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RESPIRATORY PHENOTYPES ARE DISTINCTLY AFFECTED IN MICE WITH COMMON RETT SYNDROME MUTATIONS MECP2 T158A AND R168X

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Abstract—Respiratory disturbances are a primary phenotype of the neurological disorder, Rett syndrome (RTT), caused by mutations in the X-linked gene encoding methyl-CpG-binding protein 2 (MeCP2). Mouse models generated with null mutations in *Mecp2* mimic respiratory abnormalities in RTT girls. Large deletions, however, are seen in only ~10% of affected human individuals. Here we characterized respiration in heterozygous females from two mouse models that genetically mimic common RTT point mutations, a missense mutation T158A (*Mecp2*$^{T158A/+}$) or a nonsense mutation R168X (*Mecp2*$^{R168X/+}$). MeCP2 T158A shows decreased binding to methylated DNA, while MeCP2 R168X retains the capacity to bind methylated DNA but lacks the ability to recruit complexes required for transcriptional repression. We found that both *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ heterozygotes display augmented hypoxic ventilatory responses and depressed hypercapnic responses, compared to wild-type controls. Interestingly, the incidence of apnea was much greater in *Mecp2*$^{R168X/+}$ heterozygotes, 189 per hour, than *Mecp2*$^{T158A/+}$ heterozygotes, 41 per hour. These results demonstrate that different RTT mutations lead to distinct respiratory phenotypes, suggesting that characterization of the respiratory phenotype may reveal functional differences between MeCP2 mutations and provide insights into the pathophysiology of RTT.

Key words: Rett syndrome, apnea, hypoxia, hypercapnia, MeCP2, transcriptional repression domain.

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Abbreviations: EPSCs, excitatory post-synaptic currents; MBD, methyl-CpG-binding domain; MeCP2, methyl-CpG-binding protein 2; NLS, nuclear localization signals; NMDA, *N*-methyl-D-aspartic acid; NTS, nucleus tractus solitarius; PHFD, post-hypoxic decline in respiratory frequency; RTT, Rett syndrome; T_E, expiratory time; T_I, inspiratory time; T_{TOT}, total respiratory time; TRD, transcriptional repression domain; V_T, tidal volume; XCI, X-chromosome inactivation.

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INTRODUCTION

Rett syndrome (RTT) is a neurological disorder that affects approximately 1 in 10,000 girls. It is caused by mutations in the X-linked gene encoding methyl-CpG-binding protein 2 (MeCP2), a modulator of gene transcription (Amir et al., 1999; Guy et al., 2011). To date, more than 200 different mutations in *MECP2* have been identified in RTT cases (Van den Veyver and Zoghbi, 2001; Bienvenu and Chelly, 2006). The majority of these, however, are point mutations clustered within or near one of three conserved functional domains of *MeCP2* (the methyl-CpG binding domain [MBD], the nuclear localization signals [NLS], and the transcriptional repression domain [TRD]) suggesting the importance of these domains in protein function.

Respiratory abnormalities, characterized by frequent apnea and an irregular inter-breath interval, are a main phenotype of RTT (Katz et al., 2009; Ramirez et al., 2013). Thus far, the majority of respiratory studies in mouse models of RTT have utilized males with *Mecp2* null mutations. While this strategy avoids the issue of mosaic expression of MeCP2 due to random X-inactivation, it does not address the fact that patients with the disorder are almost exclusively heterozygous females; hemizygous male patients typically die *in utero* or shortly thereafter (Bienvenu and Chelly, 2006). Respiratory studies in *Mecp2* deficient female mice (*Mecp2*$^{-/+}$) have, to date, been confined to animals with deletions of exons III and IV (referred to as *Mecp2*^{Bird}) (Bissonnette and Knopp, 2006; Abdala et al., 2010; Samaco et al., 2013) or exon III (referred to as *Mecp2*^{Jae}) (Schmid et al., 2012). These large deletions, however, are seen in only ~10% of affected individuals (Katz et al., 2012). Animal models of RTT are important tools for preclinical studies; therefore, it is desirable to expand investigations to include the respiratory phenotype of RTT mouse models with common RTT mutations. In doing so, we gain a better understanding of which breathing abnormalities are most robustly affected across mutation types. In addition, these studies may provide insight into how different MeCP2 domains contribute to respiratory phenotypes in RTT and how different RTT mutations impair the functional domains of MeCP2.

In this study, we characterized the respiratory phenotype of two mouse models whose mutations are among two of the eight most commonly found in human cases of RTT (Colvin et al., 2004; Archer et al., 2007; Bebbington et al., 2008; Neul et al., 2008). In the first

mouse model, conversion of threonine 158 to methionine or alanine (T158M or T158A) results in a mutated protein with decreased binding to methylated DNA and that is degraded more rapidly than normal MeCP2 (Goffin et al., 2011). In the second model, a knockin of a stop codon at arginine 168, mimicking a nonsense R168X mutation, yields an early truncated MeCP2 protein that retains the MBD domain, but the TRD domain is lost (Brendel et al., 2007; Lawson-Yuen et al., 2007). We characterized the respiratory patterns in female heterozygotes with either the *MeCP2* T158A (*Mecp2*$^{T158A/+}$) or *MeCP2* R168X (*Mecp2*$^{R168X/+}$) mutation. In addition, it has previously been shown that *Mecp2* deficient mice have an augmented ventilatory response to hypoxia (Bissonnette and Knopp, 2006; Voituron et al., 2009; Ward et al., 2011) and a depressed carbon dioxide chemosensitivity (Zhang et al., 2011; Toward et al., 2013). Accordingly we have also examined the hypoxic ventilatory and CO_2 responses in the *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ female mice.

EXPERIMENTAL PROCEDURES

The experiments were approved by the Institutional Animal Care and Use Committee at the Oregon Health Science University and were in agreement with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals".

Animals

Mecp2$^{T158A/+}$ and their *Mecp2*$^{+/+}$ littermates were generated by crossing *Mecp2*$^{T158A/+}$ females to wild-type males (*Mecp2*$^{+/y}$). The eight *Mecp2*$^{T158A/+}$ females were from five litters and each mouse had one or two *Mecp2*$^{+/+}$ littermates that allowed direct comparisons. These mice were on a C57Bl/6 background. *Mecp2*$^{R168X/+}$ and *Mecp2*$^{+/+}$ mice (Lawson-Yuen et al., 2007) were a kind gift from Joanne E Berger-Sweeney at the Tufts University. These mice were on a mixed C57Bl/6 × 129S6/SvEv Tac background.

