain peripheral structures such as adrenal medulla (30) where MeCP2 reactivation in our rescue mice would also be expected to occur.

Preservation of Mecp2 in catecholaminergic cells extends the lifespan of male Mecp2-deficient mice

Analysis of Kaplan–Meier survival plots revealed life expectancy to be significantly longer in male rescue mice than male non-rescue mice. In contrast to Mecp2-null non-rescue mice, which displayed a median survival age of 77 days, male rescue mice displayed a median survival age of 180 days,
40% of the rescue cohort survived longer than 200 days (Fig. 2A). Only one of the male non-rescue mice in our cohort lived longer than 100 days (Fig. 2A). In addition to increased longevity, male rescue mice displayed an overall improvement in general phenotypic severity compared with non-rescue mice. Using a phenotypic severity scale previously employed for Mecp2-deficient mice (16,17,33), we found that male rescue mice consistently displayed lower severity scores than non-rescue mice at and after 5 weeks of age (Fig. 2B, two-way ANOVA \( P < 0.05 \)). The increase in lifespan and attenuation of phenotype severity occurred in the absence of body mass normalization, however, as male rescue mice remained significantly underweighted compared with age-matched wild-type mice, and not significantly different from non-rescue mice (Fig. 2C, two-way ANOVA \( P < 0.001 \)).
Preservation of Mecp2 in catecholaminergic cells decreases the rate of sudden unexpected death in female Mecp2-deficient mice

Although early lethality is not a typical phenotype of female Mecp2^{−/−} mice (14,15), female Mecp2-deficient mice are prone to sudden and unexpected death. In our cohort, 34.5% (10 of 29) of the female Mecp2-deficient mice died suddenly and without indication before reaching 1 year of age, which is significantly higher than the rate of 4.8% (2 of 42) observed in female wild-type mice. In contrast, the spontaneous unexplained death rate in female rescue mice (light gray line) is significantly lower than that of male ‘Non-rescue mice’ (n = 13, light gray line) and after 35 days of age (P < 0.05, one-way ANOVA with Tukey’s post hoc test). The severity scores for male wild-type mice were between 0 and 1 over these ages (shown in closed circles). (C) The average body mass of male rescue mice (dark gray line, n = 19–8 at different ages) does not significantly differ from male non-rescue mice (light gray line, n = 23–27 at different ages), and both groups remain significantly underweighted compared with male wild-type mice (black line, n = 23) (P < 0.01, one-way ANOVA with Tukey’s post hoc test). (D) The spontaneous death rate in female rescue mice (3 of 33) is significantly lower than that in female non-rescue mice (10 of 29; P < 0.05, chi-square test with 1 df). (E) The gross phenotypic severity score of female non-rescue mice (light gray line, n = 8) and female rescue mice (dark gray line, n = 10) does not significantly differ at any time between 30 and 50 weeks of age (one-way ANOVA). Wild-type female mice severity scores were between 0 and 1 over these ages (shown in solid circles). (F) The average body mass of female rescue mice (dark gray line, n = 12) does not significantly differ from female non-rescue mice (light gray line, n = 13) or female wild-type mice (black line, n = 17, one-way ANOVA with Tukey’s post hoc test).

Catecholaminergic preservation of Mecp2 improves deficits in ambulatory rate, motor coordination and anxiety-like behavior in male Mecp2-deficient mice

Consistent with previous reports (21,28), 50–70-day-old male non-rescue mice displayed clear impairments relative to wild-type and TH-cre mice in general activity, balance and coordinated movement, ambulatory rate, risk-assessment behavior and nest-building performance. In the open-field test, male non-rescue mice displayed, on average, a 72.3% decrease in total activity counts, an 83.2% decrease in rearing counts and a 38.5% decrease in ambulatory rate compared with age-matched male wild-type mice. In contrast, although remaining below wild-type levels, the general activity, total rearing behavior and ambulatory rate of male rescue mice were significantly improved when compared with non-rescue mice (Fig. 3F).
of the four consecutive trial days. The latency to fall time for male rescue mice was significantly longer than non-rescue mice on days 2–4 of the trial paradigm (Fig. 3B, two-way ANOVA $P < 0.05$). In the light/dark place preference test, male rescue mice conducted fewer risk assessments while in the dark compartment compared with either wild-type or TH-cre controls ($n = 9$). (C) Anxiety-like behavior was assessed using the light–dark place preference test. The histogram shows the mean and standard error of the number of risk assessment time (head poke) taken by the different cohorts of male mice per minute of time spent in the dark chamber of the apparatus. The risk-assessment behavior of male rescue mice ($n = 9$) was significantly above that of male non-rescue mice ($n = 12$), but remained below the behavior of male wild-type ($n = 20$) or TH-cre controls ($n = 7$). (D) The nest-building test was conducted as an index of social behavior. The histogram shows the mean and standard error of the nest volume built by the different cohorts of mice 24 h after being placed in the test cage with a nestlet. Male rescue mice ($n = 14$) assembled significantly larger nests than male non-rescue mice ($n = 13$), but smaller than male wild-type ($n = 14$) or TH-cre control mice ($n = 13$). One-way ANOVA with Tukey's post hoc test for multiple comparisons was used for the open-field, light–dark place preference and nesting behavior tests, and a two-way ANOVA (genotype versus trial) with Bonferroni's post hoc test was used for the accelerating rotarod test. For each panel, the asterisk indicates $P < 0.05$ compared between rescue and non-rescue mice.

