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ORIGINAL PAPER

Breathing abnormalities in a female mouse model of Rett syndrome

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Abstract Rett syndrome (RTT) is a female neurodevelopmental disease with breathing abnormalities. To understand whether breathing defects occur in the early lives of a group of female *Mecp2*$^{+/-}$ mice, a mouse model of RTT, and what percentage of mice shows RTT-like breathing abnormality, breathing activity was measured by plethysmography in conscious mice. Breathing frequency variation and central apnea in a group of *Mecp2*$^{+/-}$ females displayed a distribution pattern similar to *Mecp2*$^{-/Y}$ males, while the rest resembled the wild-type mice. Similar results were obtained using the k-mean clustering statistics analysis. With two independent methods, about 20 % of female *Mecp2*$^{+/-}$ mice showed RTT-like breathing abnormalities that began as early as 3 weeks of age in the *Mecp2*$^{+/-}$ mice, and were suppressed with 3 % CO_2. The finding that only a small proportion of *Mecp2*$^{+/-}$ mice develops RTT-like breathing abnormalities suggests incomplete allele inactivation in the RTT-model *Mecp2*$^{+/-}$ mice.

Keywords Rett syndrome · MeCP2 · Brainstem · Breathing · Apnea · Plethysmograph

Introduction

Rett syndrome (RTT) is a neurodevelopmental disease caused by disruptions of the X-linked gene encoding methyl-CpG-binding protein 2 (MeCP2) which affects approximately 1 in 10,000 live female births [1–3]. Males with this defect are usually unable to survive after birth. In addition to autistic symptoms, people with RTT show breathing disturbances, such as irregular breathing, apnea, hyperpnea, apneusis, Valsalva breathing, air swallowing, etc. [4, 5]. The breathing disorders contribute to the high rate (26 %) of sudden and unexpected death, and developmental abnormalities in the brain [6].

Many of these breathing abnormalities exist in male mice with the *Mecp2* gene deletion (*Mecp2*$^{-/Y}$), which are typically used as mouse models of RTT. Our previous studies have shown that the *Mecp2*$^{-/Y}$ mice lose their sensitivity to moderate hypercapnia, while their sensitivity to severe hypercapnia is normal [7]. The defect seems to be, at least partially, due to the abnormal expression of pH-sensitive K^+ channels in the central nervous system [7]. As a result, CO_2 levels are detected only when hypercapnia becomes severe in the mice, leading to a transition from hypoventilation to hyperventilation. After the excessive CO_2 is removed from the body, hypoventilation is resumed. The defective response to moderate PCO_2 thus can lead to periodic hyper- and hypoventilation as seen in RTT.

Nearly all RTT patients are female, whereas the majority of previous studies on the RTT breathing phenotype were performed on male mouse models with disruption of the *Mecp2* gene. The rationale behind such an approach is that the RTT-like symptoms can be reliably produced in these *Mecp2*$^{-/Y}$ males, whereas they may be variable in female mice heterozygous with the *Mecp2* gene deletion (*Mecp2*$^{+/-}$) owing to random X-chromosome inactivation. However, the female animal models of RTT resemble more closely human RTT patients with respect to their genotypes. Therefore, information generated in *Mecp2*$^{-/Y}$ male mice needs to be validated in *Mecp2*$^{+/-}$ females.

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Indeed, breathing defects have been found in female *Mecp2*$^{+/-}$ mice older than 2 months, which is an age equivalent to full grown humans [8, 9]. However, it is unclear what percentage of *Mecp2*$^{+/-}$ females develop RTT-like breathing abnormalities, whether the breathing abnormalities occur in the early lives, and whether they deteriorate with growth. It is still challenging to address these questions, because the random X-chromosome inactivation causes only a certain number of mice to develop breathing disorders with variable symptoms, which requires subtle experimental approaches to reveal. In our previous studies, we have developed several methods for the analysis of the breathing irregularities in *Mecp2*-null mice, including population analysis of breathing variation and CO_2 sensitivity [7]. Therefore, we performed these studies to address the above questions in heterozygous *Mecp2*$^{+/-}$ female mice.

Methods

Animals

Mecp2-null and *Mecp2* heterozygous mice of strain B6.129P2 (C)-*Mecp2*tm1.1Bird, developed by Dr. Adrian Bird, were used as mouse models for Rett syndrome in this study [10]. To produce *Mecp2*$^{-/Y}$ mice, *Mecp2*$^{+/-}$ purchased from the Jackson Laboratory (Bar Harbor, ME) were crossed with Swiss Webster *Mecp2*$^{+/Y}$ males. Genotypes of females and males were identified by following protocols of the Jackson Laboratory. Female and male offspring were used in the following studies. All animal experimental procedures were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the regulation of the Institutional Animal Care and Use Committee of Georgia State University.

Measurements of breathing activity by plethysmography

Breathing activity of unanesthetized mice was measured by plethysmography. The individual mouse was kept in the plethysmograph chamber ($\sim$40 ml) connected to an empty reference chamber. The animal was allowed to adapt to the chamber for at least 20 min. After this period, movements of the mouse monitored with a digital camera were reduced. A recording was taken for 30 min under normal air-ventilation conditions. Breathing activity of the mouse was analyzed offline. Records with the interference of animal movements were rejected from further analysis. A total of 52 individual *Mecp2*$^{+/-}$ female mice were tested. Thirteen were tested on 2–4 occasions with at least a month before subsequent testing. One breath cycle consisted of a period of inspiration followed by a period of expiration. Times for inspiration and expiration were detected using Clampfit 10 software, and used for calculation of the frequency of breathing as described previously [7].