Plethysmography

Respiratory frequency, tidal volume (V_T) and their product minute ventilation (VE) were determined in a body plethysmograph (Mortola and Noworaj, 1983; Bissonnette and Knopp, 2006). Briefly, individual unanesthetized animals were placed in a 65 mL chamber with their heads exposed through a close fitting hole in Parafilm®. A pneumotachograph (Mortola and Noworaj, 1983) was connected to the chamber and a differential pressure transducer (Model PT5A, Grass Instrument Co., West Warick, RI, USA). The pressure signal was integrated to give V_T. Volume changes were calibrated by injecting known amounts of air into the chamber. The analog signal from the transducer was amplified, converted to digital, displayed on a monitor, and stored to disc by computer for later analysis. The studies were begun after the animal was given time to become adjusted to the chamber. A cone was fitted over the animal's head that allowed inspired gases to be delivered. For hypoxia experiments gas at the nose was rapidly, in 5 s, changed in succession from air to 8% O_2 for 5 min and then returned to air for a 5 min recovery period. Similarly for CO_2 sensitivity, after 5 min in air the animal was exposed in succession to 5 min of 1%, 3% and then 5% CO_2, balance air. The last 2 min of each 5 min exposure to air and to CO_2 were used for analysis as used previously in (Davis et al., 2006). Segments of the record that contained apnea were not used to calculate frequency. Minute ventilation in response to 8% O_2 and to graded CO_2 is reported as percent increase relative to the control. Recorded data was analyzed with custom functions in Chart v5.5.6 (AD Instruments, Inc., Colorado Springs, CO, USA). The hypoxia and hypercarbia studies were conducted when the animals were between 9 and 14 months of age.

Analysis

Apnea was defined as interval between breaths (T_{TOT}) $\geq$ 1.0 s. Respiratory cycle irregularity score was obtained from absolute $(T_{TOTn} - T_{TOTn+1})/T_{TOTn+1}$, and is expressed as the variance (Viemari et al., 2005). Results are given as mean ± SEM. Inspiratory time (T_I), expiratory time (T_E) and their sum (T_{TOT}) as well as V_T were determined from representative segments of 100 consecutive breaths (Fig. 1) using custom functions in Igor Pro® (WaveMetrics, Inc. Lake Oswego, OR, USA). Single comparisons between genotypes such as for brain weight and baseline respiratory parameters were analyzed with unpaired student *t*-tests. The relative effect of hypoxia and CO_2 on T_I compared to T_E within strains was determined with paired *t*-tests. Repeated measurements, such as for the three levels of CO_2, were made with a mixed model analysis of variance (ANOVA) with percentage CO_2 as the repeated measure and genotype as the between group factor. Significance was determined using Sigma stat 3.1 programs with $P < 0.05$.

RESULTS

Animal characteristics

Mecp2$^{T158A/+}$ ($n = 8$) and *Mecp2*$^{+/+}$ ($n = 8$) female mice were studied at monthly intervals from 4 months to a year of age. Average weight, 29.3 ± 1.1 gms, of the mutant mice did not differ from that of wild-type animals, 27.1 ± 1.8 g ($t = 1.034$, $p = 0.32$, unpaired *t*-test). As is characteristic of *Mecp2* null mice, brain weights at 12 to 14 months of age were significantly smaller in *Mecp2*$^{T158A/+}$ than *Mecp2*$^{+/+}$ controls (432 ± 5 vs. 500 ± 9 mg; $t = 6.508$, $p < 0.0001$). Studies in *Mecp2*$^{R168X/+}$ female mice ($n = 8$) started at ages between 11.5 and 12.4 months and continued to 15.5 months. Body weight in *Mecp2*$^{R168X/+}$ mice was not statistically different from wild type at 12.5 months (22.6 ± 0.7 vs. 24.4 ± 0.7 g, respectively). Similar to *Mecp2*$^{T158A/+}$, the brain weights of *Mecp2*$^{R168X/+}$ mice were reduced compared to *Mecp2*$^{+/+}$ controls (396 ± 5 vs. 450 ± 6 mg; $t = -7.052$, $p < 0.0001$).

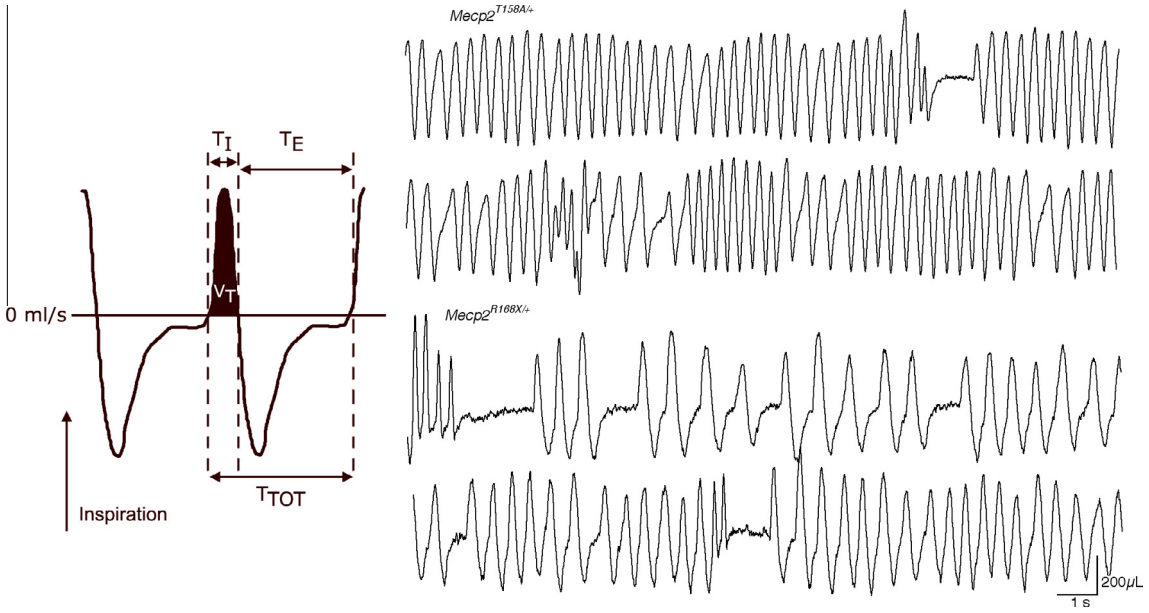

Fig. 1. Breathing in *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ mice. Diagram illustrating calculation of inspiratory (T_I), expiratory (T_E), total time (T_{TOT}), and tidal volume (V_T) of a sample breath. Representative traces in *Mecp2*$^{T158A/+}$ (top two traces continuous record) and *Mecp2*$^{R168X/+}$ bottom two traces. Calibration is the same for all traces. Apnea is more frequent and respiratory frequency is slower in *Mecp2*$^{R168X/+}$ compared to *Mecp2*$^{T158A/+}$ mice.