Catecholaminergic preservation of MeCP2 improves the ambulatory and anxiety-like behavioral deficits of adult female MeCP2-deficient mice

Although less well characterized to date, female MeCP2$^{+/−}$ mice also display significant impairments in overall activity, ambulation rates, motor coordination and anxiety-like behavior after 8–12 months of age (19,34,35), although these deficits are not as severe as seen in male MeCP2-deficient mice (15,35). Consistent with the results obtained in male rescue mice, these behavioral deficits were also largely improved in female rescue mice. In the open-field test, the general activity counts, rearing behavior and average ambulation rate were each significantly improved compared with female non-rescue mice ($n = 9$). In the accelerating rotarod test, female rescue mice showed a partial rescue; their performance was significantly improved than female non-rescue mice on the fourth day of the trial paradigm (Fig. 4B, two-way ANOVA $P < 0.05$). In the light/dark place preference test, female rescue mice displayed a significant improvement in risk-assessment behavior compared with age-matched female non-rescue mice (Fig. 4C, one-way ANOVA $P < 0.05$). In fact, the risk-assessment behavior of female rescue mice did not significantly differ from that of female wild-type mice. In the nest-building test, female rescue mice assembled nests that were significantly larger in total volume than those of female non-rescue mice (Fig. 4D, one-way ANOVA $P < 0.05$), and equivalent in volume.

Figure 3. Behavioral performances are improved in male rescue mice. (A) Histogram showing the mean and standard error of male rescue mice ($n = 16$, black) relative to male non-rescue mice ($n = 17$, dark gray) and male TH-cre control mice ($n = 24$, light gray) in the open-field test. On the histogram, the average performance of male wild-type mice is denoted as 100% (dotted line). The general activity, rearing and ambulatory rate of male rescue mice were significantly improved from male non-rescue mice. (B) Motor coordination was assessed using the accelerating rotarod test. Though remaining below the values of male wild-type ($n = 26$) or TH-cre ($n = 21$) control mice, male rescue mice ($n = 20$) displayed a significantly longer latency to fall than male ‘Non-rescue mice’ ($n = 17$) on trial days 2, 3 and 4. (C) Anxiety-like behavior was assessed using the light–dark place preference test. The histogram shows the mean and standard error of the number of risk assessment time (head poke) taken by the different cohorts of male mice per minute of time spent in the dark chamber of the apparatus. The risk-assessment behavior of male rescue mice ($n = 12$) was significantly above that of male non-rescue mice ($n = 12$), but remained below the behavior of male wild-type ($n = 20$) or TH-cre controls ($n = 7$). (D) The nest-building test was used as an index of social behavior. The histogram shows the mean and standard error of the nest volume built by the different cohorts of mice 24 h after being placed in the test cage with a nestlet. Male rescue mice ($n = 13$) assembled significantly larger nests than male non-rescue mice ($n = 13$), but smaller than male wild-type ($n = 14$) or TH-cre control mice ($n = 13$). One-way ANOVA with Tukey's post hoc test for multiple comparisons was used for the open-field, light–dark place preference and nesting behavior tests, and a two-way ANOVA (genotype versus trial) with Bonferroni’s post hoc test was used for the accelerating rotarod test. For each panel, the asterisk indicates $P < 0.05$ compared between rescue and non-rescue mice.
to those assembled by female wild-type or TH-cre mice (Fig. 4D).