CO_2 ventilation

In the experiments, mice were allowed to adapt to the chamber for 20 min when the plethysmograph chamber was ventilated with room air. After a 10-min recording of the baseline breathing activity with normal air, the ventilation air was switched to 3 % CO_2 by balancing with normal air in room air for 5 min at a speed of 50 ml/min. The gas mixture was obtained from a local supplier containing 21 % O_2. The total volume of the plethysmograph chamber was $\sim$40 ml. Since the mouse occupied most of this volume, a steady state of hypercapnia was reached within 1 min as in previous experiments [7].

Data analysis

The ventilation parameters breathing frequency (*f*) and apneas per hour were analyzed. To calculate variability of *f*, breathing activity was analyzed with the Clampfit 10 software. After an average of at least 200 successive breathing cycles, the arithmetic mean value and standard deviation (SD) were obtained. The *f* variability or variation index was then calculated as the division of SD by the mean value. Apneas were detected manually, and counted as events per hour based on the time frame of recording. Apneas were defined as at least one sustained breath equivalent to one regular breath cycle. The entire recording period (30 min) under normal air ventilation was used to analyze the number of apneas. Breathing *f* variation data is reported as mean $\pm$ SE, and apneas are presented as median $\pm$ interquartile range (IQR). Differences in *f* variation between two groups were determined using Student's *t*-test or one-way ANOVA for multiple groups. Differences in apneas between groups were determined using a Mann–Whitney *U* test for two groups or Kruskal–Wallis analysis for multiple groups. Group distribution was tested with the k-means clustering statistics analysis (http://scistatcalc.blogspot.com/2014/01/k-means-clustering-calculator.html).

Results

Breathing frequency variation in female *Mecp2*$^{+/-}$ mice

Because of the random expression of the X chromosome, *Mecp2*$^{+/-}$ heterozygous females may have breathing

phenotypes resembling either wild-type (WT) or *Mecp2*$^{-/Y}$ mice. Therefore, we measured breathing activity of the females in comparison with the WT, which consisted of normal male and female mice (*Mecp2*$^{+/+}$) without *Mecp2* deletion. The WT mice ($n = 56$) showed stable breathing activity. In contrast, a clear variation in *f* and apnea were seen in *Mecp2*-null males (*Mecp2*$^{-/Y}$) (Fig. 1b).

To analyze our data quantitatively, the variation index was used to measure the breathing *f* variations as we described previously [7]. Consistent with our previous observations [7], the *f* variation was much greater in the *Mecp2*$^{-/Y}$ mice, showing a significant difference from the WT mice. In contrast, certain *Mecp2*$^{+/-}$ females showed breathing activity like the *Mecp2*$^{-/Y}$ mice, while the breathing of others resembled the WT (Fig. 1c), suggesting that they are not a single homogenous group.

To understand whether the *Mecp2*$^{+/-}$ females consist of two different populations with respect to their *f* variation and what percentage of mice develop RTT-like *f* variation, we studied the distribution of *f* variation index against ages. Firstly, we analyzed the *f* variation distribution of WT and *Mecp2*$^{-/Y}$ mice. Figure 2a shows that the *f* variation of the *Mecp2*$^{-/Y}$ mice tends to distribute between 0.2 and 0.4, while that of the WT is mostly located around 0.1 or between 0 and 0.2. Using the mean value and standard deviation of these data, Gaussian distribution curves were made to represent the data (Fig. 2a).

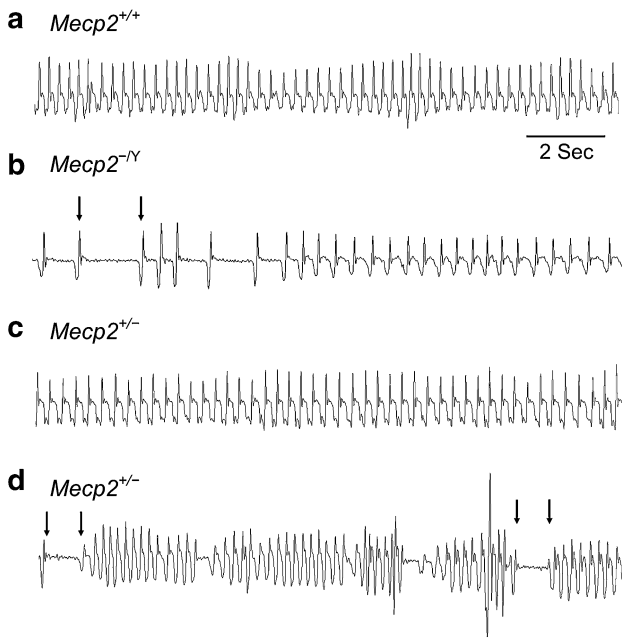

Fig. 1 *Mecp2*$^{+/+}$ and *Mecp2*$^{-/Y}$ mice show typical normal and abnormal breathing patterns, respectively. **a** *Mecp2*$^{+/+}$ mice exhibit normal inspiration (downdraft) and expiration breathing patterns. **b** *Mecp2*$^{-/Y}$ mice display stereotypical irregular breathing patterns. *Arrows* indicate an example of apnea. **c** An example trace of a *Mecp2*$^{+/-}$ mouse that exhibits normal breathing patterns. **d** An example trace of a *Mecp2*$^{+/-}$ mouse that exhibits breathing patterns similar to *Mecp2*$^{-/Y}$ mice

We then compared the *f* variation index of *Mecp2*$^{+/-}$ females with the Gaussian distribution curves obtained from WT and *Mecp2*$^{-/Y}$ mice. Figure 2b shows that although most of the *Mecp2*$^{+/-}$ females have *f* variations around 0.1 similar to the WT mice, several *Mecp2*$^{+/-}$ mice seem to have much greater *f* variations that were around 0.3. To prove that the apparent separation of the *f* variation index within the *Mecp2*$^{+/-}$ female population is statistically significant, we performed the k-means clustering analysis. Using this statistics method, the *f* variation index of the *Mecp2*$^{+/-}$ females was divided into two populations (Fig. 2c).

