Habituation without NMDA receptor-dependent desensitization of Hering–Breuer apnea reflex in a *Mecp2*^{+/−} mutant mouse model of Rett syndrome

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Non-associative learning is a basic neuroadaptive behavior exhibited in almost all animal species and sensory modalities but its functions and mechanisms in the mammalian brain are poorly understood. Previous studies have identified two distinct forms of non-associative learning in the classic Hering–Breuer inflation reflex (HBIR) induced apnea in rats: NMDA receptor (NMDAR)-independent habituation in a primary vagal pathway and NMDAR-dependent desensitization in a secondary pontine pathway. Here, we show that abnormal non-associative learning of the HBIR may underlie the endophenotypic tachypnea in an animal model of Rett syndrome (RTT), an autism-spectrum disorder caused by mutations in the X-linked gene encoding methyl-CpG-binding protein 2 (MECP2). *Mecp2*^{+/−} symptomatic mice on a mixed-strain background demonstrated significantly increased resting respiratory frequency with shortened expiration and normal inspiratory duration compared with asymptomatic mutants and wild-type controls, a phenotype that is characteristic of girls with RTT. Low-intensity electrical stimulation of the vagus nerve elicited fictive HBIR with time-dependent habituation in both *Mecp2*^{+/−} and wild-type mice. However, time-dependent desensitization of the HBIR was evidenced only in wild-type controls and asymptomatic mutant mice but was absent or suppressed in *Mecp2*^{+/−} symptomatic mice or in wild-type mice after blockade of NMDAR with dizocilpine. Remarkably, ~50% of the *Mecp2*^{+/−} mice developed these X-linked phenotypes despite somatic mosaicism. Such RTT-like respiratory endophenotypes in mixed-strain *Mecp2*^{+/−} mice differed from those previously reported in *Mecp2*[/y] mice on pure C57BL/6J background. These findings provide the first evidence indicating that impaired NMDAR-dependent desensitization of the HBIR may contribute to the endophenotypic tachypnea in RTT.

Keywords: Rett syndrome, Mecp2, NMDA receptor, non-associative learning, habituation, sensitization, desensitization, X-chromosome inactivation

INTRODUCTION

Rett syndrome (RTT) is a neurological disorder most frequently caused by sporadic mutations in the X-linked gene encoding methyl-CpG-binding protein 2 (MeCP2; Amir et al., 1999), a transcriptional activator/repressor that regulates the expression of many genes (Chahrour et al., 2008). Homozygous mutation in females is rare (Karall et al., 2007), and hemizygous males (homozygous for a single X-chromosome) usually die shortly after birth except in variant cases (Villard, 2007). Heterozygous females are viable but show rapid developmental regression between ages 1–3 years (Hagberg et al., 1983).

Among the cardinal symptoms of RTT is a highly irregular respiratory rhythm particularly during daytime (Kerr et al., 2001; Hagberg et al., 2002; Julu et al., 2008). Recent studies in these patients reveal a predominantly hyperventilatory pattern with decreased expiratory duration (T_E) and increased respiratory frequency; during wakefulness this is also punctuated by frequent episodes of breath-holding/obstructive apnea or Valsalva breathing against closed airways (Julu et al., 2001; Weese-Mayer et al., 2006, 2008). The breath-holding/obstructive apnea phenotype of

RTT is often conflated in the clinical literature with central apnea, which has similar physiological effects but fundamentally distinct neural mechanisms (Lugaresi et al., 1985; Cirignotta et al., 1986; Southall et al., 1988; Kerr et al., 1990, 2001; Marcus et al., 1994; Schluter et al., 1995; Rohdin et al., 2007; Stettner et al., 2008b). The irregular breathing pattern in RTT is reproduced in several mutant mouse models to varying degrees but the corresponding respiratory phenotype varies significantly among different mouse strains (Bissonnette and Knopp, 2006; Ogier and Katz, 2008; Katz et al., 2009). In *Mecp2*[tm1.1Jae] null (hemizygous) mice on a mixed-strain background (Chen et al., 2001) the principal phenotype is tachypnea along with hyperventilation similar to human RTT patients (Ogier et al., 2007), whereas in *Mecp2*[tm1.1Bird] null or heterozygous mice on a pure C57BL/6J background (Guy et al., 2001) the principal phenotype is repetitive spontaneous central apnea (Viemari et al., 2005; Stettner et al., 2007; Abdala et al., 2010).
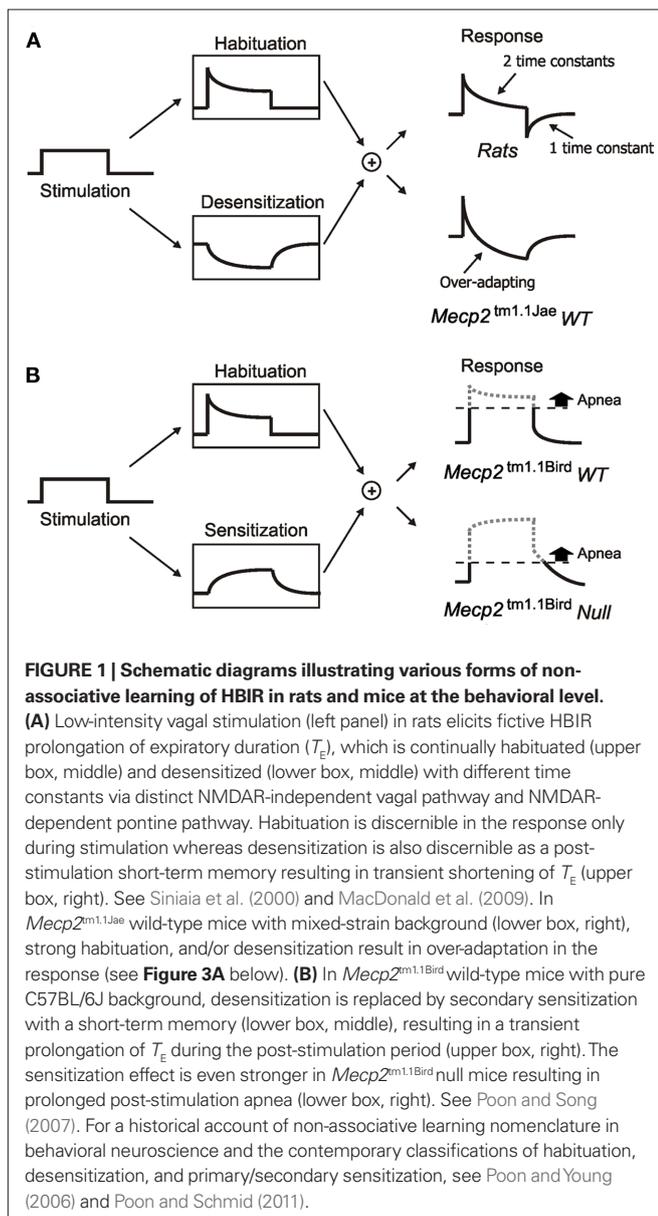
Remarkably, *Mecp2*[tm1.1Bird] null mice are reportedly highly prone to repetitive and prolonged central apneas particularly when vagal and dorsolateral pontine afferent pathways are activated to induce fictive Hering–Breuer inflation reflex (HBIR), a powerful apnea