Preservation of Mecp2 in catecholaminergic cells improves cortical electroencephalographic abnormalities in male, but not female, Mecp2-deficient mice

Previous studies have demonstrated the presence of spontaneous epileptiform discharge activity in the cortex of both male and female Mecp2-deficient mice (34,36). In our current cohort, 100% (9 of 9) of the male non-rescue mice examined displayed cortical epileptiform-like discharges (Fig. 5A and B), although no discharge activity was observed in any of the age-matched male wild-type mice (n = 5). In these male non-rescue mice, the average number of discharge events was 42.1 ± 7.9 per hour, with each discharge having an average duration of 2.8 ± 0.7 s and an average frequency of 6.2 ± 0.3 Hz (Fig. 5E and F). Each of the male rescue mice retained spontaneous cortical discharge activity (Fig. 5C and D), and there was no significant change in their average duration or frequency compared with male non-rescue mice. However, the average number of discharge events in the rescue mice was significantly decreased from that of male non-rescue mice to 20.5 ± 6.3 discharges per hour (Fig. 5G, P < 0.05, one-way ANOVA).

Although a significant decrease in average cortical discharge activity was seen in male rescue mice, it was not observed in female rescue mice. Cortical discharge activity was observed in female non-rescue mice (Fig. 6A and B), with an average incidence rate of 69.6 ± 15.5 events per hour (n = 5). Although this incidence rate is greater than in male non-rescue mice, the average duration of the discharge events is significantly less in the female non-rescue mice compared with male non-rescue mice. In female rescue mice, this cortical discharge activity was still observed (Fig. 6C and D), and there were no significant differences in its average duration or frequency compared with female non-rescue mice.
Unlike the decreased incidence rate observed in male rescue mice, however, the incidence rate of cortical discharge activity in female rescue mice \( (n = 6) \) was 67.1 ± 13.9 per hour, which did not significantly differ from the incidence rate seen in female non-rescue mice (Fig. 6G).

Preservation of Mecp2 in catecholaminergic cells improves peak hippocampal theta frequency in male, but not female, Mecp2-deficient mice

In addition to possessing epileptiform-like electroencephalographic (EEG) discharge activity, we have shown previously that neural network oscillatory activity is altered in male and female Mecp2-deficient mice (34,36). In our current cohort of male non-rescue mice, the peak hippocampal theta frequency was found to be 7.2 ± 0.1 Hz, compared with 8.7 ± 0.2 Hz for wild-type male mice \( (P < 0.05, \text{ one-way ANOVA}) \) (Fig. 7B and E). In male rescue mice, this peak theta frequency shift was partially restored. Although still slower than wild-type, the peak theta frequency of male rescue mice during exploratory behavior was 7.8 ± 0.1 Hz \( (P < 0.05 \text{ relative to non-rescue, one-way ANOVA}) \). In female non-rescue mice, the alteration in peak hippocampal theta frequency was less pronounced, but still significantly slower than that of female wild-type mice (Fig. 8B and E). Peak hippocampal theta frequency in female non-rescue mice was 7.9 ± 0.3 Hz, compared with 8.9 ± 0.2 Hz in female wild-type mice \( (P < 0.05, \text{ one-way ANOVA}) \). In contrast to the positive effect seen in males, however, the peak hippocampal theta frequency during exploratory behavior in female rescue mice was 8.0 ± 0.1 Hz, which did not significantly differ from female non-rescue mice (Fig. 8E).

Preservation of Mecp2 in catecholaminergic cells rescues deficits in hippocampal gamma-band oscillatory activity in male, but not female, Mecp2-deficient mice

Power spectrum analysis was then conducted to compare the composite power of frequencies in the theta and gamma ranges between wild-type, non-rescue and rescue mice of both genders. Analysis of total raw theta activity power revealed no significant changes in total power of the

(Fig. 6E and F). Unlike the decreased incidence rate observed in male rescue mice, however, the incidence rate of cortical discharge activity in female rescue mice \( (n = 6) \) was 67.1 ± 13.9 per hour, which did not significantly differ from the incidence rate seen in female non-rescue mice (Fig. 6G).