Respiratory pattern in *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ mice

All eight *Mecp2*$^{T158A/+}$ animals exhibited an increased incidence of apnea (Figs. 1 and 2A) that occurred between 5.6 and 10.8 months of age (mean: 7.7 ± 0.7 months); although the age at onset varied between mice. Increased apnea incidence did not persist, rather the rates returned to a level that did not differ from that in *Mecp2*$^{+/+}$ mice within 1 month in five *Mecp2*$^{T158A/+}$ mice and within 2 months in the remaining three *Mecp2*$^{T158A/+}$ mice. Since the onset of apnea did not occur at the same age in individual *Mecp2*$^{T158A/+}$ mice, we normalized the peak incidence across age and plotted apnea rates in the months preceding and following the peak (Fig. 2A). Breath interval irregularity at the time of peak apnea incidence was significantly greater in *Mecp2*$^{T158A/+}$ mice compared to *Mecp2*$^{+/+}$ ($F = 12.93$, $p = 0.029$; Fig. 2B), but not in the months preceding or following peak incidence (data not shown). Baseline T_E in *Mecp2*$^{T158A/+}$ mice was significantly prolonged compared to WT, but T_I, T_{TOT} and frequency were not significantly different (Fig. 2C and Table 1).

The respiratory pattern in *Mecp2*$^{R168X/+}$ mice was followed from 11.5 to 15.5 months. Due to age variation in the acquired *Mecp2*$^{R168X/+}$ cohort, respiration was assessed in five *Mecp2*$^{R168X/+}$ mice at 11.5 months and in eight mice at 12.5 months and after. Respiration in wild type mice ($n = 7$) from the R168X colony was determined at 11.5 and 14.5 months. The incidence of apnea in wild type mice at 11.5 months (9.7 ± 5.3/h) and 14.5 months (1.6 ± 0.9/h) was compared to apnea in *Mecp2*$^{R168X/+}$ at 11.5 and 12.5 months and 13.5, 14.5 and 15.5 months, respectively. At 11.5 months, apnea

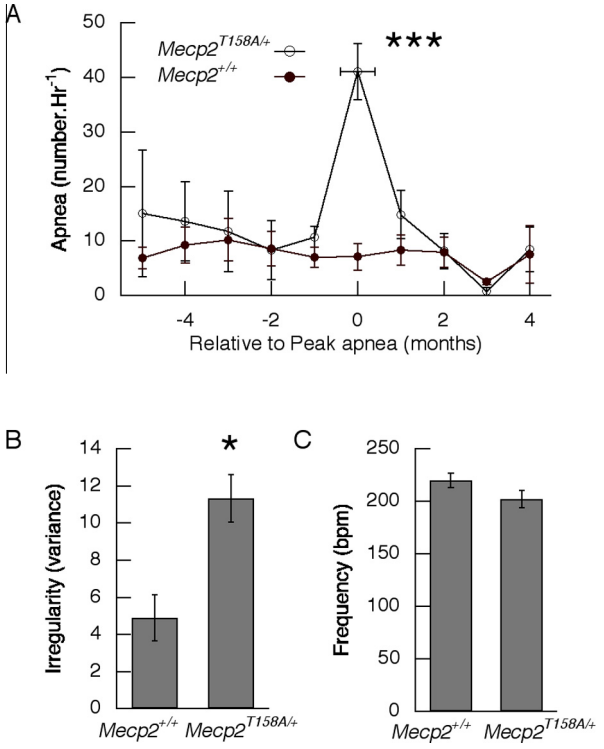

Fig. 2. Respiratory pattern in *Mecp2*$^{T158A/+}$ mice. (A) Apnea incidence normalized to the peak incidence that occurred at 7.7 ± 0.7 months shown as the months preceding and following the peak $n = 8$ for *Mecp2*$^{T158A/+}$ and for *Mecp2*$^{+/+}$; $^{***}F = 35.59$; $p < 0.0001$ (ANOVA, Tukey post hoc test). (B) Irregularity score at time of peak incidence in apnea. Score calculated from absolute $(T_{TOTn} - T_{TOTn+1})/T_{TOTn+1}$, and expressed as the variance (Viemari et al., 2005). $^{*}F = 12.93$; $p = 0.029$. (C) Respiratory frequency.

Table 1. Respiratory pattern in *MeCP2*$^{T158A/+}$ and *MeCP2*$^{R168X/+}$ mice

Strain	*N*	T_I ms	T_E ms	T_{TOT} ms	Rate bpm
Mecp2$^{+/+}$	8	135 ± 5	139 ± 4	275 ± 8	220 ± 7
Mecp2$^{T158A/+}$	8	145 ± 6	157 ± 7	301 ± 12	202 ± 8
F		1.22	5.09	2.12	3
P		0.29	0.041	0.099	0.105
Mecp2$^{+/+}$	7	135 ± 5	147 ± 5	283 ± 10	214 ± 7
Mecp2$^{R168x/+}$	8	212 ± 15	276 ± 2	487 ± 31	127 ± 7
F		21.13	35.24	33.29	64.62
p		0.0005	< 0.0001	< 0.0001	< 0.0001

T_I, inspiratory time; T_E, expiratory time; T_{TOT}, total respiratory time; bpm, breaths per minute.

was greater in all *Mecp2*$^{R168X/+}$ (Figs. 1 and 3A) than wild type and remained elevated throughout all 5 months of assessment ($F = 10.6$, $p = 0.0004$ for 11.5–12.5 months and $F = 3.59$, $p = 0.041$ for 13.5–15.5 months). Furthermore, inter-breath interval was irregular ($F = 16.67$, $p = 0.013$) (Fig. 3B). *Mecp2*$^{R168X/+}$ mice show significantly prolonged T_I, T_E and T_{TOT} and significantly reduced frequency ($F = 51.71$, $p < 0.0001$) relative to wild-type littermates (Table 1 and Fig. 3C).

In marked contrast to *Mecp2*$^{T158A/+}$ mice, the incidence of apnea in *Mecp2*$^{R168X/+}$ animals was significantly greater (41 ± 5 vs. 189 ± 43/h; $t = 69.55$ $p = < 0.0001$; unpaired *t*-test). The irregular breathing patterns and decreased frequency were also more severely affected in *Mecp2*$^{R168X/+}$ mice, relative to wild type, than in *Mecp2*$^{T158A/+}$ animals. Irregularity was 7.2 ± 0.7-fold greater than wild type in *Mecp2*$^{R168X/+}$ compared to 3.5 ± 0.4 in *Mecp2*$^{T158A/+}$ ($t = 1.55$, $p = 0.016$; unpaired *t*-test). Respiratory frequency was significantly lower in *Mecp2*$^{R168X/+}$ (0.59 ± 0.04 that of wild type) than in *Mecp2*$^{T158A/+}$ (0.92 ± 0.05 that of wild type) ($t = 5.43$, $p = 0.0001$; unpaired *t*-test).