reflex in mammals (Stettner et al., 2007; Abdala et al., 2010). In rats, it has been shown that the HBIR apnea induced by abrupt lung inflation or low-intensity vagal stimulation is typically counteracted centrally by progressive habituation and desensitization, two distinct decrementing forms of non-associative learning (Poon et al., 2000; Siniaia et al., 2000; MacDonald et al., 2007, 2009). Behaviorally, desensitization is distinguished from habituation by the manifestation of a memory trace (engram) of the adaptation effect in a secondary pathway post-stimulation independent from the primary stimulus (**Figure 1A**; Poon and Young, 2006; Poon and Schmid, 2011). Functionally, desensitization of the HBIR is abolished by lesion of the dorsolateral pontine pneumotaxic center or blockade of NMDA receptor (NMDAR) while the habituation component remains unaffected by these interventions, suggesting that habituation is ascribable to an NMDAR-independent primary afferent pathway that is directly activated by the vagal input, and

desensitization to an indirect NMDAR-dependent pontine pathway that is driven by a latent secondary input (Poon et al., 2000; Siniaia et al., 2000; MacDonald et al., 2007). By contrast, the vagal-induced HBIR in the *Mecp2*[tm1.1Bird] null mice appeared to exhibit secondary sensitization (rather than desensitization), an incrementing (rather than decrementing) form of non-associative learning characterized by the manifestation of a memory trace of the sensitization effect in a secondary pathway post-stimulation (**Figure 1B**; Poon and Young, 2006; Poon and Schmid, 2011). Such secondary sensitization effect tended to exacerbate instead of mitigate the HBIR-induced apnea in the *Mecp2*[tm1.1Bird] null mice (Poon and Song, 2007).

Interestingly, wild-type mice with pure C57BL/6J background also demonstrate similar repetitive spontaneous central apneas (Han et al., 2002; Stettner et al., 2007, 2008a,c; Yamauchi et al., 2008) and secondary sensitization of the HBIR albeit to a lesser degree (Poon and Song, 2007; Stettner et al., 2007; **Figure 1B**). C57BL/6 inbred mouse strains are known to be vulnerable to slight variations in genetic background, such that behavioral phenotypes may vary significantly even among C57BL/6 substrains (Matsuo et al., 2010). A critical question arising is whether the pronounced spontaneous and HBIR-induced central apneas and sensitization of the HBIR in *Mecp2*[tm1.1Bird] null mice are intrinsic to the *Mecp2* mutation or specific to the mouse strain used. Resolution of this question is crucial in pinpointing the respiratory endophenotypes of *Mecp2* mutation in order to elucidate the underlying neural mechanisms or develop proper endophenotype-specific therapeutic strategies for RTT (Katz et al., 2009; Cobb et al., 2010). For example, treatments with certain neurotrophins have been shown to reverse the hyperventilation/tachypnea and other RTT-like symptoms in *Mecp2*[tm1.1Jae] null mice (Ogier et al., 2007; Tropea et al., 2009), whereas boosting the levels of certain monoamines and/or GABA have been suggested to effectively suppress spontaneous central apnea in *Mecp2*[tm1.1Bird] null mice and prolong their survival (Roux et al., 2007; Zanella et al., 2008; Abdala et al., 2010).