Figure 5. The incidence rate of cortical epileptiform discharge activity is reduced in male rescue mice. (A and B) Representative EEG recording traces showing 10 min of cortical activity (A), or 10 s of activity surrounding a discharge event (B), from a male non-rescue mouse. The upper and lower traces in (A) are contiguous in time, and individual discharge events are highlighted above the trace. Representative EEG traces from a male rescue mouse. As above, (C) 10 min of continuous activity, with discharges highlighted. (D) A single discharge event. Cortical EEG activity in wild-type mice \( (n = 6) \) was also assessed, and no discharge activity was seen in any of the individual mice (traces not shown). (E) Histogram showing the mean and SEM of the discharge duration for cortical epileptiform events in male non-rescue \( (n = 9) \) and male rescue \( (n = 8) \) mice. As shown, no differences in the average discharge duration of the individual discharge events were observed. (F) Histogram showing the mean and SEM of the frequency observed for individual discharges in male rescue and non-rescue mice. No differences in the average individual discharge frequency were observed between groups. (G) Histogram showing the mean and SEM for the number of epileptiform discharge events per hour in male rescue mice \( (n = 8) \) and male non-rescue mice \( (n = 9) \). The number of discharge events in the rescue mice per hour was significantly decreased from those of male non-rescue mice \( (*P < 0.05, \text{ Student’s unpaired t-test}) \).
6–12 Hz theta frequency range between groups (Figs 7D and 8D). Male wild-type mice displayed total theta power of 385 ± 56 mV^2/Hz, which was not significantly different from non-rescue male mice, whose total theta power was 295 ± 9 mV^2/Hz (P = 0.2, one-way ANOVA). The total theta power for male rescue mice was 345 ± 29 mV^2/Hz, which also did not differ significantly from either wild-type or non-rescue male mice (Fig. 7D). In contrast to the preservation of total theta power, however, significant changes were observed between groups for total gamma-band power. Analysis of total raw EEG power from 35–60 Hz revealed a significant decrease in total gamma-band power in male non-rescue compared with wild-type mice (124 ± 6 versus 235 ± 25 μV^2/Hz, respectively; P < 0.01, one-way ANOVA). In male rescue mice, however, total gamma power in this range was restored to 261 ± 31 μV^2/Hz, which was significantly improved from non-rescue mice, and not significantly different from wild-type mice (Fig. 7F).

As with male mice, no significant differences in total hippocampal theta power were observed between any of the female mouse groups during exploratory behavior (Fig. 8D). Total theta power in female wild-type mice was 396 ± 44 μV^2/Hz (n = 4), was 424 ± 101 μV^2/Hz for female non-rescue mice (n = 5) and was 415 ± 74 μV^2/Hz for female rescue mice (n = 5). Consistent with what was observed in male mice, the total hippocampal gamma-band power was also significantly diminished in female non-rescue mice compared with female wild-type mice (118 ± 11 versus 295 ± 47 μV^2/Hz, respectively, P < 0.01, one-way ANOVA). However, unlike the improvement in gamma power seen in male rescue mice, no improvement in total gamma-band power was observed. The total gamma power in female rescue mice during exploratory behavior was found to be 164 ± 16 μV^2/Hz, which did not differ significantly from female non-rescue mice, and remained significantly below the value of female wild-type mice (Fig. 8F).

**DISCUSSION**

Several of the impairments seen clinically in RTT patients show similarities with disorders involving dopaminergic or...
noradrenergic disturbances (23–25,37), and recent studies have shown MeCP2-deficient catecholaminergic neurons display altered morphology and function (38–41). Collectively, these observations suggest that deficits caused by the lack of MeCP2 in catecholaminergic cells may play a significant role in RTT pathophysiology. Recent data support this possibility, as the targeted restoration of Mecp2 function within HoxB1-expressing cells, which include populations of catecholaminergic cells of the hindbrain, significantly improved the RTT-like phenotype of Mecp2-deficient mice (21). Here, we extend upon this study by illustrating that the selective preservation of Mecp2 in catecholaminergic cells of both male and female Mecp2-deficient mice can rescue specific RTT-like behavioral and neurophysiological deficits. Our

