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Brain-Derived Neurotrophic Factor Expression and Respiratory Function Improve after Ampakine Treatment in a Mouse Model of Rett Syndrome

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Rett syndrome (RTT) is caused by loss-of-function mutations in the gene encoding methyl-CpG-binding protein 2 (MeCP2). Although MeCP2 is thought to act as a transcriptional repressor of brain-derived neurotrophic factor (BDNF), *Mecp2* null mice, which develop an RTT-like phenotype, exhibit progressive deficits in BDNF expression. These deficits are particularly significant in the brainstem and nodose cranial sensory ganglia (NGs), structures critical for cardiorespiratory homeostasis, and may be linked to the severe respiratory abnormalities characteristic of RTT. Therefore, the present study used *Mecp2* null mice to further define the role of MeCP2 in regulation of BDNF expression and neural function, focusing on NG neurons and respiratory control. We find that mutant neurons express significantly lower levels of BDNF than wild-type cells *in vitro*, as *in vivo*, under both depolarizing and nondepolarizing conditions. However, BDNF levels in mutant NG cells can be increased by chronic depolarization *in vitro* or by treatment of *Mecp2* null mice with CX546, an ampakine drug that facilitates activation of glutamatergic AMPA receptors. Ampakine-treated *Mecp2* null mice also exhibit marked functional improvement, characterized by restoration of normal breathing frequency and minute volume. These data demonstrate that BDNF expression remains plastic in *Mecp2* null mice and raise the possibility that ampakine compounds could be of therapeutic value in the treatment of RTT.

Key words: Mecp2 null mice; respiratory frequency; minute volume; nodose ganglion; neurotrophin expression; AMPA receptors modulator

Introduction

Rett syndrome (RTT) is an X-linked neurodevelopmental disorder caused by mutations in the methyl-CpG-binding protein 2 gene (*MECP2*) (Amir et al., 1999). Six to 18 months after birth, RTT patients begin a neurological decline characterized by regression of acquired skills, behavioral disturbances with autistic features (Hagberg et al., 1983), motor stereotypies, seizures, autonomic dysfunction, and severely disordered breathing (Shahbazian and Zoghbi, 2002). Respiratory abnormalities in RTT include alternating periods of hyperventilation and breath holds and forced and apneustic breathing (Weese-Mayer et al., 2006, and references therein) and may contribute to up to 26% of deaths in RTT (Kerr et al., 1997). The primary cause of these breathing alterations is unknown, and current hypotheses include cortical dysfunction (Elian and Rudolf, 1991; Marcus et al., 1994), brainstem immaturity (Julu et al., 2001), decreased noradrenergic transmission in ponto-medullary respiratory networks (Viemari et al., 2005), and hyperexcitability in pontine and vagal afferent pathways (Stettner et al., 2007). There is no treatment currently available for respiratory dysfunction in RTT.

Recent studies suggest that alterations in brain-derived neurotrophic factor (BDNF) signaling contribute to RTT pathophysiology. For example, *Mecp2* null mice exhibit progressive deficits in BDNF levels after birth (Chang et al., 2006; Wang et al., 2006), and genetic restoration of BDNF in the forebrain improves somatomotor function and extends lifespan (Chang et al., 2006). Moreover, neural structures important for cardiorespiratory control, including the nodose cranial sensory ganglia (NGs) and brainstem, exhibit the earliest and most significant known deficits in BDNF expression in the *Mecp2* null mouse brain (Wang et al., 2006). Because BDNF is required for the development of NG and brainstem respiratory neurons, as well as breathing (Katz, 2005), we hypothesize that BDNF deficits contribute to the RTT-like respiratory phenotype of *Mecp2* null mice.