Hypoxic ventilatory response in *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ mice

The hypoxic ventilatory response was augmented in females with both the T158A and R168X MeCP2 mutation. *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ heterozygous females exhibited an increase in minute ventilation in the first minute of breathing 8% oxygen that significantly exceeded that of wild type mice (Figs. 4A, B and 5). The greater increase in minute ventilation was entirely due to a larger increase in respiratory rate in heterozygous females with no difference in V_T (Fig. 5). On return to air, after the 5 min hypoxic exposure, ventilation remained elevated in *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ mice while it rapidly returned to baseline in wild type mice (Fig. 4). Additionally, respiratory frequency in *Mecp2*$^{+/+}$ mice decreased below that of the pre-hypoxia baseline (Fig. 5), a characteristic of rodents termed post-hypoxic frequency decline (Dick and Coles, 2000). In the 1st minute of recovery, frequency fell 22 ± 8 bpm in wild-type while it remained elevated 17 ± 7 bpm above baseline in *Mecp2*$^{T158A/+}$ animals ($F = 4.757$; $p = 0.047$). Similarly in *Mecp2*$^{R168X/+}$ mice respiratory frequency was 31 ± 23 bpm above baseline in the 1st min of recovery while it fell 38 ± 18 bpm in wild type ($F = 5.66$; $p = 0.037$). Moreover, the decrease in frequency during recovery in wild-type mice tended to be due to longer T_Es than at baseline (T_E: 30 ± 7% for T158A *Mecp2*$^{+/+}$ and 35.5 ± 18.9% for R168X *Mecp2*$^{+/+}$) than in lengthened T_Is (T_I: 12.6 ± 4.2% for T158A *Mecp2*$^{+/+}$ and 21.9 ± 17.3% for R168X *Mecp2*$^{+/+}$).

CO_2 chemosensitivity in *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ mice

Chemosensitivity was depressed in both *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ mice. The relative increase in minute ventilation in both mutant strains was significantly less at 1%, 3% and 5% CO_2 (Fig. 6) (*Mecp2*^{T158A}: $F = 20.589$; $p < 0.0001$ and *Mecp2*^{R168X}: $F = 21.81$; $p < 0.0001$). The depression was mostly pronounced at 1% CO_2 (71.4%) compared to that at 3% and 5% (55.8% and 55.5%, respectively) in *Mecp2*$^{T158A/+}$ mice. In *Mecp2*$^{R168X/+}$ mice, depression at the lowest CO_2 was also most prominent, 78.2% at 1% CO_2 compared to 51.2% and 30.2% at 3% and 5% CO_2, respectively. In general, wild type mice showed increased respiratory rate and V_T above baseline at all levels of CO_2 (Fig. 7). In contrast, respiratory rate only increased at 5% CO_2 in *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ mice (Fig. 7). CO_2 did not elicit an increase in V_T in any CO_2 level in mutant animals (Fig. 7). In comparison to wild type mice, the increase in frequency for *Mecp2*$^{T158A/+}$ mice tended to be less at 3% CO_2 and was significantly less at 5% CO_2, while it was significantly less in *Mecp2*$^{R168X/+}$ at 3% CO_2 (Fig. 7).

The increase in respiratory rate at 5% CO_2 across strains and genotypes was mainly the result of a significant shortening of T_E as compared to T_I. T_E was shortened to a greater extent than TI in *Mecp2*$^{T158A/+}$ (16.7 ± 0.9% vs. 11.6 ± 1.6%; $t = 3.196$, $p = 0.015$, paired *t*-test) and *Mecp2*$^{R168X/+}$ heterozygous females (23 ± 5.6% vs. 12.2 ± 4.3%; $t = 3.11$, $p = 0.021$) as well as their wild-type littermates (T158A *Mecp2*$^{+/+}$:18.1 ± 2.9% vs. 10.7 ± 2.5%; $t = 3.716$, $p = 0.0075$ and R168X *Mecp2*$^{+/+}$: 25.9 ± 2.4% vs.14.6 ± 3.1%; $t = 5.11$, $p = 0.002$).

DISCUSSION

The principal findings in this study are that heterozygous female mice with RTT mutation MeCP2 T158A: (1) develop a modest increase in the incidence of apnea between 5.6 and 10.8 months of age, followed by a normal breathing pattern thereafter; (2) have an augmented hypoxic ventilatory response and (3) depressed CO_2 chemosensitivity. In contrast, mice with RTT mutation MeCP2 R168X: (1) have a pronounced incidence of apnea equal to that in *Mecp2* null heterozygotes that is sustained from 11.5 through 15.5 months of age and (2) display hypoxic and hypercapnic ventilatory responses similar to *Mecp2*$^{T158A/+}$ mice and *Mecp2* null heterozygotes.