Figure 7. The peak hippocampal theta frequency and the total hippocampal gamma activity power are significantly improved in male rescue mice. (A) A representative power spectrum of hippocampal EEG activity during exploration from male wild-type (black), rescue (dark gray) and non-rescue (light gray) mice. For this plot, the highest peak in the theta range of the raw data was set at the 100% level, and the remaining frequency powers were normalized to this peak value. (B) Representative normalized hippocampal power spectra showing specifically the theta range from male wild-type (black), rescue (dark gray) and non-rescue (light gray) mice during periods of exploratory behavior. Note the shift in peak theta power frequency seen between the different mice. (C) Representative spectral plots showing raw activity power (e.g. non-normalized) within the gamma-frequency range (35–60 Hz) in male wild-type (black), rescue (dark gray) and non-rescue (light gray) mice. (D) The histogram showing the mean and SEM of the total hippocampal non-normalized spectral power of the theta range during exploratory behavior of male wild-type ($n = 5$), non-rescue ($n = 9$) and rescue ($n = 7$) mice. No significant differences in total theta power were observed, indicating that the MeCP2-deficiency does not cause a ubiquitous decrease in hippocampal EEG spectral power. (E) The histogram showing the mean and SEM for the observed peak theta frequency during exploratory behavior for wild-type, non-rescue and rescue mice. The $n$ numbers for each group are the same as above. The peak theta frequency is significantly lower in non-rescue mice than in wild-type mice, whereas the peak theta frequency of rescue mice is significantly greater than non-rescue mice. Although improved, the peak theta frequency in the rescue mice remained significantly below the peak frequency of wild-types. (F) The histogram showing the mean and SEM of the total hippocampal non-normalized spectral power in the gamma range during exploratory behavior of male wild-type ($n = 5$), non-rescue ($n = 5$) and rescue ($n = 7$) mice. Unlike the preservation of total theta range power, total gamma-range power was significantly diminished in male non-rescue mice. In rescue mice, total gamma power was significantly improved when compared with non-rescue mice, and did not significantly differ from the levels of wild-type mice. For these panels, the asterisk indicates significance levels of $P < 0.05$ between the indicated groups (one-way ANOVA with Tukey’s post hoc correction).
results, therefore, not only add to the growing evidence that Mecp2 dysfunction in catecholaminergic cells plays a significant role in RTT pathophysiology, but also provide the proof-of-principle that the selective targeting of these cells is sufficient to improve several of the cardinal RTT-like deficits seen in Mecp2-deficient mice.

The positive effect on lifespan and survival rates associated with preserving Mecp2 function in catecholaminergic cells is consistent with previous suggestions that deficits in catecholaminergic neuronal function play a key role in the early lethality and sudden unexpected death seen in both this mouse model and clinical RTT (11,42). For example, treating Mecp2-null mice with the norepinephrine uptake inhibitor desipramine significantly extends lifespan (43,44), and as discussed above, the preservation of Mecp2 function in HoxB1-expressing neurons of the brain stem and spinal cord also significantly extends the lifespan of male Mecp2-deficient mice (21). Although our data show clearly that preserving Mecp2 expression in catecholaminergic cells significantly extend survival times in male Mecp2-deficient mice, it is worth noting that the lifespan extension seen by Mecp2 reactivation in HoxB1 neurons was more robust than what we observed in the

Figure 8. The peak hippocampal theta frequency and the total power of hippocampal gamma activity are not improved in female rescue mice. (A) The representative normalized power spectrum of hippocampal EEG activity during exploration from female wild-type (black), rescue (dark gray) and non-rescue (light gray) mice. (B) Representative normalized hippocampal power spectra of the theta range from male wild-type (black), rescue (dark gray) and non-rescue (light gray) mice during periods of exploratory behavior. (C) Representative spectral plots showing raw gamma-frequency range power distribution in female wild-type (black), rescue (dark gray) and non-rescue (light gray) mice. (D) The histogram showing the mean and SEM of the total non-normalized hippocampal theta spectral power during exploratory behavior of female wild-type (n = 4), non-rescue (n = 5) and rescue (n = 7) mice. No significant differences in total theta power were observed between groups. (E) The histogram showing the mean and SEM for the peak theta frequency during exploratory behavior for wild-type, non-rescue and rescue mice. Consistent with what was seen in male non-rescue mice, the peak theta frequency is significantly lower in female non-rescue mice than in female wild-type mice. However, the peak theta frequency of female rescue mice is not significantly altered and remains significantly below the peak theta frequency of female wild-type mice. (F) The histogram showing the mean and SEM of the total hippocampal non-normalized gamma-range spectral power during exploratory behavior in female wild-type, non-rescue and rescue mice. Total gamma-band power is significantly reduced from wild-type levels in non-rescue mice. In contrast to the effect seen in male rescue mice, however, total gamma power was not significantly improved in female rescue mice and remained significantly below the levels of wild-type mice. For these panels, the asterisk indicates significance levels of $P < 0.05$ between the indicated groups (one-way ANOVA with Tukey’s post hoc correction).
ponent of our study was the examination of cortical and catecholaminergic neural and peripheral systems of mice. We then determined the impact of a global loss of Mecp2 function across non-catecholaminergic cells. Our results showed that impairments of non-catecholaminergic neural circuits (at least some of which express HoxB1) also contribute significantly to the longevity of male Mecp2-deficient mice, and that full rescue of this phenotype will require less restrictive cell-type preservation of Mecp2 function.