The fact that *Mecp2* null mice exhibit decreased BDNF expression contrasts with the prevailing view that *Mecp2* is a transcriptional repressor of *Bdnf* (Chen et al., 2003). One model proposed to explain this apparent discrepancy is that decreased neuronal activity in *Mecp2* null mutants (Dani et al., 2005) reduces activity-dependent BDNF expression, thereby masking any effect of derepression (Chang et al., 2006). To test this hypothesis, we examined BDNF expression in NG neurons cultured under

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depolarizing and nondepolarizing conditions. Because the NG comprises a single neuronal cell type (sensory neurons) and exhibits the *Mecp2* null BDNF phenotype *in vitro* as *in vivo* (Wang et al., 2006), it provides a simple model for exploring mechanisms that underlie BDNF regulation by MeCP2. Our data indicate that *Mecp2* null cells exhibit significantly lower levels of BDNF expression than wild type, under both depolarizing and nondepolarizing conditions. However, BDNF levels in mutant cells can be elevated to wild-type resting levels by depolarizing stimuli *in vitro*. Similarly, we find that treatment of *Mecp2* null mice with the ampakine drug 1-(1,4-benzodioxan-6-yl-carbonyl)piperidine (CX546), which enhances activation of glutamatergic AMPA receptors (Nagarajan et al., 2001), elevates NG BDNF levels *in vivo*. Moreover, ampakine treatment significantly improves respiratory function in *Mecp2* null mice, suggesting that this class of compounds may be of therapeutic value in RTT.

Materials and Methods

Animals. *Mecp2$^{tm1-1Jae}$* mice (Chen et al., 2001), developed by Dr. R. Jaenisch (Whitehead Institute, Massachusetts Institute of Technology, Cambridge, MA) and obtained from the Mutant Mouse Regional Resource Center (University of California Davis, Davis, CA), were maintained on a mixed background (129Sv, C57BL/6, BALB/c). Male *Mecp2* nulls (*Mecp2$^{-/y}$*) were generated by crossing heterozygous *Mecp2$^{tm1-1Jae}$* knock-out females with *Mecp2$^{tm1-1Jae}$* wild-type males (*Mecp2$^{+/y}$*). All experimental procedures were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University.

Cell cultures. Wild-type and *Mecp2* null mice were killed with CO_2 on postnatal day 35 (P35). The NGs were removed, digested in 0.1% collagenase (Sigma, St. Louis, MO) in Earle's balanced salt solution (Invitrogen, San Diego, CA) for 70 min at 37°C, triturated in culture medium (see below) containing 0.15% BSA, and plated at a density of one NG per well into 96-well flat-bottom ELISA plates coated with poly-D-lysine. Cultures were grown for 3 d in DMEM/F-12 medium supplemented with 5% fetal bovine serum (Invitrogen) and 1% penicillin–streptomycin–neomycin, with or without 40 mM potassium chloride (KCl) or 1.5 μM tetrodotoxin (TTX).

Ampakine treatment. Beginning on P25, wild-type and *Mecp2* null littermates were acclimatized to the injection protocol to reduce stress, first by handling for 10 min/d for 3 d, followed by saline injections (0.9% NaCl, i.p., b.i.d.) at 8:00 A.M. and 8:00 P.M. for an additional 3 d. Subsequently, mice were assigned either to drug treatment (40 mg/kg CX546 in 16.5% 2-hydroxypropyl-β-cyclodextrin, i.p., b.i.d.) or vehicle injections (cyclodextrin alone). On the day of their last injection, mice were trained in the plethysmograph recording chamber for 1 h. Eighteen to 24 h after their last injection, on P35, mice were returned to the chamber for recording of respiratory activity.

Plethysmography. Breathing was recorded in unrestrained mice using a whole-body flow plethysmograph (Buxco II; Buxco Research Systems, Wilmington, NC) in which a constant bias flow supply connected to the animal recording chamber ensured continuous inflow of fresh air (1 L/min). Ambient temperature was maintained between 23 and 25°C. Breathing traces were analyzed using Biosystem XA software (Buxco Research Systems). After the recording sessions, mice were euthanized with CO_2 and tissue was processed for BDNF immunoassay.