Most previous studies were conducted on *Mecp2*[−/y] male mice for their phenotypic homogeneity and early manifestation of respiratory abnormalities (reviewed in Ogier and Katz, 2008; Katz et al., 2009). However, whereas *Mecp2*[−/y] mice are viable and may live to adulthood, most human males with *MECP2* mutations die perinatally (Hagberg et al., 1983) indicating differential vulnerability of humans and mice to loss of *Mecp2*. Indeed, mice with a less severe *Mecp2* mutation, such as a mutation that results in a truncated protein instead of null mutation, could live even longer (Shahbazian et al., 2002). Recent case studies have shown that male patient survivors with RTT-like phenotypes do not carry pathogenic mutations in the *MECP2* gene (Santos et al., 2009). Conversely, there is evidence that *MECP2* null mutations in males may be responsible for a wide spectrum of neurological disorders that are distinctly different from RTT (Villard, 2007). In particular, it has been suggested that MeCP2 null males displaying a congenital encephalopathic phenotype, and not females with RTT, represent the human equivalent of the *Mecp2*[tm1.1Bird] hemizygous male mouse model (Schule et al., 2008). Here, we show that *Mecp2*[tm1.1Jae] mutant mice surprisingly showed Mendelian-like distribution for respiratory symptoms despite reputed somatic mosaicism of the X-linked *Mecp2* gene, with heterozygous symptomatic mice demonstrating very different RTT-like breathing patterns at rest and during

FIGURE 1 | Schematic diagrams illustrating various forms of non-associative learning of HBIR in rats and mice at the behavioral level.
(A) Low-intensity vagal stimulation (left panel) in rats elicits fictive HBIR prolongation of expiratory duration (T_E), which is continually habituated (upper box, middle) and desensitized (lower box, middle) with different time constants via distinct NMDAR-independent vagal pathway and NMDAR-dependent pontine pathway. Habituation is discernible in the response only during stimulation whereas desensitization is also discernible as a post-stimulation short-term memory resulting in transient shortening of T_E (upper box, right). See Siniaia et al. (2000) and MacDonald et al. (2009). In *Mecp2*[tm1.1Jae] wild-type mice with mixed-strain background (lower box, right), strong habituation, and/or desensitization result in over-adaptation in the response (see **Figure 3A** below). **(B)** In *Mecp2*[tm1.1Bird] wild-type mice with pure C57BL/6J background, desensitization is replaced by secondary sensitization with a short-term memory (lower box, middle), resulting in a transient prolongation of T_E during the post-stimulation period (upper box, right). The sensitization effect is even stronger in *Mecp2*[tm1.1Bird] null mice resulting in prolonged post-stimulation apnea (lower box, right). See Poon and Song (2007). For a historical account of non-associative learning nomenclature in behavioral neuroscience and the contemporary classifications of habituation, desensitization, and primary/secondary sensitization, see Poon and Young (2006) and Poon and Schmid (2011).

fictive HBIR than previously reported for hemizygous mice of the *Mecp2*$^{tm1.1Bird}$ strain. Results reveal distinct abnormalities in the neural pathways mediating NMDAR-dependent desensitization of the HBIR in the *Mecp2*$^{tm1.1Jae}$ female mouse model of RTT that may be responsible for their tachypneic pattern.

MATERIALS AND METHODS
ANIMAL PREPARATION
Heterozygous mice of the *Mecp2*$^{tm1.1Jae}$ strain (Chen et al., 2001) and their wild-type female littermates were maintained on a mixed background (129Sv, C57BL/6, BALB/c). Genotyping was performed as previously described (Chen et al., 2001). Eight adult heterozygous mice (age = 102 ± 24 days; mean ± SD) and five wild-type littermates (age = 103 ± 31 days) were studied. All of the mutant mice appeared healthy at the time of study and none of them exhibited behavioral and motor abnormalities which typically begin to develop after 5 weeks of age in these animals (Chen et al., 2001). All experimental methods and procedures were as approved by the Animal Care and Use Committee at Massachusetts Institute of Technology and conformed to National Institutes of Health guidelines. Briefly, The mouse was anesthetized with urethane (1.5 g/kg, i.p.), paralyzed with pancuronium bromide (0.1 mg in 0.1 ml, i.p.) and artificial ventilated (Minivent-856, Harvard Apparatus) with humidified and oxygen-enriched medical air (O_2 at 40%) through a tracheal cannula. Ventilator tidal volume (80–120 µl) and frequency (100–120 cycles/min) were carefully adjusted to obtain stable phrenic discharge. The animal was kept warm with a heating pad (set at 37.5°C) while lying in supine position. EKG was monitored with subcutaneous needle electrodes. Ringer's solution (0.5 ml) was subcutaneously injected every hour to keep the animal from dehydration. The depth of anesthetization was regularly checked. Whenever a noxious stimulus (clamping the hind paw) caused changes in respiration and heart rate or elicited a withdrawal reflex, a supplementary dose of urethane (1/10 original dosage) was given (i.p.) to maintain adequate anesthesia.

ELECTROPHYSIOLOGY
A phrenic nerve and both vagi at the cervical level were isolated from surrounding tissues and severed. The central cut-ends of the phrenic nerve and a vagus nerve were mounted onto bipolar platinum electrodes for recording or electrical stimulation. Phrenic discharges were amplified (CyberAmp 380, Axon Instruments, Union City) and sampled into a Dell PC with LabView (National Instruments, Austin, TX, USA) at a sampling rate of 10 kHz. The phrenic discharges were also integrated with a Paynter filter (time constant 15 ms) and sampled into the computer in a similar fashion. The vagus nerve was stimulated with square-wave pulses (pulse duration 0.1 ms) generated by a voltage pulse generator (A.M.P.I., Master 8) through a stimulus-isolation unit (A.M.P.I., ISO-Flex). The stimulation threshold for each animal was defined as the lowest stimulus current that produced a discernible reflex inhibition of phrenic activity over a 5-s interval. Stimulus pulses (80 Hz) with lowest currents possible (typically between 1.5–2× threshold, or roughly 10–70 µA) were applied repetitively to the vagus nerve for 1 min to evoke fictive HBIR and its resultant habituation and desensitization, as described previously (Siniaia et al., 2000). All exposed nerves were protected from dehydration by immersion in warm paraffin mineral oil pools.