The beneficial effect on longevity was not restricted to male mutants, as heterozygous female rescue Mecp2-deficient mice, which are the gender-appropriate model for clinical RTT, also displayed a significant diminution in spontaneous death rates. In fact, the sudden and unexpected death rates in our female rescue cohort did not differ significantly from those of wild-type female mice up to 1 year of age. This outcome is not necessarily surprising given the mosaic nature of female Mecp2-deficient mice, where, on average, endogenous Mecp2 is expressed in half of the cells in the body, which by itself prevents the early lethality seen in male Mecp2-deficient mice. Based on the average reactivation efficiency for Mecp2 observed in TH-positive neurons of the substantia nigra and locus ceruleus in male subjects (85%), the expression of Mecp2 in catecholaminergic cells of female rescue mice would be expected to increase from ~50% on average to >90% in these target neuronal populations. Thus, our data suggest that this increase in Mecp2, occurring on the already existing mosaic expression pattern of Mecp2 in female Mecp2-deficient mice, is able to completely abrogate the sudden and unexpected death seen in this gender-appropriate RTT mouse model.

In addition to improving longevity and diminishing spontaneous death rates, our data also show that the catecholaminergic-specific preservation of Mecp2 partially rescues behavioral deficits in ambulation, anxiety-like behavior, motor coordination and nest-building in both male and female Mecp2-deficient mice. As pharmacologic and genetic manipulations have established a role for noradrenergic and dopaminergic systems in these behaviors (45–50), the observed improvements in the respective rescue mice are consistent with heightened overall catecholaminergic function in the rescue mice. Intriguingly, Samaco et al. (28) demonstrated that the selective ablation of Mecp2 from only catecholaminergic cells using a similar TH-cre transgenic mouse was sufficient to cause hypoactivity, impaired motor performance and elevated anxiety-like levels in mice. Our results complement this study, therefore, by showing that the preservation of Mecp2 in only catecholaminergic cells is sufficient to improve the performance of both male and female Mecp2-deficient mice in a host of behavioral tasks. Collectively, these results highlight the importance of Mecp2 function in the catecholaminergic system and show that retaining Mecp2 function in these relatively small numbers of neurons and peripheral cells is able to partially overcome the impact of a global loss of Mecp2 function across non-catecholaminergic neural and peripheral systems of mice.

In addition to assessing behavioral outcomes, a novel component of our study was the examination of cortical and hippocampal EEG activity in the male and female rescue mice. Our previous work revealed the presence of EEG abnormalities in both male and female Mecp2-deficient mice, which include the presence of spontaneous high-amplitude epileptiform discharges in somatosensory cortex (34,36), and a shift in the average peak frequency of hippocampal theta wave activity during exploratory behavior (36). In this study, we also showed that hippocampal gamma-band power during exploratory behavior is significantly reduced in both male and female Mecp2-deficient mice, an observation that comports with data from a recent report that also showed gamma-band abnormalities in a related strain of Mecp2+-/- mice (51). Consistent with male Mecp2-deficient mice being more severely affected than female Mecp2-deficient mice, the average discharge duration, the peak theta frequency and the magnitude of total gamma power attenuation were more pronounced in male Mecp2-null mice than heterozygous female Mecp2+-/- mice. Intriguingly, however, significant improvements in EEG abnormalities were evident only in male rescue mice. This gender-specific outcome was unexpected, as we anticipated the less robust phenotypic deficits of female Mecp2-deficient mice would be more amenable to correction by selective catecholaminergic Mecp2 reactivation. One possible explanation for the selective improvement in male rescue mice might be a 'ceiling' response, where the restoration of Mecp2 in catecholaminergic neurons was sufficient to partially improve the severe EEG phenotypes of male Mecp2-null mice, but not sufficient to completely restore these phenotypes to wild-type levels. In fact, the improved theta frequency and gamma power of the male rescue mice remained below their wild-type levels, but interestingly, these values in the male rescue mice were not significantly different from their respective values in female non-rescue mice. Thus, the EEG phenotypes of female non-rescue mice might already be at the ceiling level that could be afforded to male mice by catecholaminergic-specific Mecp2 preservation, as their phenotype arises from their, on average, 50% mosaic nature. Irrespective of the mechanism, however, these results do highlight an important point: they provide evidence that interventions showing positive effects in male Mecp2-deficient mice may not show the same beneficial statistically significant effects in mosaic female Mecp2+-/- mice. Given that the vast majority of clinical RTT cases affect females, these results highlight the importance of including female Mecp2-deficient mice in future translational studies.