BDNF reverse transcription-PCR. Total RNA was isolated from intact P35 NG using the RNeasy Mini kit (Qiagen, Valencia, CA). For each sample, 500 ng of total RNA was digested with DNase I (Invitrogen, Carlsbad, CA) and reverse transcribed by oligodT priming using SuperScriptIII (Invitrogen). The amount of each *Bdnf* transcript present in the sample was measured by quantitative real-time PCR (qRT-PCR) using SYBR Green detection (Applied Biosystems, Foster City, CA). *Bdnf* mRNA levels were normalized to *β-tubulin III* mRNA levels to adjust for small differences in input RNA. The following primers were used for qRT-PCR: *Bdnf* exon 8 (coding exon), forward (F) 5′-gatgccgcaaacatgtctatga-3′ and reverse (R) 5′-taatactgtcacacacgctcagctc-3′; *Bdnf* exon 1, F 5′-cactgagcaaagccgaacttctc-3′ and R 5′-tcacctggtggaacattgtggc-3′; *Bdnf* exon 2, F 5′-agcggtgtaggctggaatagactc-3′ and R 5′-ggtggaacttctttgcggcttac-3′; *Bdnf* exon 4, F 5′-cgccatgcaatttccactatcaataatttaac-3′ and R 5′-cgccttcatgcaaccgaagtatg-3′; *Bdnf* exon 5, F 5′-gatccgagagctttgtgtggac-3′ and R 5′-gccttcatgcaaccgaagtatg-3′; *β-tubulin* III, F 5′-cgacaatgaagccctctacgac-3′ and R 5′-atggtggcagacacaaggtggttg-3′.

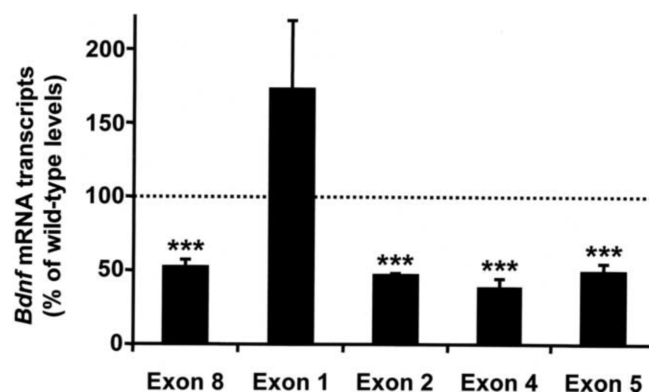

Figure 1. *Mecp2* null mutation is associated with decreased expression of specific *Bdnf* transcripts in nodose neurons. *Bdnf* transcript levels in intact NG from wild-type and *Mecp2* null mice were determined using qRT-PCR. The *Bdnf* gene has a complex structure in which multiple promoters drive the expression of different mRNA isoforms containing alternative noncoding 5′ exons spliced to a common downstream coding exon [exon 8; nomenclature of Liu et al. (2006)]. Total *Bdnf* mRNA levels (Exon 8), as well as transcripts containing exons 2, 4, and 5 were markedly decreased in mutant NG compared with wild type, whereas transcripts containing exon 1 were expressed at levels that were not significantly different from wild type. Results are the mean ± SEM ($n = 4$). ***$p < 0.001$, ANOVA I with *post hoc* Tukey's test.

BDNF immunoassay. BDNF protein levels in intact NGs or in cultured NG cells were measured by ELISA using the BDNF Emax Immunoassay System (Promega, Madison, WI). Protein extracts from one intact NG or from an equivalent number of cultured cells were used for ELISA.

MeCP2 and β-tubulin III double staining. Mice were killed with CO_2 and perfused with 4% paraformaldehyde, and the head was sectioned at 10 μm with a cryostat. Sections were stained with rabbit polyclonal anti-MeCP2 (Upstate Biotechnology, Lake Placid, NY) and chicken polyclonal anti-β-tubulin III (Aves Labs, Ft. Lauderdale, FL).

Statistical analysis. Differences between wild-type and mutant mice, and between vehicle-treated and CX546-treated mice, were tested using an unpaired *t* test or ANOVA I with Tukey's multiple comparison *post hoc* analysis. A *p* value <0.05 was considered statistically significant. Data are presented as mean ± SEM.