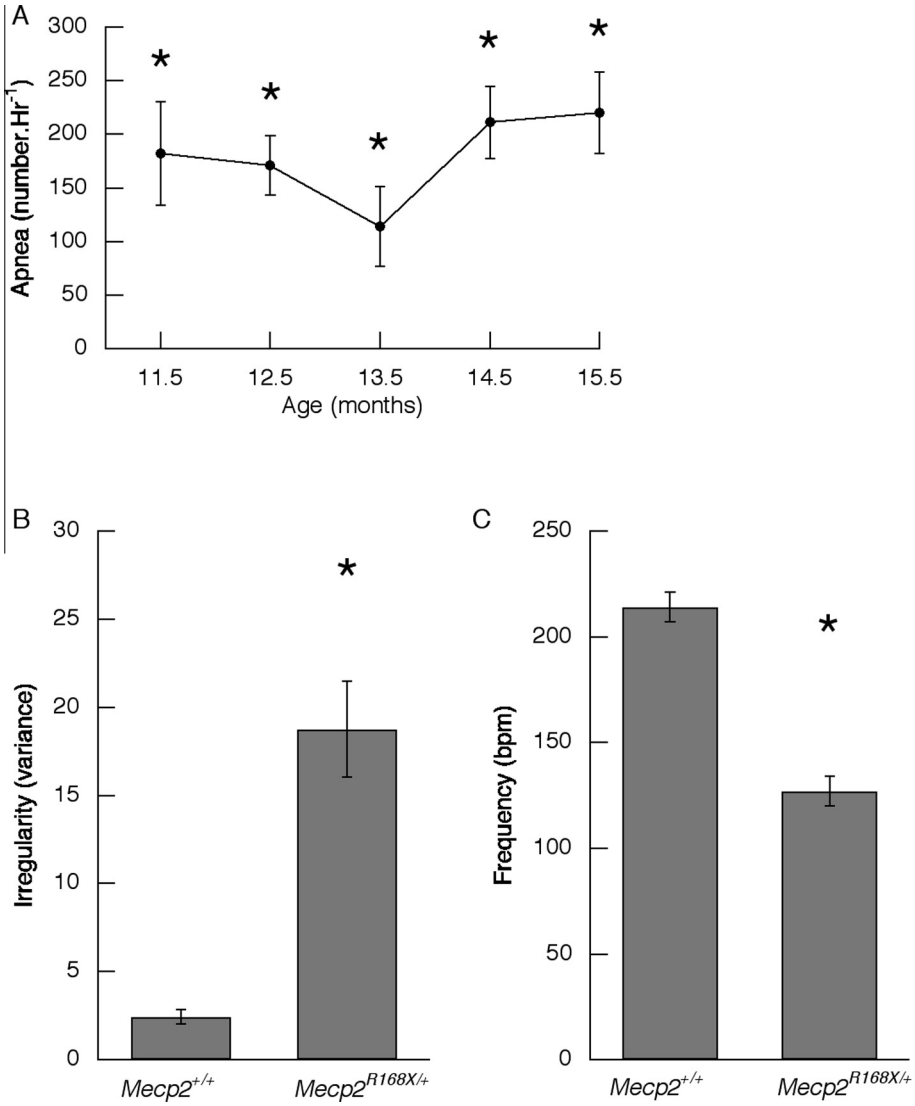

Fig. 3. Respiratory pattern in $Mecp2^{R168X/+}$ mice. (A) Incidence of apnea; $Mecp2^{R168X/+}$ n varied from 5 to 8. Apnea in $Mecp2^{+/+}$ averaged 9.7 ± 5.3/h at 11.5 months and 1.6 ± 0.9/h at 14.5 $n = 7$; $^{*}F = 10.607$, $p = 0.0004$ for 11.5 and 12.5 months; $F = 3.594$, $p = 0.041$ for 13.5–15.5 months. (B) Average irregularity score between 11.5 and 15.5 months. $^{*}F = 16.67$; $p = 0.013$ (C) Average respiratory frequency between 11.5 and 15.5 months, $^{*}F = 51.71$; $p < 0.0001$.

Thus, different RTT mutations result in similar age-dependent breathing abnormalities but with distinct severity.

$Mecp2^{T158/+}$ and $Mecp2^{R168X/+}$ mice have distinct respiratory phenotypes

The respiratory pattern was less significantly affected in $Mecp2^{T158A/+}$ than $Mecp2^{R168X/+}$ heterozygous mice. Interestingly, the incidence of apnea (range 24–68/h) in the $Mecp2^{T158A/+}$ mice was considerably less than that in $Mecp2^{R168X/+}$ (range 64–421/h) and in older $Mecp2^{Bird}$ or $Mecp2^{Jae}$ females measured in prior studies (average 80–160/h) (Abdala et al., 2010) (Knopp, Bissonnette unpublished). Thus, $Mecp2^{R168X/+}$ female mice had an incidence of apnea more similar to that measured in mice with large deletions. The irregular breathing pattern in $Mecp2^{R168X/+}$, sevenfold that of wild type, is greater than that observed in $Mecp2^{Jae/+}$ and $Mecp2^{Bird/+}$ heterozygous females (fivefold and fourfold, respectively) (Knopp, Bissonnette unpublished); while that in $Mecp2^{T158A/+}$ (3.5-fold) is somewhat less. Differences in apnea incidence and respiratory irregularity between mutation types suggest that the respiratory phenotypes are closely coupled to the functions of MeCP2. While breathing problems are reported to be common in patients with both T158M and R168X mutations (de Lima et al., 2009; Halbach et al., 2012), a distinction with respect to severity has not been documented in clinical studies.

The divergent respiratory phenotypes of $Mecp2^{T158A/+}$ and $Mecp2^{R168X/+}$ heterozygous mice share similarities and differences with the phenotype in RTT patients as well as other mouse models of RTT. RTT subjects have

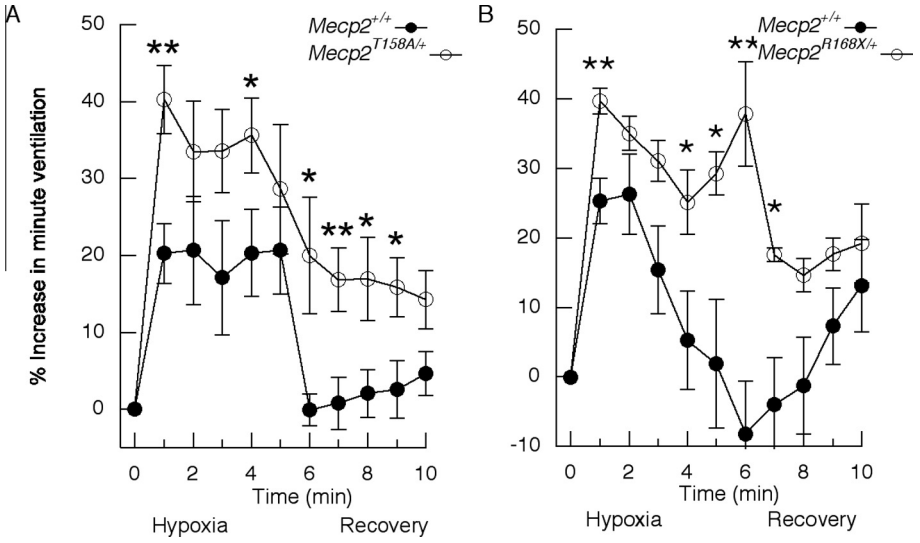

Fig. 4. Hypoxic ventilatory response in *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ mice. (A) *Mecp2*$^{T158A/+}$ and *Mecp2*$^{+/+}$ mice, $n = 8$ for each genotype; hypoxia: $F = 10.87$; min 1 $p = 0.0044$; min 3; $p = 0.043$. Recovery: min 6, $p = 0.041$; min 7 $p = 0.018$; min 10 $p = 0.048$. (B) *Mecp2*$^{R168X/+}$ mice $n = 6$ and *Mecp2*$^{+/+}$ mice $n = 7$: Hypoxia: $F = 5.16$; min 1 $p = 0.0039$; min 3 $p = 0.057$; min 4 $p = 0.046$; min 5 $p = 0.024$. Recovery: min 6 $p = 0.0013$; min 7 $p = 0.014$; min 8 $p = 0.068$.