A characteristic of the *f* variation distribution is the overlap without a clear boundary, which was seen not only among *Mecp2*$^{+/-}$ females (Fig. 2b) but also between WT and *Mecp2*$^{-/Y}$ mice (Fig. 2a). Therefore, we chose to use the interception of the two Gaussian distribution curves as the threshold to separate the RTT-like phenotype (*Mecp2*$^{-/Y}$[R]) from the normal (*Mecp2*$^{-/Y}$[N]), which is 0.2 in *f* variation as shown in Fig. 2b. Statistically, the *f* variation of *Mecp2*$^{-/Y}$[R] mice was not different from that of RTT-like phenotype mice isolated by the k-means clustering (*Mecp2*$^{-/Y}$[r]) (Fig. 2d). So was *Mecp2*$^{-/Y}$[N] mice in comparison with *Mecp2*$^{-/Y}$[n] mice. Therefore, the interception of the two Gaussian distribution curves seems to be a useful way to separate the *Mecp2*$^{+/-}$ females with different breathing *f* variations.

With such a pooling, 10 of 75 female *Mecp2*$^{+/-}$ mice tested (13 %) were separated from the rest. These *Mecp2*$^{-/Y}$[R] mice had an *f* variation index of 0.24 that was highly significantly different from the WT (*f* variation index = 0.11). The *f* variation of the *Mecp2*$^{-/Y}$[R] had no significant difference from the *Mecp2*$^{-/Y}$ males (*f* variation index = 0.27) (Fig. 2e). The *f* variation index of the other group of *Mecp2*$^{+/-}$ females, *Mecp2*$^{+/-}$ [N] (0.10), resembled more the WT (Fig. 2e). Similar results were obtained by pooling *Mecp2*$^{+/-}$ mice using the k-means clustering, in which 13 (17 %) showed significantly high *f* variation (Fig. 2f). Statistical analysis of these two results showed no significant difference ($P > 0.05$, χ^2 test) although the k-means clustering seems more sensitive.

Based on these results obtained with two independent methods, it is likely that the *Mecp2*$^{+/-}$ females are not made of a homogenous group regarding breathing irregularity, and approximately 15 % of *Mecp2*$^{+/-}$ females show the RTT-like phenotype.

Apnea

A prolonged period of breathing cessation known as apnea is a hallmark of breathing dysfunction in RTT patients.