Results

Bdnf gene expression is reduced in *Mecp2* null cells *in vivo*

We previously found that BDNF protein content of peripheral and CNS tissues is markedly reduced in *Mecp2* null mice by 5 weeks of age (Wang et al., 2006; see also Chang et al., 2006). To determine whether these deficits are reflective of decreased *Bdnf* gene expression, we compared *Bdnf* mRNA levels in wild-type and mutant animals, using the NG as a model. This analysis revealed that on P35, total *Bdnf* mRNA was reduced by 50% in the *Mecp2* null NG compared with wild-type controls (Fig. 1), paralleling the deficit in BDNF protein (Wang et al., 2006). However, not all *Bdnf* transcripts were similarly affected. For example, although *Bdnf* splice variants containing exon 2, 4, or 5 were all decreased by ~50% in mutant tissue compared with wild type, transcripts containing exon 1 were unchanged. These data indicate that MeCP2 function is required to maintain normal levels of BDNF expression by regulating specific isoforms of *Bdnf* mRNA.

The marked deficit in BDNF content found in P35 *Mecp2* null NG neurons *in vivo* is maintained in dissociate cell culture (Wang et al., 2006), suggesting that it may be a cell-autonomous effect of MeCP2 loss. However, MeCP2 expression in peripheral neurons

has not previously been described. Therefore, initial studies examined the localization of MeCP2 immunoreactivity in the NG and found robust expression in all neurons at P0 through P35 (Fig. 2 and data not shown).

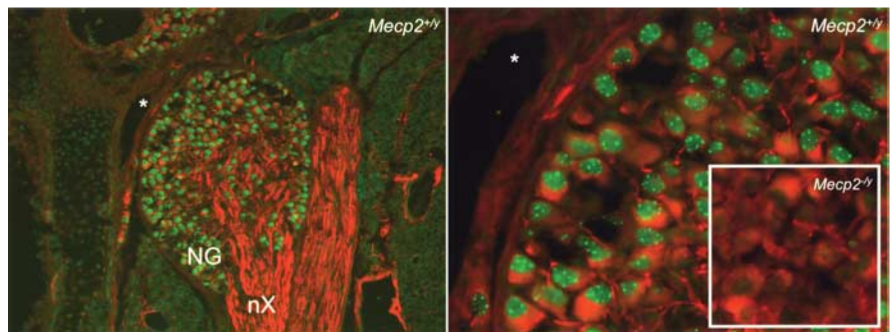

Figure 2. MeCP2 protein is expressed in nodose neurons. Left, Double immunostaining for MeCP2 (green) and β-tubulin III (red) in the newborn wild-type (*Mecp2*$^{+/y}$) mouse NG. nX, Vagal nerve. Right, Higher magnification of the same section shown on the left, illustrating the concentration of MeCP2-immunoreactive protein in heterochromatin foci. The inset shows that the MeCP2 antibody used in these studies does not produce any specific staining in the NG from a *Mecp2* null mouse (*Mecp2*$^{-/y}$). The asterisk represents an anatomical landmark shared by both panels.

To test the hypothesis that differences in BDNF content between wild-type and *Mecp2* null cells result from different levels of activity (Chang et al., 2006), BDNF levels were compared in P35 NG neurons from wild-type and *Mecp2* null mice grown in dissociated culture for 3 d under control (nondepolarizing) and depolarizing (40 mM KCl) conditions. Under control conditions, NG neurons exhibit resting membrane potentials of approximately −70 mV and are not spontaneously active (Schild and Kunze, 1997; Brosenitsch et al., 1998). However, to eliminate any possible depolarizing influence of voltage-gated sodium channels, some cultures were grown in the presence of 1.5 μM TTX [NG neurons also express TTX-insensitive Na channels; however, these activate at substantially more positive membrane potentials (Schild and Kunze, 1997)]. In both control and TTX-treated cultures, *Mecp2* null neurons exhibit 40–50% less BDNF than wild-type neurons (Fig. 3*A*), as *in vivo*, without any change in cell survival (Fig. 3*B*).

To further test the role of membrane depolarization in the BDNF phenotype of *Mecp2* null neurons, NG cultures were grown in the absence and presence of a depolarizing concentration of KCl (40 mM). In both wild-type and mutant cultures, KCl depolarization resulted in a significant increase in BDNF protein compared with unstimulated controls (Fig. 3*C*), with no change in cell survival (Fig. 3*D*). However, even under depolarizing conditions, mutant cells expressed significantly lower levels of BDNF than wild-type cells. These data indicate that *Mecp2* is required for normal levels of BDNF expression in NG neurons under both resting and depolarizing conditions. In addition, these experiments show that chronic depolarization of mutant neurons can stimulate BDNF protein expression to wild-type resting levels. This observation is consistent with previous observations showing increased expression of *Bdnf* exon 4 mRNA in cultured newborn *Mecp2* null cortical cells after KCl treatment (Chen et al., 2003).