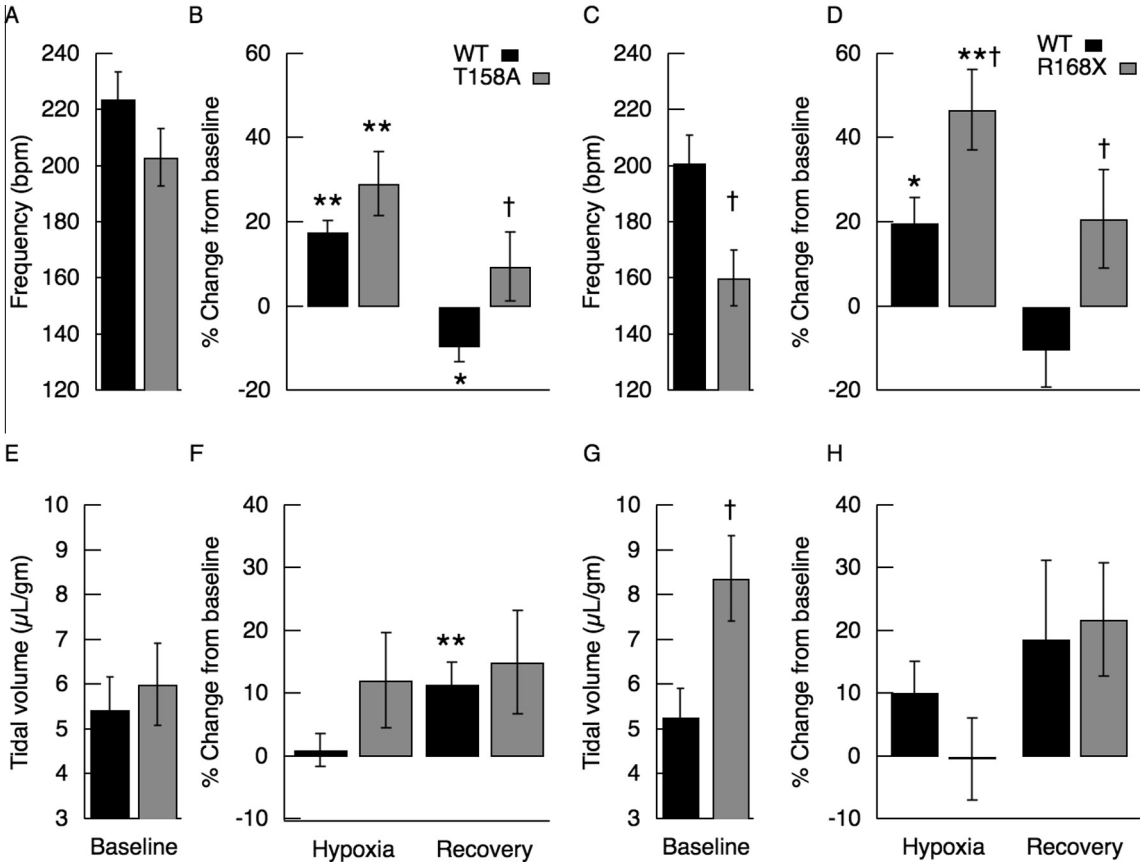

Fig. 5. Respiratory frequency and tidal volume in hypoxia and recovery. Data from the 1st min of hypoxia and 1st min of recovery in air are presented. A. Baseline frequency values in *Mecp2*$^{T158A/+}$ mice (n = 8) and *Mecp2*$^{+/+}$ (n = 8). B. Frequency response to hypoxia and recovery in T158A mice. C. Baseline frequency values for *Mecp2*$^{R168X/+}$ (n = 6) and *Mecp2*$^{+/+}$ (n = 7). D. Frequency response to hypoxia and recovery in *R168X* mice. E. Baseline tidal volume values in *Mecp2*$^{T158A/+}$ mice. F. Tidal volume response to hypoxia and recovery in *T158A* mice. G. Baseline tidal volume values in *Mecp2*$^{R168X/+}$ mice. H. Tidal volume response to hypoxia in R168X mice. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to baseline. † $p < 0.05$ compared to *Mecp2*$^{+/+}$.

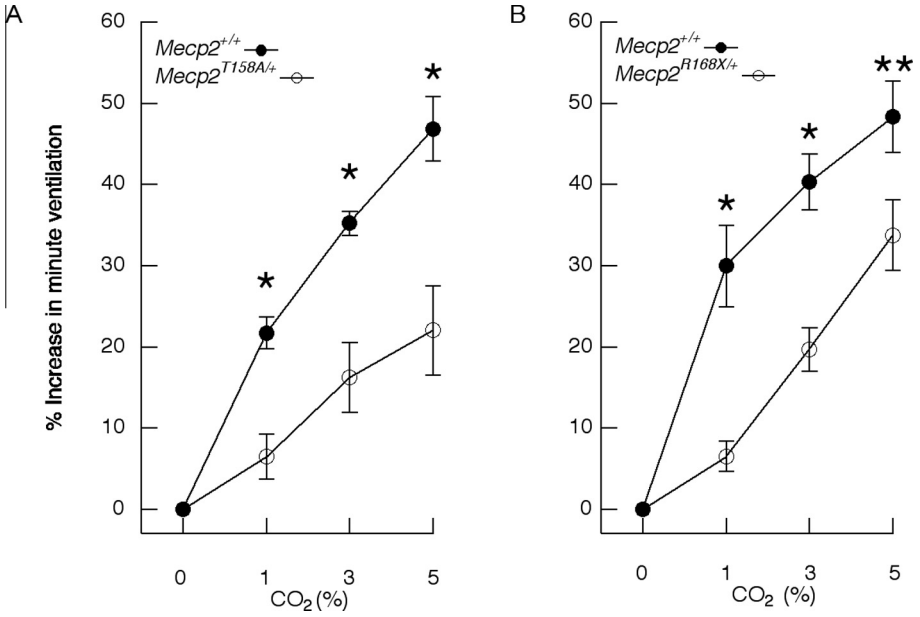

Fig. 6. Hypercapnic ventilatory response in *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ mice. (A) *Mecp2*$^{T158A/+}$ and *Mecp2*$^{+/+}$, $n = 8$ for each genotype. $F = 20.59$; 1% $p = 0.019$; 3% $p = 0.0019$; 5% $p < 0.0001$. (B) *Mecp2*$^{R168X/+}$ $n = 6$ and *Mecp2*$^{+/+}$ $n = 7$. $F = 21.81$; 1% $p = 0.0002$; 3% $p = 0.001$; 5% $p = 0.033$.