**MATERIALS AND METHODS**

**Animal subjects**

All animal procedures were reviewed and approved by the Canadian Council on Animal Care and Toronto Western Research Institute. Mice were housed together with littermates in a controlled facility on a 12:12 h light–dark cycle. TH-cre mice were obtained as a gift from Dr Joseph Savitt (Johns Hopkins University) and maintained on a pure C57Bl/6 background. Mecp2^tm2Bird^ mice (16) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and also maintained on a pure C57Bl/6 background. TH-Cre male mice were crossed with Mecp2^tm2Bird^ female mice to
generate experimental subjects of both genders. Polymerase chain reaction was used to identify the genotype of these mice. DNA samples were prepared using the HotSHOT genomic DNA preparation method (52) on tissues collected from ear punches. The floxed-stop sequence is identified using the primer set: 5′-CTTCAGTGACACGTCGAGC and 5′-CATTCTGACGCCTTCAAATG. The presence of cre recombinase sequence was identified using the primer set: 5′-AAATGCTGCCTGAGTATGTTTATCTGC and 5′-GGAAAGTGCACATTACTGACGTA.

Behavioral assessments
Animals were assessed in the open-field and accelerating rotarod tests as described previously (19). For the open-field ambulation test, subjects were placed in a Plexiglas cage (20 × 30 cm²), and an automated movement detection system (AM1053 activity monitors; Linton Instruments, UK) was used to record the motor activities of the animals for a 1 h period. For the accelerating rotarod test, subjects were placed on a rotating rod (Med Associates, Inc., #ENV-575M, St Albans, VT, USA) that accelerates linearly from 3.5 to 35 r.p.m. over a 5 min period. The time at which the subject fell from the rotating rod was recorded via a laser beam sensor. Each subject was assessed on the accelerating rotarod three times a day for 4 consecutive days. Consecutive trials were separated by at least 1 h to allow the animals to recover from physical fatigue. Animals that circumnavigated the rod for three consecutive times were scored as having fallen off the apparatus upon the third rotation. For the light–dark placement preference test, mice were placed into a box consisting of a dark compartment (20 × 14 cm²) and a light compartment (20 × 28 cm²) connected through a single small opening (4 cm²). The amount of time the animal spent in the dark and light compartments and the number of risk-assessment behaviors (head pokes out of the dark compartment) each subject took while in the dark compartment was calculated the following day. All behavior tests were conducted between 9:00 a.m. and 13:00 p.m. to minimize circadian effects. Female subjects were assayed from ear punches. The

Phenotypic severity scoring
Animals were scored using the deficit scoring system described previously (16,17,33). In short, mice were scored from 0 to 2 according to the following scheme: mobility score: 0 = same as wild-type, 1 = slower movements than wild-type with intermittent freezing periods, 2 = severely reduced or no movement at all; gait score: 0 = same as wild-type, 1 = hindlimbs are spread wider than wild-types and slips or double tapping of the same feet is observed, 2 = dragging of hindlimbs or constant slips; hindlimb clasp score: 0 = hindlimbs are spread out when lifted by the tail, same as wild-type, 1 = one hindlimb is pulled toward the body and clasped or both hindlimbs are mildly pulled inward toward the body, 2 = both hindlimbs are clasped and pulled tightly into the body; breathing score: 0 = no noticeable respiratory abnormalities, same as wild-type, 1 = occasional respiratory jerks or some irregularity in breathing patterns, 2 = severe respiratory difficulties involving strong body jerks; tremor score: 0 = no tremor, 1 = intermittent mild tremors, 2 = severe and constant tremors; general condition score: 0 = well-groomed and shiny fur, opened eyes and normal body posture, 1 = slightly disheveled fur, squinty eyes and slightly hunched posture, 2 = extremely disheveled fur, eyes closed and severely hunched posture.