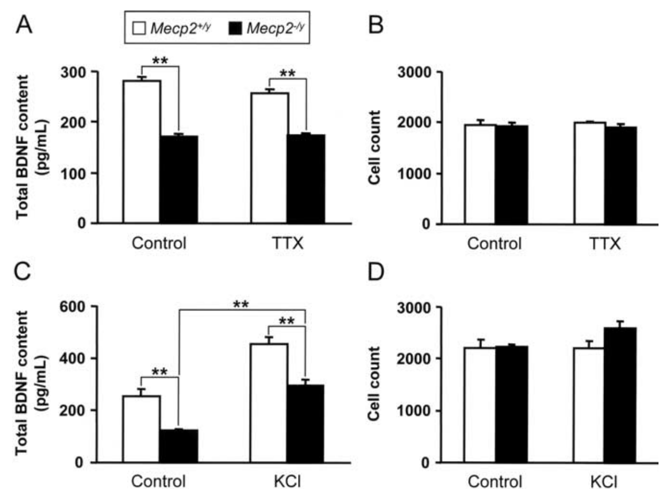

Figure 3. BDNF levels are depressed in P35 *Mecp2*$^{-/y}$ NG neurons under resting and depolarizing conditions. ***A***, ***C***, Summary data showing that BDNF content is decreased by 40–50% in NG cultures from *Mecp2* null mutants, regardless of the activity state of the cells [i.e., electrically silent (***A***; treated with TTX) or chronic depolarization (***C***, treated with KCl)]. Results show that KCl treatment can increase the BDNF level in mutant cells as in wild-type controls. ***B***, ***D***, Neuron survival was unaffected by either TTX (***B***) or KCl (***D***). Results are the mean ± SEM ($n = 6$). $^{**}p < 0.01$, ANOVA I with *post hoc* Tukey's test.

Ampakine stimulation of BDNF expression *in vivo*

The fact that depolarization of *Mecp2* null NG neurons could increase BDNF expression *in vitro* raised the possibility that neuronal activation could rescue the BDNF deficit *in vivo*. To approach this issue, we examined the effect of an ampakine drug, CX546, on BDNF protein expression in the NG in intact P35 wild-type and *Mecp2* null mice. Ampakines are fast-acting molecules that acutely lengthen the duration of AMPA receptor-mediated inward currents and thereby increase the activity of neurons that express AMPA receptors (Nagarajan et al., 2001). As a result, repeated ampakine treatment leads to an increase in activity-dependent expression of BDNF, *in vivo* and *in vitro* (Lauterborn et al., 2000, 2003; Rex et al., 2006).

P35 wild-type and *Mecp2* null littermates were treated for 3 d with CX546 (40 mg/kg in cyclodextrin, i.p., b.i.d.) or vehicle. Twenty-four hours after the last injection, respiratory activity was measured (see below), the mice were killed, and the NG was removed for BDNF ELISA. NG BDNF content in vehicle-treated *Mecp2* null mice was significantly reduced compared with vehicle-treated wild-type controls, as described previously in naive untreated animals (wild type, 170 ± 14 pg BDNF/ml vs mutant, 72 ± 3 pg BDNF/ml; $n = 6$; $p < 0.001$, ANOVA I). Treatment of wild-type mice with CX546 had no effect on NG BDNF content. However, treatment of *Mecp2* null mice resulted in a significant 42% increase in BDNF protein content compared with vehicle-treated mutants (wild-type CX546, 167 ± 5 pg BDNF/ml vs mutant, CX546 114 ± 4 pg BDNF/ml; $n = 6$; $p < 0.001$, ANOVA I).