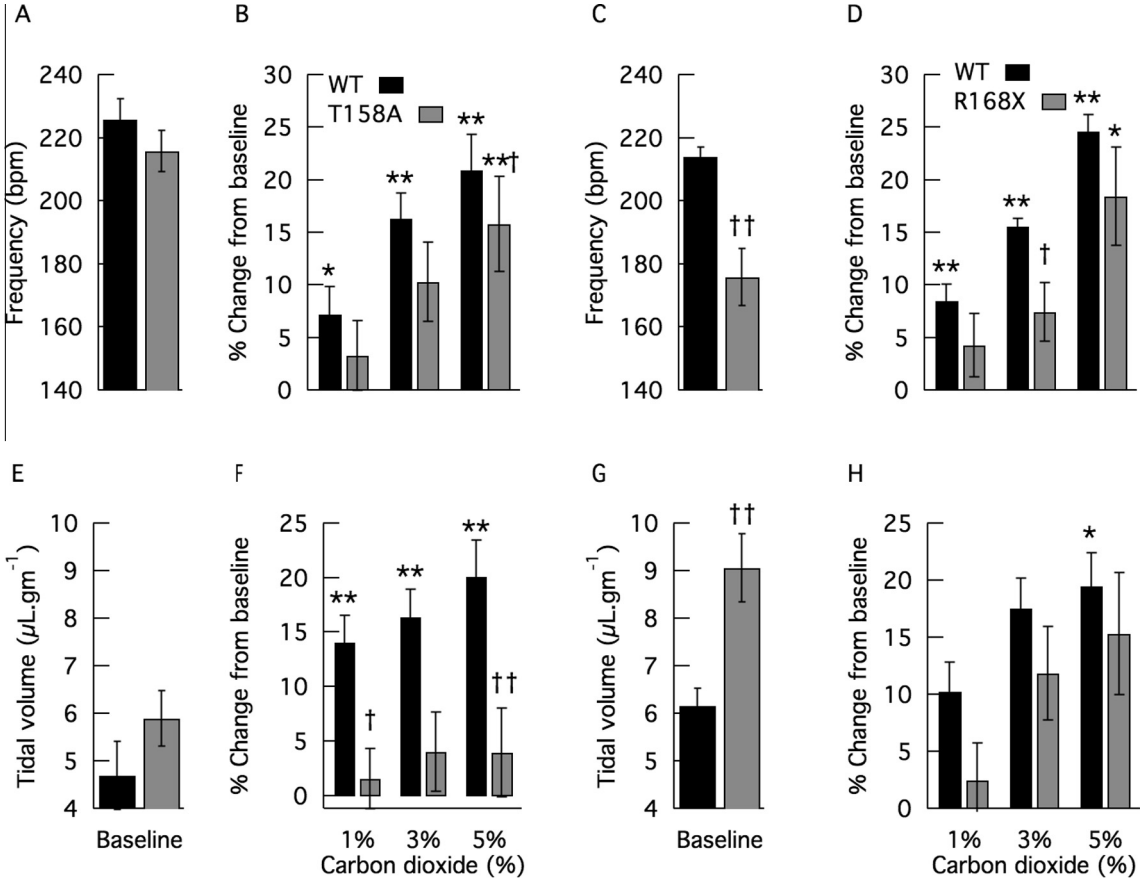

Fig. 7. Respiratory frequency and tidal volume response to carbon dioxide (CO_2). A. Baseline frequency values in *Mecp2*$^{T158A/+}$ mice (n = 8) and *Mecp2*$^{+/+}$ (n = 8). B. Frequency response to CO_2 in T158A mice. C. Baseline frequency values for *Mecp2*$^{R168X/+}$ (n = 7) and *Mecp2*$^{+/+}$ (n = 7). D. Frequency response to CO_2 in *T158A* mice. E. Baseline tidal volume values in *Mecp2*$^{T158A/+}$ mice. F. Tidal volume response to CO_2 in T158A mice. G. Baseline tidal volume values in *Mecp2*$^{R168X/+}$ mice. H. Tidal volume response to CO_2 in *R168X* mice. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to baseline. $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ compared to *Mecp2*$^{+/+}$.

been reported to have irregular breathing and increased respiratory frequency with a significantly shorter T_{TOT}, in which the shortening of T_E contributes more than shortening of T_I, compared to normal girls (Weese-Mayer et al., 2006). Similar to the irregular breathing patterns reported in RTT subjects, breath-to-breath irregularities were prominent in both the *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ heterozygous mice. However, the normal respiratory frequency we observed in *Mecp2*$^{T158A/+}$ mice and the decrease in respiratory frequency in *Mecp2*$^{R168X/+}$ mice differ from the human findings. In contrast, an increase in frequency was found in *Mecp2*$^{Jae/+}$ heterozygous female mice (Song et al., 2011; Schmid et al., 2012). The frequency in *Mecp2*$^{Bird/+}$ heterozygous female mice, on two different background strains, however, was the same as their respective wild type littermates (Samaco et al., 2013). While breathing irregularity is a common phenotype, respiratory frequency in mouse models of RTT appears to depend on the nature of the MeCP2 mutation.

Prolonged pauses in breathing are another common feature of the respiratory phenotype in RTT. A variety of types of breathing arrests have been reported in awake RTT subjects. Traces of chest wall movement in conjunction with respiration showed that arrest of breathing most commonly occurs at the end of inspiration with the chest wall held in an expanded position or a neutral position (Julu et al., 2001; Weese-Mayer et al., 2006; Stettner et al., 2008). Cheyne-stokes respiration and central apnea are far less common in awake RTT subjects whereby breathing arrest occurs following expiration (Southall et al., 1988; Julu et al., 2001). The pattern of apnea following expiration in *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ female mice (Fig. 1) differs from the common human observations, indicating that these mice may better model the more uncommonly described apneas.

Two other studies have found an asymptomatic as well as a symptomatic population of *Mecp2* deficient female mice (Song et al., 2011; Schmid et al., 2012). As noted above both studies used *Mecp2*$^{Jae/+}$ females. Song and co-authors reported that, when studied under anesthesia plus vagotomy, half of the mice were symptomatic, defined as an increase in respiratory frequency. These mice did not have apnea. Schmid et al. examined unanaesthetized mice and found that at 12 weeks of age half had an incidence of apnea that exceeded wild type. These results differ from that we have observed in *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ mice, all of which were symptomatic. Given that *Mecp2* is an X-linked gene, random X-chromosome inactivation (XCI) results in somatic mosaicism and the pattern of XCI likely varies between different cells types and individual subjects. A previous study reported that as many as 72% of neurons may express the wild type allele in the *Mecp2*$^{Jae/+}$ heterozygous mice (Braunschweig et al., 2004). Thus, unbalanced XCI and/or the XCI state of progenitor cells giving rise to critical cell mass in the brain stem may contribute to the lack of respiratory symptoms in a subpopulation of *Mecp2*$^{Jae/+}$ mice.

The differences in severity of the respiratory phenotype between *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ female mice are consistent with differences in severity of other behavioral phenotypes. Previous work reported that mice with the MeCP2 T158A mutation develop behavioral phenotypes that are similar to, but less severe than the phenotype of *Mecp2* null mice (Goffin et al., 2011). The MeCP2 T158A protein shows reduced stability and decreased binding to methylated DNA, but retains the ability to interact with co-repressor complexes (Ho et al., 2008; Goffin et al., 2011). Both the mild respiratory phenotype, described here, and the less severe behavioral phenotype, reported previously, support that T158A is a partial loss-of-function mutation. The R168X mutation, however, results in respiratory abnormalities and a behavioral phenotype more similar, but not identical, to that of *Mecp2* null mice (Schaevitz et al., 2013). The largely truncated R168X protein carries a partial NLS and lacks the TRD domain for interaction with co-repressors (Stancheva et al., 2003; Kumar et al., 2008). Given that the respiratory phenotype of *Mecp2*$^{R168X/+}$ females is similar to that of *Mecp2*$^{Jae/+}$ or *Mecp2*$^{Bird/+}$ with large deletions of the MBD or MBD and TRD respectively, it suggests that the complete loss of either of these functional domains results in a severe respiratory phenotype. Additionally, it is possible that the truncated R168X protein, though expressed at a low level (Lawson-Yuen et al., 2007), may have a dominant negative effect on MeCP2 function. Recently, Bird and colleagues reported that mice carrying a MeCP2 R306C missense mutation in the TRD domain, in which the interaction between MeCP2 and NCoR repressor complex is specifically disrupted, develop RTT-like phenotypes (Lyst et al., 2013), supporting the functional significance of the TRD domain. The respiratory phenotypes in those R306C knockin mice have yet to be characterized.