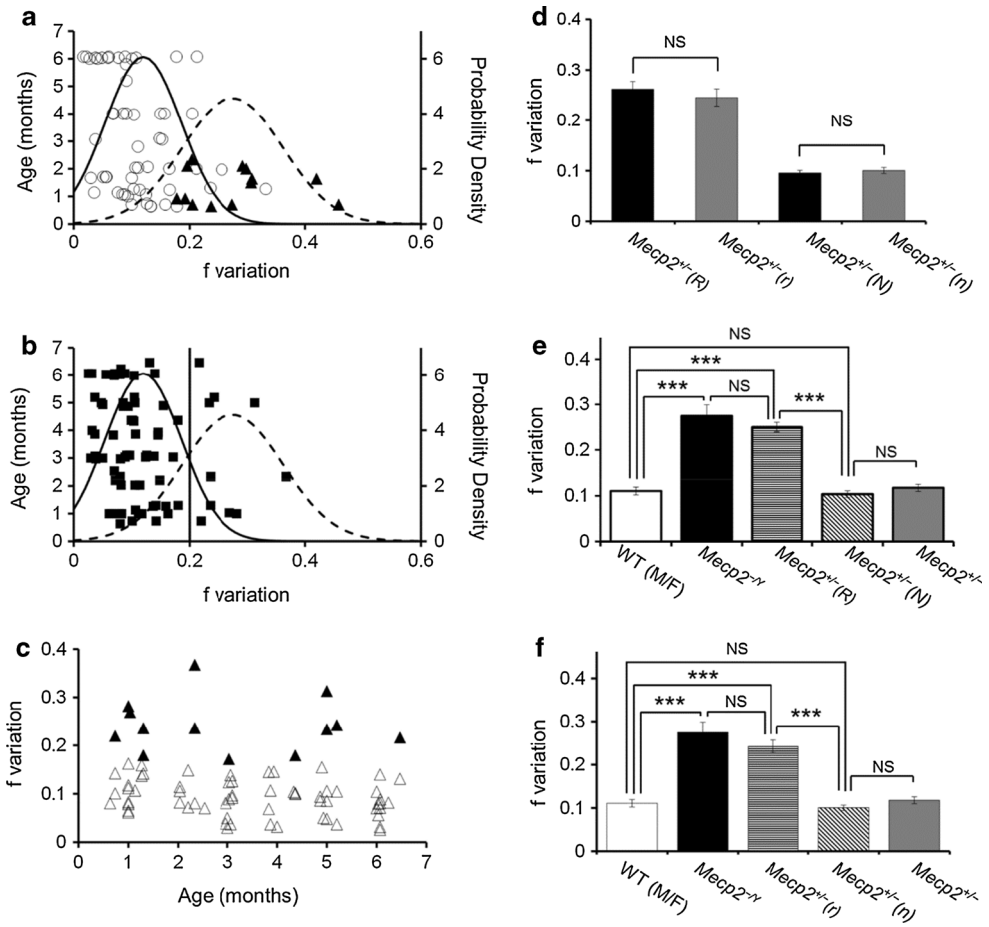

Fig. 2 Separation of *Mecp2*$^{+/-}$ mice based on *f* variation. **a** The *f* variation distribution of both WT ($n = 56$) and *Mecp2*$^{-/Y}$ ($n = 13$) mice against ages showed typical Gaussian distributions. WT mice are represented as *open circles*, *Mecp2*$^{-/Y}$ mice are represented as *black triangles*. **b** The *f* variation distribution of *Mecp2*$^{+/-}$ mice show *two peaks* that fit fairly to the Gaussian distributions of WT and *Mecp2*$^{-/Y}$ mice in (**a**). The *vertical line* at 0.20 indicates the intersection point that was used as a threshold to *Mecp2*$^{+/-}$ mice with and without breathing *f* variation. Of 75 *Mecp2*$^{+/-}$ mice, 52 individual *Mecp2*$^{+/-}$ female mice were tested. Thirteen were tested 2 to 4 times with no less than a month between tests. **c** *Mecp2*$^{+/-}$ mice were separated based on the k-means clustering method. RTT-like *Mecp2*$^{+/-}$ mice are represented as *black triangles*, and WT-like *Mecp2*$^{+/-}$ mice are represented as *open triangles*. **d** RTT-like *Mecp2*$^{+/-}$ and WT-like *Mecp2*$^{+/-}$ mice indicated by the threshold level show no significant differences from those indicated by the k-means clustering method. **e** With the threshold separation, 13 *Mecp2*$^{+/-}$ mice (*Mecp2*$^{+/-}$[R]) showed *f* variation like the *Mecp2*$^{-/Y}$, which have significant difference from the WT but not the *Mecp2*$^{-/Y}$ mice. The rest of the WT-like *Mecp2*$^{+/-}$ mice (*Mecp2*$^{+/-}$[N], $n = 62$) showed no significant difference in *f* variation from the WT. **f** When the *f* variation was separated using the k-means clustering method, the RTT-like *Mecp2*$^{+/-}$ mice ($n = 13$, *Mecp2*$^{+/-}$[r]) have significant difference from WT ($n = 56$) but not from *Mecp2*$^{-/Y}$ mice. Vice versa for the WT-like *Mecp2*$^{+/-}$ mice (*Mecp2*$^{+/-}$[n], $n = 62$). Data are presented as mean ± SE ($^{*}P < 0.05$, $^{***}P < 0.005$)

Although apneas are seen in the WT mice as well, the frequency of the apneas was extremely low. In contrast, *Mecp2*$^{-/Y}$ mice displayed high frequencies of apneas. Therefore, the apnea count was measured as number of events per hour (apneas/h), and used as another indication of the severity of breathing abnormality. Apnea was defined as at least one breath cycle missing as described above. The entire recording period (30 min) under normal air ventilation was used to analyze the number of apneas.

Similar to *f* variations, apnea counts showed normal (Gaussian) distributions in the WT and *Mecp2*$^{-/Y}$ mice, in which most WT and *Mecp2*$^{-/Y}$ mice were separate from each other, although overlaps were also found (Fig. 3a). At the intercept of two Gaussian distribution curves, a threshold value (38 apneas/h) was obtained. Using the threshold value, the *Mecp2*$^{+/-}$ females were divided into two groups (Fig. 3b). Of 75 female *Mecp2*$^{+/-}$ mice tested, 17 (23 %) were isolated as *Mecp2*$^{+/-}$[R] with RTT-like phenotype from the rest (*Mecp2*$^{+/-}$[N]). A similar number (17, 23 %) was isolated using the k-means clustering method (Fig. 3c). Statistical analysis of these two results showed no significant difference ($P > 0.05$, χ^2 test). Consistently, apnea counts of the *Mecp2*$^{+/-}$[R] were not significantly different from *Mecp2*$^{+/-}$[r] ($P > 0.05$, Fig. 3d).

After the median and IQR were calculated, a Kruskal–Wallis test showed that the *Mecp2*$^{+/-}$[R] females had no significant difference from *Mecp2*$^{-/Y}$ mice, but were significantly different from the WT in their medians (Fig. 3e); and vice versa for the rest of the WT-like *Mecp2*$^{+/-}$ females, i.e., *Mecp2*$^{+/-}$[N]. Separation of apneas/h with the k-means clustering analysis was identical to the threshold results (Fig. 3f).

Since *Mecp2*$^{+/-}$ mice with significant *f* variations were not completely identical to those with severe apneas, *Mecp2*$^{+/-}$ mice with either alone could have a mild breathing abnormality. Therefore, we further examined these mice. Using k-means clustering analysis, 9 of 75 *Mecp2*$^{+/-}$ mice (*Mecp2*$^{+/-}$ [v]) showed significant *f* variation and apneas (Fig. 4a, b). With respect to *f* variation and apnea, the *Mecp2*$^{+/-}$ [v] mice were not significantly different from the *Mecp2*$^{+/-}$[R] mice, but significantly different from the *Mecp2*$^{+/-}$[N] females (Fig. 4c, d).

Similar results were obtained based on the threshold levels determined, in which 7 of 75 *Mecp2*$^{+/-}$ mice showed both significantly high *f* variation and apneas. The *Mecp2*$^{+/-}$[v] mice isolated with the threshold did not show more severe *f* variation and apnea than the *Mecp2*$^{+/-}$[R] mice, either (not shown). These results thus suggest that either *f* variation or apnea may be used to isolate *Mecp2*$^{+/-}$ female mice with RTT-like breathing abnormalities from the rest of the females.