PHARMACOLOGY
Dizocilpine (MK-801, Sigma-Aldrich Co., St. Louis, MO, USA), which is a non-competitive NMDAR antagonist that can pass through the blood–brain barrier, was administered i.p. (1.5 mg/kg).

DATA ANALYSES
Inspiratory duration (T_I), T_E and respiratory frequency were measured for each respiratory cycle from the phrenic discharge. Inspiratory amplitude was measured as the peak of the integrated phrenic signal. Baseline values of these respiratory pattern variables were averaged over 1 min before vagal stimulation. The effects of HBIR were measured by the changes in T_E and respiratory frequency in the first respiratory cycle upon abrupt low-intensity vagal stimulation compared with corresponding baseline values. Combined effects of habituation and desensitization of the HBIR were measured by the decreases in T_E and respiratory frequency in the last 10 s of the 1-min vagal stimulation compared with those in the first respiratory cycle. The effect of desensitization of the HBIR was measured by the changes in T_E and respiratory frequency in the first 5 s upon termination of vagal stimulation compared with corresponding baseline values.

Experiments and data analyses were performed blind to the animal genetic background except during final statistical analysis. Two-tailed Student's *t*-test (paired or unpaired where appropriate) was used to determine statistical significance ($p < 0.05$) of differences in respiratory pattern between animal genetic backgrounds and experimental conditions.
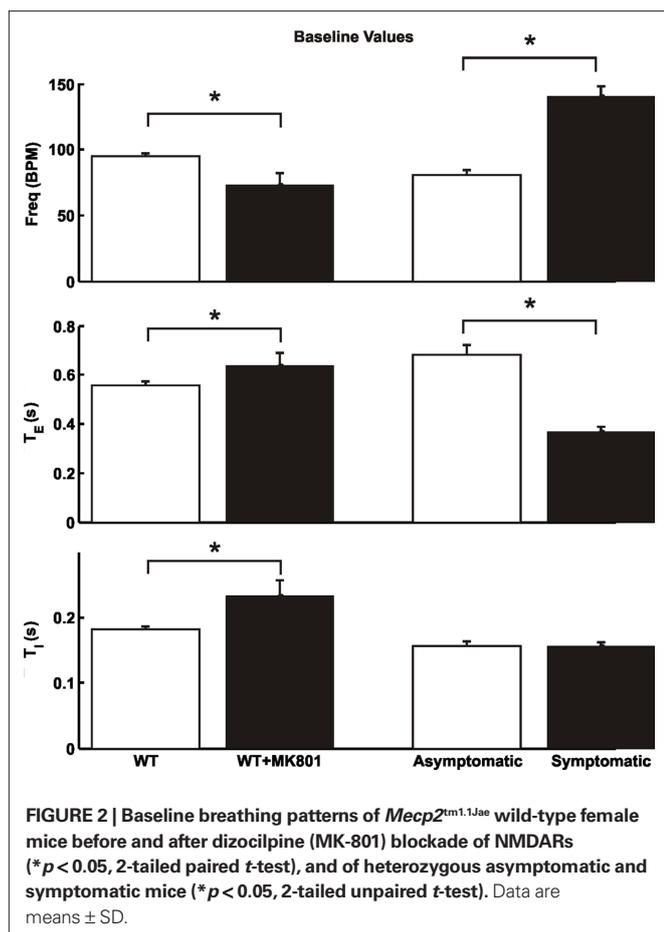
RESULTS
EXPIRATORY-SHORTENING PHENOTYPE IN *Mecp2*$^{tm1.1Jae}$ HETEROZYGOUS MICE
In contrast to the profound spontaneous apnea and periodic breathing in *Mecp2*$^{tm1.1Bird}$ mutant or wild-type mice (Viemari et al., 2005; Stettner et al., 2007; Abdala et al., 2010), we found no signs of such abnormalities in any of the *Mecp2*$^{tm1.1Jae}$ wild-type or heterozygous female mice. Half (asymptomatic females, $n = 4$; age = 102 ± 7 days; mean ± SD) of the *Mecp2*$^{tm1.1Jae}$ heterozygous mice were found to exhibit breathing patterns similar to the wild-type animals whereas the other half (symptomatic females, $n = 4$; age = 103 ± 35 days) showed significantly increased respiratory frequency (**Figure 2**), in agreement with previous results in unanesthetized *Mecp2*$^{tm1.1Jae}$ null mice (Ogier et al., 2007). Importantly, the increased frequency was attributable mainly to a shortening of T_E with little or no change in T_I (**Figure 2**), in exact opposite to the spontaneous apnea or prolongation of T_E reported in *Mecp2*$^{tm1.1Bird}$ heterozygous or null mice (Viemari et al., 2005; Stettner et al., 2007; Abdala et al., 2010).