Immunohistochemistry
Animals were anesthetized through inhalation of 2% isoflurane, and transcardially perfused with 0.9% NaCl saline solution followed by perfusion with an ice-cold 2% paraformaldehyde–PBS solution. Intact brains were dissected from the skull and equilibrated overnight in a 30% sucrose/PBS solution at 4°C. The brain was then stored at −80°C until further assessment. For sectioning, the brain was cut in the sagittal plane at midline, and one hemisphere was embedded in OCT compound (Sakura, Torrance, CA, USA), and coronal sections (15 µm) were collected with a Leica cryostat (model Jung CM 3000, Wetzlar, Germany) at −24°C. Sections were blocked with 10% NGS + 2% BSA in 0.1% PBS-T for 1 h and then incubated with mouse anti-TH (1:1000, Millipore, #NG1752067) and rabbit anti-MeCP2 (1:500 Cell Signaling, #3456S) antibodies in 0.1% PBS-T supplemented with 2% normal goat serum overnight at 4°C. The sections were then washed using 0.1% PBS-T at room temperature three times and incubated with secondary antibodies conjugated to either DyLight 488 (Invitrogen, goat anti-mouse, #A11001) or DyLight 568 (Invitrogen, goat anti-rabbit, #A11011) for 1 h at room temperature. Following incubation, the sections were washed with PBS and then incubated briefly with DAPI (5 µg/ml, Roche Diagnostics, Indianapolis, IN, USA, #10236276001) for 3 min. Sections were then rinsed with PBS and mounted atop slides with Dako Fluorescent Mounting Media (Burlington, Ontario, Canada, #S302380). Imaging was done using a Zeiss Axiosplan 2 deconvolution microscope (Carl Zeiss, Göttingen, Germany).

Cell counting
MeCP2 and TH expression was determined through random sampling of every fifth section in brain slices containing substantia nigra or locus ceruleus. Substantia nigra was identified from the bregma −2.48 to −3.88 mm, and the locus ceruleus was identified from the bregma −5.83 to −5.85 mm. MeCP2 expression was assessed only in clearly identified nuclei that displayed DAPI staining. MeCP2 antibody specificity was confirmed by comparing the staining patterns of wild-type and MeCP2+/− animals. Counts were made for cells displaying either TH or MeCP2 immunoreactivity alone and for cells expressing both TH and MeCP2. Cell counts were conducted by two independent examiners blinded to condition and their individual counts averaged for analysis.
Electrode implantation

Animals were implanted with electrode cap assemblies as described previously (53). Briefly, animals were anesthetized under 2–4% isoflurane through inhalation. Electrodes made from polyimide-insulated stainless steel were implanted in the hippocampal CA1 (bregma, −2.3 mm; lateral, 1.7 mm; depth, 2.0 mm) and contralateral somatosensory cortex (bregma, −0.8 mm; lateral, 1.8 mm; depth, 1.5 mm). A reference electrode was implanted in the frontal cortex (bregma, −3.8 mm; lateral 1.8 mm; depth, 1.5 mm). Male mice were implanted between 40 and 60 days of age, which corresponds to a time when symptoms are beginning to manifest. Female mice were implanted after 250 days of age and after symptom onset. The implanted mice were allowed to recover for at least 7 days before any further experimentation was conducted. Baytril antibiotics (Bayer Healthcare, Toronto, Ontario, Canada) were added to the water supply 2 days before surgery and 7 days after surgery to minimize infections.

EEG recordings and analysis

EEG recordings were collected as described previously (36). Briefly, the implanted electrodes were connected to two independent head stages (Model-300, AM Systems, Inc., Carlsberg, WA, USA). EEG signals were amplified 1000 times, bandpass-filtered (0.01–1000 Hz) and digitized (Digitgard 1300, Axon Instruments, Weatherford, TX, USA). EEG data were collected at 60 kHz and analyzed using the Clampfit software (Axon Instruments). Recording sessions were at least 2 h in duration, and each subject was recorded for a minimum of two sessions on different days. The EEG recordings were decimated 10 times via the Clampfit 10.2 software before analysis. Epileptiform discharge-like events were counted manually using the following criteria: frequency between 6 and 12 Hz, minimum duration of 0.5 s and at least 1.5 times the baseline amplitude and high rhythmicity. Hippocampal theta epochs during exploratory behaviors were bandpass-filtered (0.5–200 Hz), and spectral plots (50% window overlap and frequency resolution of 0.25 Hz) were generated. A minimum of 10 epochs from at least two recording sessions were averaged to obtain peak theta frequency, total theta power and total gamma power for each animal. The frequency between 6 and 12 Hz with the greatest power was taken as the peak theta. Total theta wave power was calculated by taking the area underneath the spectral plot between 6 and 12 Hz. Similarly, total gamma-wave activity was taken as the area underneath the spectral plot between 35 and 60 Hz. All EEG data were calculated and analyzed using the Clampfit 10.2 software.

Spontaneous death rate was compared using population chi-square tests with 1 df. For all cases, the threshold for statistical significance was set at $P < 0.05$.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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