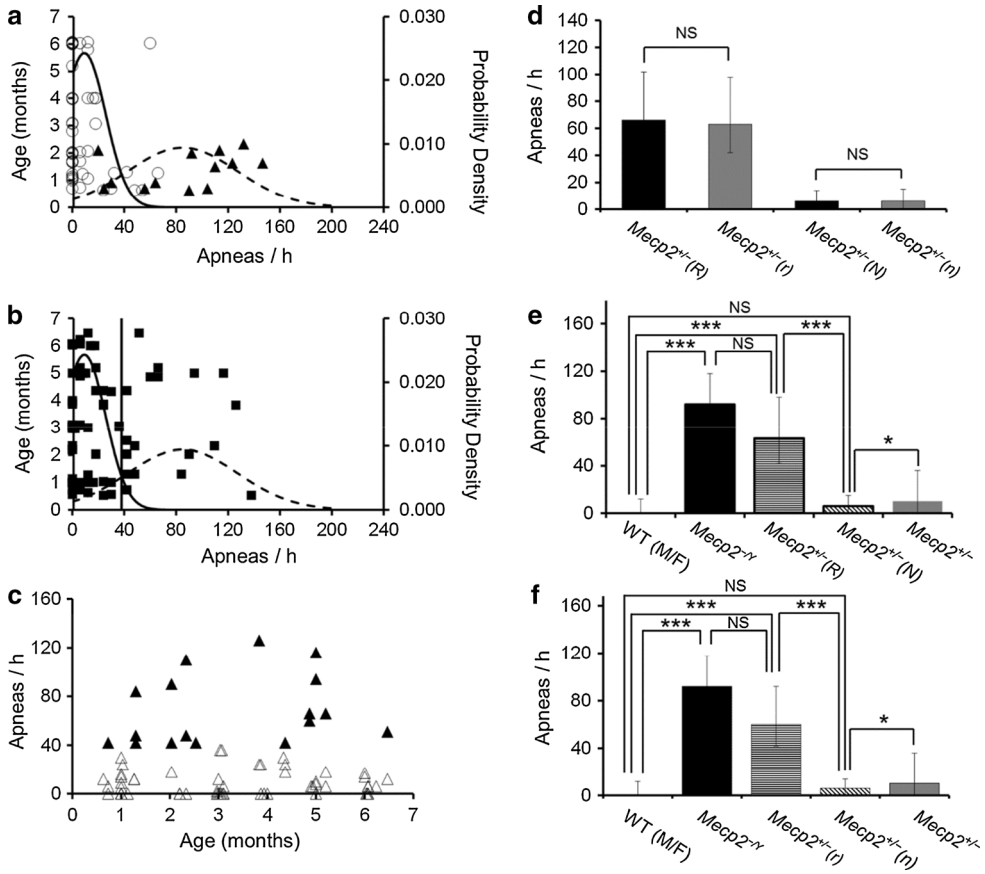

Fig. 3 Separation of *Mecp2*$^{+/-}$ mice based on number of apneas. **a** Both WT ($n = 56$, *open circles*) and *Mecp2*$^{-/Y}$ ($n = 13$, *black triangles*) mice show typical Gaussian distributions of the number of apneas. **b** *Mecp2*$^{+/-}$ mice are represented as *black squares*. The *vertical line* represents the separation level at 38 apneas/h. **c** *Mecp2*$^{+/-}$ mice were separated based on the k-means clustering method. RTT-like *Mecp2*$^{+/-}$ mice ($n = 17$) are represented as *black triangles*, and WT-like *Mecp2*$^{+/-}$ ($n = 58$) mice are represented as *open triangles*. **d** RTT-like *Mecp2*$^{+/-}$ and WT-like *Mecp2*$^{+/-}$ mice indicated by the threshold level show no significant differences from those indicated by the k-means clustering method. **e** When separated using the determined threshold level, *Mecp2*$^{-/Y}$ mice and the RTT-like *Mecp2*$^{+/-}$ mice have no significant difference and WT ($n = 56$) and the WT-like *Mecp2*$^{+/-}$ mice ($n = 58$) have no significant difference. Data is presented as median ± IQ (*$P < 0.05$, ***$P < 0.005$). **f** When separated based on the number of apneas using the k-means clustering method, *Mecp2*$^{-/Y}$ ($n = 13$) mice and the RTT-like *Mecp2*$^{+/-}$ mice ($n = 17$) have no significant difference and WT ($n = 56$) and the WT-like *Mecp2*$^{+/-}$ mice ($n = 58$) have no significant difference. Data is presented as median ± IQ (*$P < 0.05$, ***$P < 0.005$)