PRONOUNCED HABITUATION AND DESENSITIZATION OF FICTIVE HBIR IN WILD-TYPE MICE
To test whether the reported sensitization of HBIR prolongation of T_E in the *Mecp2*$^{tm1.1Bird}$ wild-type mice (Poon and Song, 2007; Stettner et al., 2007) was specific to the C57BL/6J strain, we examined the use-dependent learning and memory of the HBIR in the mixed-strain *Mecp2*$^{tm1.1Jae}$ wild-type mice using an established protocol that has been shown to reproduce the habituation and desensitization of the HBIR induced by sustained lung inflation

FIGURE 2 | Baseline breathing patterns of *Mecp2*^{tm1.1Jae} wild-type female mice before and after dizocilpine (MK-801) blockade of NMDARs (*$p < 0.05$, 2-tailed paired *t*-test), and of heterozygous asymptomatic and symptomatic mice (*$p < 0.05$, 2-tailed unpaired *t*-test). Data are means ± SD.

in rats (MacDonald et al., 2009). Low-intensity vagal stimulation (10–70 µA) in *Mecp2*^{tm1.1Jae} wild-type mice elicited the classic HBIR response characterized by immediate cessation of phrenic discharge and prolongation of T_E (**Figure 3A**). Thereafter, rhythmic phrenic discharge gradually reappeared and T_E gradually shortened in adaptation to the initial reflex apnea response. As a result, respiratory frequency exhibited an initial abrupt decrease followed by gradual adaptive increase. As the stimulation continued, the shortening of T_E and increase in respiratory frequency eventually over-adapted and went beyond their baseline levels. By the end of the 1-min vagal stimulation, T_E was 13–39% below (-17.0 ± 3.5%, mean ± SD) its pre-stimulation value and respiratory frequency was 18–57% above (25.3 ± 4.2%). Upon cessation of vagal stimulation, the over-adaptations in T_E and respiratory frequency persisted with a short-term memory indicating desensitization, and gradually returned to baselines in ~20 s. The time-dependent adaptations of T_E and respiratory frequency during and after low-intensity vagal stimulation (**Figure 3A**) are characteristic of, and even stronger than, the habituation and desensitization of the HBIR seen in rats (**Figure 1A**).

DIZOCILPINE SUPPRESSES DESENSITIZATION OF FICTIVE HBIR IN WILD-TYPE MICE

Next, we tested the effects of blockade of NMDARs by systemic administration of dizocilpine (MK-801). Fifteen minutes after systemic administration (1.5 mg/kg, i.p.) of this chemical in wild-type
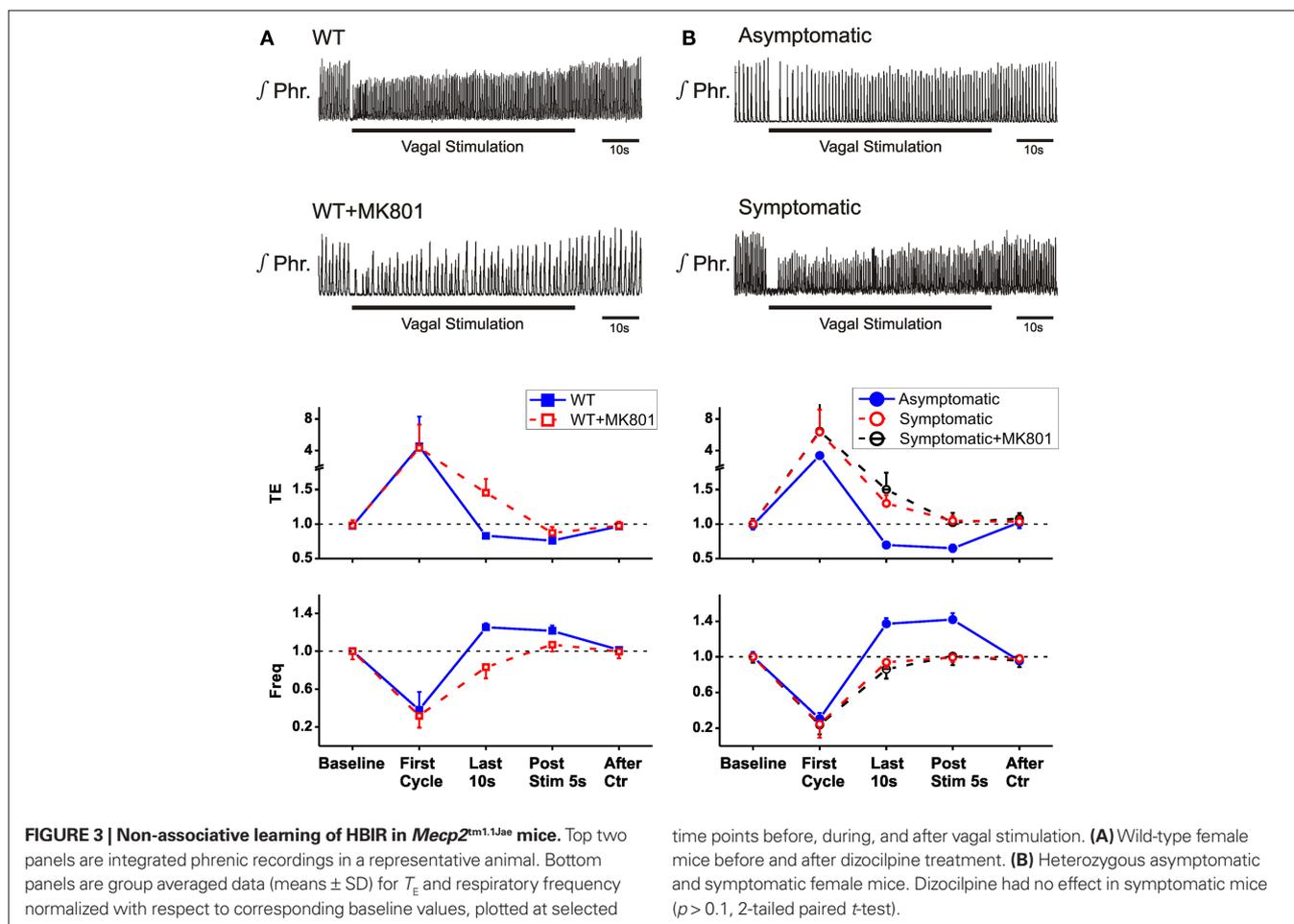
mice of the *Mecp2*^{tm1.1Jae} strain, respiratory frequency was significantly decreased and T_I was significantly increased (**Figure 2**), as previously reported in vagotomized rats and mice (Cassus-Soulanis et al., 1995). Under dizocilpine, low-intensity vagal stimulation still elicited fictive HBIR response with immediate cessation of phrenic discharge and prolongation of T_E (**Figure 3A**). However, the ensuing time-dependent adaptations of T_E and respiratory frequency responses during vagal stimulation were much weaker than before dizocilpine treatment as measured in the last 10 s of vagal stimulation ($p < 0.05$, 2-tailed paired *t*-test). Importantly, the responses in T_E and respiratory frequency within the first 5-s post-stimulation were not significantly different from the corresponding baseline values ($p > 0.1$) indicating suppressed desensitization of the HBIR, although possible attenuation of the habituation component cannot be ruled out (**Figure 3A**).