Unexpectedly, the longitudinal study of respiration in *Mecp2*$^{T158A/+}$ heterozygous females revealed a novel transient increase in apneas. Longitudinal studies of respiration in heterozygous *Mecp2*$^{Jae/+}$ and *Mecp2*$^{Bird/+}$ female mice have, to our knowledge, not been reported over the time span included in this study. Thus it is difficult to determine if the transient increase in apneas is a general feature of *Mecp2* deficient female mice or is unique to *Mecp2*$^{T158A/+}$ animals. A crossectional retrospective examination of apneas in *Mecp2*$^{Bird/+}$ mice between 5.5 and 18.5 months of age showed a consistent pattern of apneas (Bissonnette and Knopp, unpublished) ($n = 32$). While we were only able to follow *Mecp2*$^{R168X/+}$ mice from 11.5 to 15.5 months of age, there were no significant changes in apnea incidence suggesting that, of the mouse models studied to date, the transient increase and improvement in apneas over time may be unique to the *Mecp2*$^{T158A/+}$ heterozygous females. Importantly, in RTT patients, cross sectional observations suggest that abnormal breathing patterns improve with advancing age (Lugaresi et al., 1985; Julu et al., 2001). Thus *Mecp2*$^{T158A/+}$ heterozygous female mice may be a

useful model for understanding the molecular mechanisms behind improvements in respiration.

Hypoxic ventilatory response is altered in *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ mice

The augmented response to hypoxia noted in both *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ mutants is due to an exaggerated increase in respiratory frequency as compared to wild-type littermates and is similar to that reported previously in *Mecp2*^{Bird} female (Bissonnette and Knopp, 2006) and male mutant mice (Bissonnette and Knopp, 2006; Voituron et al., 2009; Ward et al., 2011; Ren et al., 2012). An increase in excitability of nucleus tractus solitarius (NTS) neurons may underlie the augmented response. Kline and coauthors (Kline et al., 2010) demonstrated that stimulation of the tractus produced larger excitatory post-synaptic currents (EPSCs) in slices from *Mecp2* null male mice compared to controls. Bath-applied brain-derived neurotrophic factor (BDNF) reduced the exaggerated EPSCs in the *Mecp2*-null mice. Furthermore, the absence of a post-hypoxic decline in respiratory frequency (PHFD) (Fig. 4) robustly distinguished both *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ mice from their respective wild type controls. In wild-type mice, PHFD is mediated mainly through the prolongation of T_E compared to baseline as shown previously (Dick and Coles, 2000; Song and Poon, 2009). PHFD requires intact signaling from neurons in the ventrolateral pons and depends on *N*-methyl-D-aspartic acid (NMDA) receptor function (Dick and Coles, 2000). While impaired NMDA receptor signaling has been demonstrated in symptomatic, but not asymptomatic, *Mecp2*$^{Jae/+}$ female mice (Song et al., 2011), the extent to which alterations in NMDA function in the ventrolateral pons and EPSCs in the NTS contribute to hypoxic ventilatory response in *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ mice has yet to be studied.

CO_2 chemosensitivity response is altered in *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ mice

The depressed hypercapnic ventilatory response displayed by both *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ is similar to that reported in *Mecp2*^{Bird} null male and female heterozygous mice (Zhang et al., 2011; Toward et al., 2013). Both breath frequency and V_T changes were significantly lower in *Mecp2*^{Bird} null males than wild types when mice were exposed to low concentrations of CO_2 (1–3%), but not at 6% and 9% CO_2 (Zhang et al., 2011). Similarly, in this study, the increase in minute ventilation was most depressed at lower CO_2 concentrations (~70% at 1% CO_2 versus 30–50% at 3% and 5% CO_2) in both *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ heterozygous females. The depressed ventilatory response to CO_2 in heterozygous mutants is the result of minimal increases in both respiratory rate and V_T as compared to baseline. In wild-type mice of both strains, respiratory rate and V_T increased at all levels of CO_2. In contrast, in *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ mutant mice, respiratory rate significantly increased only at 5% CO_2 while V_T remained unchanged. The increased respiratory frequency demonstrated only at 5% CO_2 in both wild type and heterozygous mutants was the result of decreased T_E rather than T_I. The blunted chemosensitivity in *Mecp2*-deficient mice appears to be connected to low levels of the neuromodulators norepinepherine and serotonin. Blocking norepinephrine reuptake markedly improved the response to CO_2 (Zhang et al., 2011) and increasing brain serotonin restored the CO_2 response to the level of wild-type mice (Toward et al., 2013). Thus, it is likely that elevation of norepinepherine and/or serotonin may restore CO_2 response in *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ mice as well. During episodes of hyperventilation, CO_2 concentration significantly decreases in RTT subjects (Southall et al., 1988; Smeets et al., 2006). As in mouse models, if RTT individuals have an elevated CO_2 apnea threshold, they would experience a cessation of breathing at carbon dioxide levels that sustain ventilation in unaffected individuals.

SUMMARY AND CONCLUSIONS

In conclusion this detailed characterization of the respiratory phenotype in heterozygous *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ mice shows that they share a number of similarities with mice containing large deletions in *Mecp2* but with distinct severity. Mice with both T158A and R168X mutations exhibit an increased incidence of apnea, and an irregular breath cycle. The irregular breathing pattern is consistent with what has been found in RTT subjects, but the reduced respiratory frequency in *Mecp2*$^{R168X/+}$ mice and the onset of apnea after expiration in both strains differ from the common breathholding following inspiration in RTT subjects. In addition, we found that abnormal respiration is ameliorated with advancing age in the *Mecp2*$^{T158A/+}$ strain. By comparing *Mecp2*$^{T158A/+}$ mice at their pre-symptomatic, symptomatic and recovered stages, these mice may prove to be very valuable tools for understanding the pathophysiology of respiratory disorders in RTT. The severe phenotype seen in *Mecp2*$^{R168X/+}$ heterozygous females are well suited for pre-clinical treatment trials that utilize respiration as a primary outcome measure, though classification of individual animal respiratory severity is necessary prior to treatment trials. Furthermore, the augmented hypoxic ventilatory response and depressed hypercapnic response exhibited by both *Mecp2*$^{T158A/+}$ and *Mecp2*$^{R168X/+}$ and previously in mice containing large deletions suggest that these respiratory phenotypes in particular, may be robust outcome measures for pre-clinical trials.

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