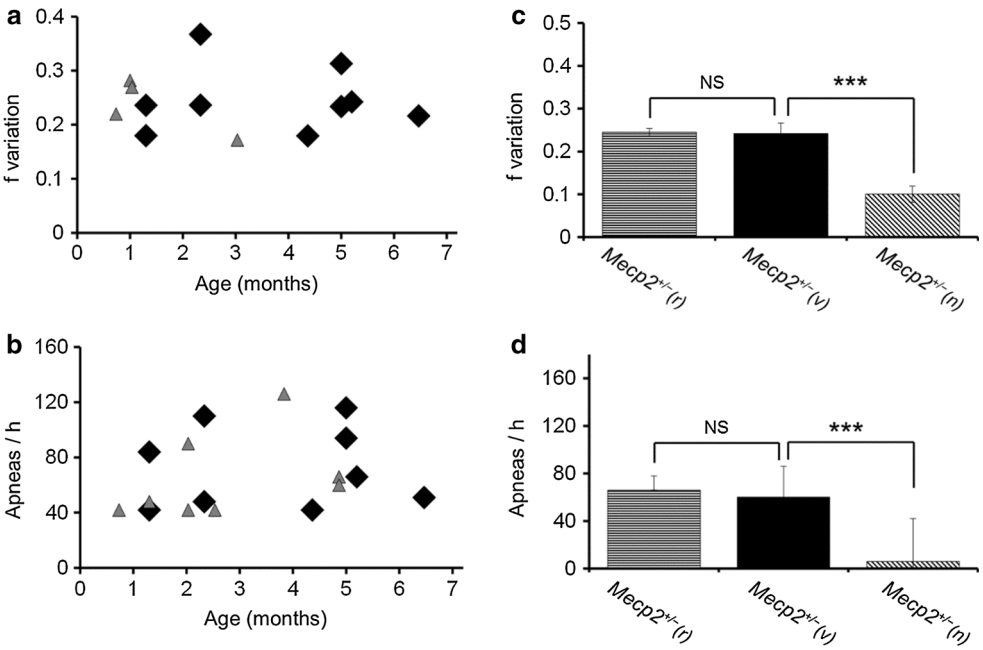

Fig. 4 RTT-like *Mecp2*$^{+/-}$ mice with both significant *f* variation and apneas. **a**, **b** *Mecp2*$^{+/-}$ mice with significant *f* variations and apneas (*Mecp2*$^{+/-}$[v], *large diamond*) indicated by the k-means clustering analysis were compared with *Mecp2*$^{+/-}$ mice showing significant *f* variation only (*Mecp2*$^{+/-}$[r], *gray triangles*). Regarding severity of *f* variations, *Mecp2*$^{+/-}$[v] mice were not significantly different from *Mecp2*$^{+/-}$[r]. **c**, **d** Severity of apnea was not significantly different between *Mecp2*$^{+/-}$[v] and *Mecp2*$^{+/-}$[r], either. Data are presented as mean ± SE (***$P < 0.005$) **d** Data is presented as median ± IQ (***$P < 0.005$)

Age contribution

The severity of breathing disorders seen in *Mecp2*$^{+/-}$ mice may change with the progression of age. Based on the threshold levels identified, age differences between *Mecp2*$^{+/-}$ mice at ages of 3 weeks to 6 months were analyzed between RTT-like and normal *Mecp2*$^{+/-}$ mice with respect to *f* variation and apnea (Fig. 5a, b). Both breathing abnormalities were seen in mice of 3 weeks of age, and the occurrence ratio of breathing abnormalities did not increase with aging (from 3 weeks to 6 months). Approximately 13 % of *Mecp2*$^{+/-}$ females displayed RTT-like breathing *f* variations, and approximately 28 % of *Mecp2*$^{+/-}$ mice showed RTT-like levels of apnea in this age range in these groups of mice, which were very close to our observations in the general mouse population above. The *f* variation and apnea counts were compared in the three age groups between RTT-like and normal *Mecp2*$^{+/-}$ mice. The RTT-like *Mecp2*$^{+/-}$ mice showed significantly higher levels of *f* variation and apnea counts than the normal *Mecp2*$^{+/-}$ mice (Fig. 5c, d). Out of the 13 *Mecp2*$^{+/-}$ mice that were retested, 5 initially displayed apnea counts above the determined threshold of 38 apneas/h, but upon subsequent testing did not display apnea numbers above the threshold. One *Mecp2*$^{+/-}$ mouse did not display apneas above the threshold, but upon subsequent testing showed a number of apneas above the threshold. The other 7 *Mecp2*$^{+/-}$never displayed a number of apneas above the threshold (data not shown).

Response to CO_2

To determine whether exposing RTT-like *Mecp2*$^{+/-}$ mice to an elevated level of CO_2 reduces the severity of their breathing abnormalities, a concentration of 3 % CO_2 was delivered to the animals in a plethysmograph chamber. Apneas and the *f* variation were analyzed in the same way as described above. RTT-like *Mecp2*$^{+/-}$ mice displayed a significant reduction in *f* variation when exposed to 3 % CO_2 (Fig. 6a). Their irregular breathing was resumed when the high CO_2 ventilation stopped. In normal air, the *f* variation was 0.24 in RTT-like *Mecp2*$^{+/-}$ mice. These breathing variations were suppressed with 3 % CO_2, leading to an *f* variation of 0.14 (Fig. 6b), which was not significantly different from the baseline *f* variation (0.12) of WT mice. The RTT-like *Mecp2*$^{+/-}$ mice also displayed a significant reduction in apneas from 56 counts/h to 18 counts/h when exposed to 3 % CO_2 (Fig. 6c, d). Frequent apneas returned when the plethysmograph chamber was ventilated with room air.

Discussion

It is known that RTT-like breathing disorders start to manifest themselves in *Mecp2*$^{-/Y}$ males at 3 weeks of age [10, 11], while a fraction of *Mecp2*$^{+/-}$ mice also begin to display the same symptoms at this age as shown in the present study. Since most *Mecp2*$^{+/-}$ mice do not develop breathing irregularities due to random X-chromosome

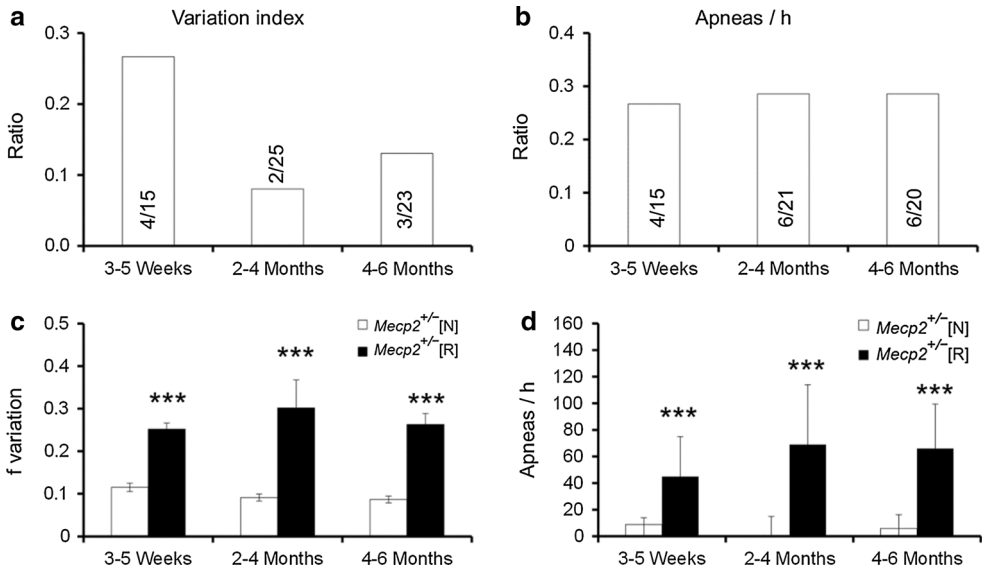

Fig. 5 Severity and occurrence rate of breathing irregularities in *Mecp2*$^{+/-}$ mice. **a** The ratio of RTT-like *Mecp2*$^{+/-}$ mice to WT-like *Mecp2*$^{+/-}$ mice based on *f* variation separation during the different age groups. **b** The ratio of RTT-like *Mecp2*$^{+/-}$ mice to WT-like *Mecp2*$^{+/-}$ mice based on the number of apneas/h separation during the different age groups. **c** The severities of *f* variation of the *Mecp2*$^{+/-}$ during the different age groups. Data is presented as mean ± SE (***$P < 0.005$). **d** The numbers of apneas/h of the *Mecp2*$^{+/-}$ during the different age groups. Data is presented as median ± IQ (***$P < 0.005$)