ABSENCE OF SENSITIZATION/DESENSITIZATION OF FICTIVE HBIR IN *Mecp2*^{tm1.1Jae} HETEROZYGOUS MICE

To investigate whether the *Mecp2* mutation demonstrated similar effects as dizocilpine, we applied the above low-intensity vagal stimulation protocol to *Mecp2*^{tm1.1Jae} heterozygous mice. In heterozygous asymptomatic mutants the HBIR responses in T_E and respiratory frequency (**Figure 3B**) were similar to those of their wild-type littermates (**Figure 3A**). In heterozygous symptomatic mutants characterized by decreased T_E and increased respiratory frequency (**Figure 2**), low-intensity vagal stimulation also elicited immediate HBIR prolongation of T_E and decrease of respiratory frequency. However, the ensuing time-dependent adaptations of T_E and respiratory frequency were much weaker ($p < 0.01$, 2-tailed unpaired *t*-test) and never exceeded the corresponding baselines. Neither of these variables exhibited post-stimulation short-term memory such that responses within the first 5-s post-stimulation were indistinguishable from corresponding baseline values ($p > 0.1$), indicating that the adaptations are now ascribable solely to habituation without desensitization or sensitization (**Figure 3B**). These effects were similar before or after dizocilpine application ($p > 0.1$, **Figure 3B**) and resembled those resulting from dizocilpine in wild-type animals (**Figure 3A**).

DISCUSSION

The foregoing results demonstrate that respiratory abnormalities are different in adult *Mecp2*^{tm1.1Jae} symptomatic female mice (~P100 of age) than previously reported in hemizygous male or heterozygous female mice of the *Mecp2*^{tm1.1Bird} strain (P40 to 14 months of age). A salient respiratory symptom of the *Mecp2*^{tm1.1Jae} heterozygous mutant mice was a shortening of T_E and resultant increase in respiratory frequency, which is diametrically opposite to the repetitive spontaneous central apnea or prolongation of T_E in the *Mecp2*^{tm1.1Bird} null mice (Viemari et al., 2005; Stettner et al., 2007; Abdala et al., 2010). The tachypnea phenotype in the *Mecp2*^{tm1.1Jae} heterozygous mutants under anesthesia and bivagotomy is consistent with a previously reported increase in mean respiratory frequency in *Mecp2*^{tm1.1Jae} null mice (P35 of age) during wakefulness (Ogier et al., 2007) and more importantly, it is in agreement with the documented tachypneic breathing pattern with a shortened T_E in girls with RTT during sleep (Weese-Mayer et al., 2008) as well as during wakefulness amidst intermittent breath-holding/obstructive apnea

FIGURE 3 | Non-associative learning of HBIR in *Mecp2*tm1.1Jae mice. Top two panels are integrated phrenic recordings in a representative animal. Bottom panels are group averaged data (means ± SD) for T_E and respiratory frequency normalized with respect to corresponding baseline values, plotted at selected time points before, during, and after vagal stimulation. **(A)** Wild-type female mice before and after dizocilpine treatment. **(B)** Heterozygous asymptomatic and symptomatic female mice. Dizocilpine had no effect in symptomatic mice ($p > 0.1$, 2-tailed paired *t*-test).

episodes (Weese-Mayer et al., 2006). As with RTT patients during sleep, breath-holding/obstructive apnea and Valsalva maneuvers were not observed in these anesthetized animals.

Another important, albeit more subtle, respiratory endophenotype of the *Mecp2*tm1.1Jae heterozygous symptomatic mice was a degenerate non-associative learning characterized by a significant habituation of the HBIR but absent the NMDAR-dependent desensitization that is found in wild-type or asymptomatic female mice. None of these wild-type and heterozygous mutant mice evidenced the sensitization of HBIR seen in the *Mecp2*tm1.1Bird null and wild-type mice (**Figure 1B**). This suggests that secondary sensitization of the HBIR is specific to the *Mecp2*tm1.1Bird strain.