inactivation, the demonstration of the breathing disorders in the present study should have an impact on the understanding of RTT with female animal models.

Like human patients with RTT, *Mecp2*$^{-/Y}$ male mice show hypoventilation, apneas followed by brief hyperventilation and increased variability in breathing frequency [12]. Several different studies have shown the alleviation of these irregularities by increasing the availability of neurotransmitters or their precursors such as norepinephrine, GABA and serotonin [8, 13, 14]. Our previous studies have indicated that breathing patterns of the *Mecp2*$^{-/Y}$ mice can improve in response to elevated CO_2. We have found that these mice lose their sensitivity to moderate hypercapnia, while their sensitivity to severe hypercapnia appears normal. The *Mecp2*$^{-/Y}$ mice do not respond to moderate hypercapnia, and display hypoventilation and breathing irregularities as under normocapnic conditions. The breathing patterns may not allow an adequate clearance of CO_2, leading to a buildup of systemic CO_2 or the development of severe systemic hypercapnia. Because the mice have a decent sensitivity to severe hypercapnia, they then hyperventilate so that the excess CO_2 is removed from the body. These events seem to occur periodically in the *Mecp2*$^{-/Y}$ mice as seen in patients with RTT [7].

In one study, the percentage of *Mecp2*$^{+/-}$ mice that showed apnea levels significantly greater than WT mice were studied from 8 to 12 weeks of age. The percentage of these *Mecp2*$^{+/-}$ increased from 20 % at 8 weeks to 50 % at 12 weeks of age. [15]. The occurrence of apneas unaffected by age were detected in female *Mecp2*$^{+/-}$ mice 4–14 months old and periodic breathing defects have been demonstrated in 9-month-old female *Mecp2*$^{+/-}$ mice [8]. Concerning the lifespan of mice, it is unclear whether the defects in the old female mice might involve biological processes that are rather different from the early development of RTT patients. A recent study has demonstrated a great number of apneas in *Mecp2*$^{+/-}$ female mice when compared to WT [9], in which breathing irregularities were found in *Mecp2*$^{+/-}$ mice approximately 2 months old. At this age, mice are fully mature, and *Mecp2*$^{-/Y}$ male mice have a low survivability rate. Apparently, the age does not correlate well with the early onset of RTT in humans. In another study, breathing abnormalities were observed in *Mecp2*$^{+/-}$ mice 6–17 months, which were reduced by the selective 5HT-1a agonist, F15599 [14].

Mecp2$^{+/-}$ mice display a range of symptoms, while the severity of RTT-like abnormalities is typically more difficult to characterize compared to *Mecp2*$^{-/Y}$ males that have a clean knockout of the *Mecp2* gene with more obvious symptoms. To identify *Mecp2*$^{+/-}$ females with breathing abnormalities we have studied the distribution patterns using two independent methods. Our data suggest that two populations of *Mecp2*$^{+/-}$ mice exist, one with a phenotype resembling WT and another displaying a significant RTT-like breathing phenotype. These two populations can be separated based on objective and quantifiable measures of breathing parameters. With these measures, a substantial number of *Mecp2*$^{+/-}$ mice display breathing abnormalities as severe as those seen in *Mecp2*$^{-/Y}$ males. These breathing abnormalities, though, appear to be less severe in the