To put these findings in perspective, a current working model (Siniaia et al., 2000; Poon, 2004; Song and Poon, 2004; MacDonald et al., 2007, 2009) postulates that the HBIR is mediated by two parallel vagally modulated afferent pathways acting in concert to modulate T_E: a primary (NMDAR-independent) habituation-prone direct pathway via the nucleus tractus solitarius (NTS), and a secondary (NMDAR-dependent) desensitization-prone indirect pathway via both the NTS and the Kölliker-Fuse/parabrachial complex ("pneumotaxic center;" Lumsden, 1923; Song et al., 2006) in dorsolateral pons (**Figure 4A**). In the absence of an extrinsic vagal input (such as post-bivagotomy), the secondary pathway provides an intrinsic (pontine) expiratory-promoting signal that facilitates

the inspiratory off-switch, perhaps via pontine post-inspiratory activity (Dutschmann and Herbert, 2006) which has been postulated to suppress inspiratory rhythm generation in the preBötzinger complex (Wittmeier et al., 2008) and has been shown to correlate with prolonged apneas in the *Mecp2*tm1.1Bird null mice (Stettner et al., 2007). A strong lung volume-related vagal input elicits the HBIR by providing an extrinsic (vagal) expiratory-promoting signal and simultaneously triggering the intrinsic expiratory-promoting signal. The ensuing HBIR is continuously attenuated in a time-dependent manner by use-dependent habituation and desensitization; the latter is manifested as a post-stimulation short-term memory (**Figure 1A**). Under this scheme, inactivation of the pneumotaxic center by pontine lesion or NMDAR blockade disrupts the intrinsic expiratory-promoting signal and its desensitization but not the extrinsic expiratory-promoting signal and its habituation, as observed experimentally in rats (Poon et al., 2000; Siniaia et al., 2000; MacDonald et al., 2007).
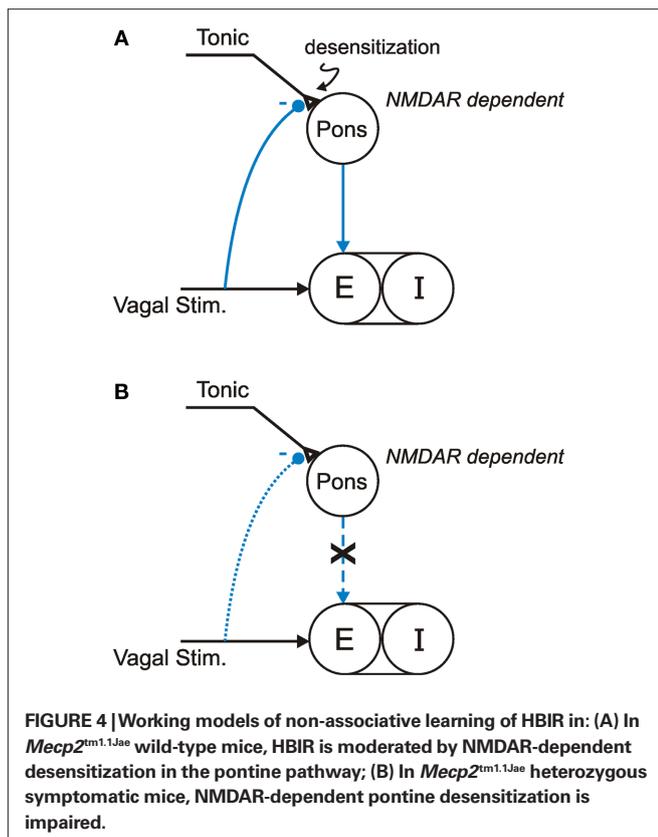
In the present study, use-dependent non-associative learning modulation of the HBIR was seen to be similar, and even stronger, in the wild-type mice compared to rats. Further, as has been observed in rats, administration of dizocilpine diminished the desensitization without affecting the habituation of HBIR in wild-type mice, indicating similar vagal-pontine neural organization of the HBIR and its non-associative learning in these species

(**Figure 4A**). Accordingly, the absence of desensitization in the HBIR response presently found in the *Mecp2*tm1.1Jae heterozygous symptomatic mice suggests that the intrinsic NMDAR-dependent expiratory-promoting mechanism in the pneumotaxic center was impaired in these animals (**Figure 4B**). This is further supported by the lack of effect of dizocilpine in the symptomatic mice (**Figure 3B**). Such a correlation between *Mecp2* mutation and disruption of NMDAR-dependent pontine desensitization provides a mechanistic explanation of the significant decrease in T_E with no change in T_I presently observed in the symptomatic mutant mice, as with RTT patients.

Given the general variability of phenotypes in X-linked genetic mutations such as in RTT due to somatic mosaicism, it is remarkable that *Mecp2*+/− mutants expressed the respiratory phenotypes with ~50% probability. To understand this, it should be noted that X-chromosome inactivation takes place early in embryonic development before gastrulation, not in individual somatic cells. Type-specific neurons with lineage to the same post-gastrulation progenitor cell inherit the X-inactivation state of the progenitor cell that does not change throughout life (Dvash and Fan, 2009). Therefore, although the X-inactivation state may vary between different cell types giving rise to somatic mosaicism, the X-inactivation state within the same cell type could remain uniform. An endophenotype has a 50% chance to express if it is dependent on the genotype of a single cell type. This is in contrast to phenotypes (non-endophenotypes) that are dependent on multiple cell types or tissues (or even multiple organs), where the phenotype-genotype relationship is less distinct and more susceptible to somatic mosai-

cism. The distinct 50–50 expression ratio of NMDAR-dependent desensitization of the HBIR in asymptomatic-symptomatic mutants therefore implies that disruption of NMDAR signaling in the pons is the single respiratory-related endophenotype in *Mecp2*+/− symptomatic mutants. This also explains the consistent expression of the hyperventilation and shortened T_E phenotypes in RTT girls as opposed to the variability of other phenotypes in these patients (Julu et al., 2001; Weese-Mayer et al., 2006, 2008). The distinct respiratory phenotype-genotype relationship in *Mecp2*+/− symptomatic mutants provides a unique opportunity for studying the effect of *Mecp2* mutation in the mammalian brain.

The present results corroborate the notion that the spontaneous apnea and prolonged HBIR-induced apnea previously reported in the *Mecp2*tm1.1Bird null mice are mediated by an overexpression of the intrinsic expiratory-promoting signal, as indicated by the pronounced pontine post-inspiratory activity in those animals (Stettner et al., 2007). Rather than desensitize, the intrinsic post-inspiratory activity in the secondary pathway appears to sensitize upon vagal stimulation instead – thereby sustaining and prolonging the induced apnea, overshadowing any habituation of the HBIR in the primary pathway (Poon and Song, 2007). The cause of such divergent mal-adaptations of the HBIR in the *Mecp2*tm1.1Bird null mice and the *Mecp2*tm1.1Jae heterozygous mice is not clear. However, the fact that *Mecp2*tm1.1Bird null and wild-type mice displayed similar propensity for spontaneous apnea and secondary sensitization of the HBIR albeit to varying degrees suggests that such abnormalities may be intrinsic to the C57BL/6J strain and are exacerbated in the *Mecp2*tm1.1Bird null mice. Indeed, the C57BL/6J mouse strain is known to be predisposed to spontaneous deletion mutation in the gene encoding nicotinamide nucleotide transhydrogenase, an inner mitochondrial membrane transmembrane proton-translocating protein involved in regenerating intramitochondrial NADPH (Freeman et al., 2006; Huang et al., 2006; Rydstrom, 2006), which plays an important role in mitochondrial metabolism of reactive oxygen species (ROS; Andreyev et al., 2005; Kowaltowski et al., 2009). Recent evidence reveals that *Mecp2*tm1.1Bird null mice are susceptible to many other mitochondrial abnormalities that may further promote mitochondrial production of ROS (Kriaucionis et al., 2006). The latter has been shown to contribute to the expression of long-term facilitation of carotid body chemosensory activity and phrenic nerve respiratory motor activity in normal animals after exposure to intermittent hypoxia (Peng et al., 2003; MacFarlane and Mitchell, 2009). We speculate that such ROS-induced long-term facilitation of chemoreflex afferent and efferent signaling may be intrinsic to the C57BL/6J mice and *Mecp2*tm1.1Bird null mice, effectively increasing the respiratory controller gain in these animals. The resultant amplifications in respiratory system loop gain could potentially leave these animals with ROS excess at high risk of periodic breathing and spontaneous central apnea (Khoo, 2000). Alternatively, there is evidence that increased ROS production may directly modulate the respiratory rhythm in preBötzinger complex neurons (Garcia et al., 2011). Further studies are needed to investigate whether the intermittent apnea in the *Mecp2*tm1.1Bird null mice and the C57BL/6J wild-type strain represents ROS-induced chemoreflex instability in the respiratory system or abnormal rhythmogenesis in pacemaker neurons.

FIGURE 4 | Working models of non-associative learning of HBIR in: (A) In *Mecp2*tm1.1Jae wild-type mice, HBIR is moderated by NMDAR-dependent desensitization in the pontine pathway; (B) In *Mecp2*tm1.1Jae heterozygous symptomatic mice, NMDAR-dependent pontine desensitization is impaired.

To our knowledge, this is the first experimental demonstration of abnormal non-associative learning caused by a specific genetic mutation that is linked to a well-defined clinical phenotype of a congenital neurological disease. Although the cellular bases of non-associative learning paradigms such as habituation and sensitization have been extensively studied in invertebrate sensorimotor systems (Kandel, 1978; Glanzman, 2009; Ardiel and Rankin, 2010), details of their counterparts in mammalian brain systems are only beginning to emerge recently (Siniaia et al., 2000; MacDonald et al., 2009; Wilson, 2009; Schmid et al., 2010). The present results provide a novel mammalian model of studying the structure-function correlations of two contrasting forms of non-associative learning (sensitization and desensitization) from genetic to behavioral levels.

In conclusion, we have shown that mutation in the *Mecp2* gene may lead to disparate respiratory endophenotypes in the *Mecp2*tm1.1Jae and *Mecp2*tm1.1Bird mouse strains, indicating possible interaction of the *Mecp2* gene with animal genetic background. Whereas the *Mecp2*tm1.1Bird male mice provide an excellent animal model of spontaneous central apnea and possibly obstructive apnea (Voituron et al., 2010), the present study confirmed that a clinically relevant RTT endophenotype – tachypnea with shortened T_E – is more faithfully reproduced in *Mecp2*tm1.1Jae female mice. Importantly, the shortening of T_E was found to correlate with the lack of NMDAR-dependent desensitization of the HBIR in these mixed-strain heterozygous mutants compared to wild-type mice, in sharp contrast to the abnormal prolongation of T_E and secondary sensitization of the HBIR reported in the *Mecp2*tm1.1Bird null mice. These findings shed new light on the mechanisms of disordered breathing in RTT and corroborate a working model of non-associative learning in the mammalian brain. This non-associative learning perspective provides a new dimension for further investigation of the pathogenesis of breathing abnormalities in these mutant animals with impaired methylated DNA binding or those with DNA hypomethylation (Fan et al., 2001), and in patients with RTT caused by mutations of the *MECP2* gene.

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