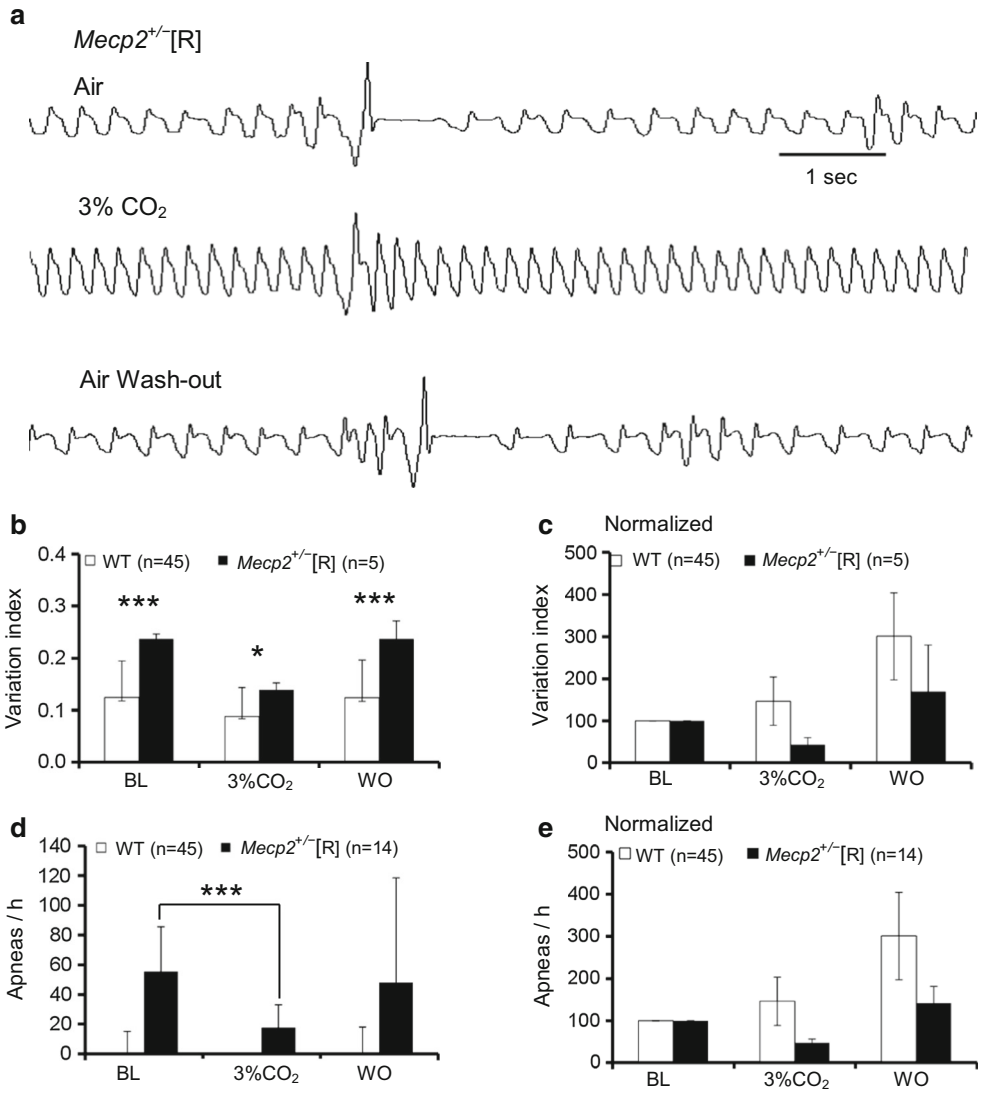

Fig. 6 *Mecp2*$^{+/-}$ mice show reduced severities of breathing irregularities in response to 3 % CO_2. **a** Representative traces of a *Mecp2*$^{+/-}$ mouse during normal air ventilation, 3 % CO_2, and normal air washout. **b**, **c** Statistical analysis was performed in *Mecp2*$^{+/-}$ mice and WT mice. Significant suppression of *f* variation (**b**, **c**) and apnea (**d**, **e**) was found with 3 % CO_2. Normalized *f* variation (**c**) and apnea (**e**). Data is presented as mean ± SE ($*P < 0.05$) and median ± IQ ($*P < 0.05$, $***P < 0.005$), respectively. *BL* baseline, *WO* washout, *WT* wild-type

general population of *Mecp2*$^{+/-}$ mice by comparison to *Mecp2*-null males due to random X-inactivation and varying MeCP2 protein levels.

Our results have shown that severe breathing defects take place as early as 3 weeks postnatal age in female *Mecp2*$^{+/-}$ mice. These abnormalities were not studied at an earlier age because of technical limitations in handling premature pups of both sexes. Despite this, our results suggest that the occurrence age for the breathing abnormalities seems comparable to that of *Mecp2*$^{-/Y}$ males. About 13 % of *Mecp2*$^{+/-}$ mice develop irregular breathing, less than 25 % of mice show frequent apneas, and ~10 % of females have both defects. Since none of these figures are close to the prediction of random X-inactivation (~50 %), it is possible that the *Mecp2* gene is not completely inactivated in 50 % of female heterozygous mice.

Our previous studies have shown that *Mecp2*$^{-/Y}$ mice have impaired central chemosensitivity. The mice do not respond to moderate CO_2 levels, but their sensitivity to high PCO_2 is normal. This defect leads to periodic hyper- and hypoventilation as seen in RTT patients [7]. Hypoventilation, frequent apneas and irregular/ineffective breathing tend to produce systemic hypoxia, while hypoxia is known to be a major risk factor for the maldevelopment of the central nervous system that occurs in RTT patients. Hypoxia has also been shown to cause vasoconstriction by the suppression of Kv2.1 channels [16]. Similar to *Mecp2*$^{-/Y}$ mice, the *Mecp2*$^{+/-}$ female mice with severe RTT-like symptoms respond well to the hypercapnic challenge. Their breathing becomes regular with a significant reduction in apnea events when their breathing air contains 3 % CO_2. Therefore, the application of high CO_2 may alleviate the deleterious consequences of breathing

abnormalities in RTT patients. Interestingly, female *Mecp2*$^{+/-}$ mice between 4 months and 19 months are found to have a higher hypercapnia-response threshold, and the CO_2 chemosensitivity is improved in *Mecp2*-null male mice by increasing the availability of serotonin [17]. Together, these findings suggest the feasibility of correcting the breathing disorders with a CO_2 intervention, which seems useful in the therapeutic design for RTT, as the consequence of the breathing disorders may not be limited to systemic hypercapnia.

Conclusions

In conclusion, *Mecp2*$^{+/-}$ female mice can be separated into two groups resembling WT and *Mecp2*$^{-/Y}$ males, respectively. In mice with RTT-like disorders, breathing irregularities begin to manifest themselves during the early lives of the *Mecp2*$^{+/-}$ mice before reaching adulthood. In the other group, the breathing phenotype remains similar to WT mice. We have shown that breathing abnormalities in *Mecp2*$^{+/-}$ mice occur earlier than previous studies have shown and the occurrence rate is less than 20 %.The variable severities and occurrence rate of breathing irregularities in the general population of *Mecp2*$^{+/-}$ mice may be due to random X-inactivation, resulting in variable amounts of MeCP2 protein. These breathing irregularities can be largely reduced under hypercapnic ventilation. Therefore, these findings indicate that the severity of breathing abnormalities can be reliably distinguished from non-disease breathing phenotypes and CO_2 intervention can reduce the severity of these breathing defects.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted. This article does not contain any studies with human participants performed by any of the authors.

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