Ampakine treatment restores wild-type mean respiratory frequency and minute volume in *Mecp2*$^{tm1-1Jae}$ null mice

NG neurons secrete BDNF in an activity-dependent manner (Balkowiec and Katz, 2000), and BDNF acutely modulates glutamatergic transmission at second-order neurons in the nucleus tractus solitarius (nTS) (Balkowiec et al., 2000), the primary relay for peripheral afferent input to the brainstem respiratory rhythm generating network. Therefore, we hypothesize that BDNF deficits in NG neurons contribute to the

pathogenesis of respiratory dysfunction in RTT by disrupting synaptic modulation in nTS.

To examine whether or not ampakine enhancement of BDNF expression in *Mecp2* null NG neurons is associated with recovery of neural function, we compared respiratory activity in wild-type and mutant mice after treatment with CX546 *in vivo* as described above. Respiratory function was monitored by whole-body plethysmography 18–24 h after the last drug injection as described in Materials and Methods. Analysis of naive untreated wild-type and mutant animals revealed a highly disordered breathing pattern in the mutants compared with wild-type controls (Fig. 4). The mutant breathing pattern is characterized by a highly variable frequency (coefficient of variation of breathing frequency: wild type, 18.8 ± 0.7% vs mutant, 22.0 ± 1.2%; $n = 6$ for wild type and $n = 7$ for mutants; $p < 0.05$, unpaired *t* test) and occasional long breathing pauses compared with wild types, similar to human RTT patients (Julu et al., 2001; Weese-Mayer et al., 2006) and other models of RTT (*Mecp2*$^{tm1-1Bird}$ null mice) (Viemari et al., 2005; Stettner et al., 2007). More detailed analysis of breathing parameters revealed that the phenotype observed in mutant mice is associated with repetitive episodes of very high breathing frequency (Fig. 4), resulting in a 23% increase in mean respiratory frequency compared with wild-type controls ($p < 0.001$, unpaired *t* test; $n = 6$ for wild type and $n = 7$ for mutants), similar to RTT patients (Weese-Mayer et al., 2006). Consequently, the mean value for minute volume/weight (tidal volume/weight × breathing frequency) is also increased in mutants (Fig. 4) (wild type, 0.97 ± 0.11 ml/min/g vs mutant, 1.38 ± 0.13 ml/min/g; $n = 6$ for wild-types and $n = 7$ for mutants; $p < 0.05$, unpaired *t* test). In contrast, there was no significant difference in tidal volume/weight alone between wild-type and mutant animals (wild type, 5.4 ± 0.6 μl/g vs mutant, 6.4 ± 0.5 μl/g; $n = 6$ for wild types and $n = 7$ for mutants).

Three-day treatment with CX546 did not significantly affect breathing frequency, tidal volume/weight, and minute volume/weight in P35 *Mecp2*$^{tm1-1Jae}$ wild-type mice (vehicle vs CX546: frequency, 179 ± 3 vs 177 ± 6 breaths/min; tidal volume/weight, 6.1 ± 0.4 vs 6.4 ± 0.5 μl/g; minute volume/weight, 1.09 ± 0.07 ml/min/g vs 1.14 ± 0.09 ml/min/g; $n = 8$ for vehicle and $n = 7$ for CX546). In contrast, ampakine treatment of mutant animals sharply decreased the episodes of high breathing frequency, leading to restoration of wild-type mean breathing frequency (Fig. 5*A*, *C*) (wild-type CX546, 177 ± 6 breaths/min vs mutant CX546, 176 ± 8 breaths/min; $n = 7$ for wild types and $n = 9$ for mutants) and minute volume/weight (Fig. 5*B*, *D*) (wild-type CX546, 1.14 ± 0.09 ml/min/g vs mutant CX546, 1.13 ± 0.07 ml/min/g; $n = 7$ for wild types and $n = 9$ for mutants). However, ampakine treatment did not decrease the higher variability in breathing frequency characteristic of mutant animals (coefficient of variation of breathing frequency: wild-type CX546, 18.5 ± 1.2% vs mutant CX546, 23.4 ± 1.5%; $n = 7$ for wild types and $n = 9$ for mutants). Tidal volume/weight was not affected in mutants by ampakine treatment and was similar to wild type (wild-type CX546 vs mutant CX546, 6.4 ± 0.5 vs 6.5 ± 0.3 μl/g; $n = 7$ for wild types and $n = 9$ for mutants).

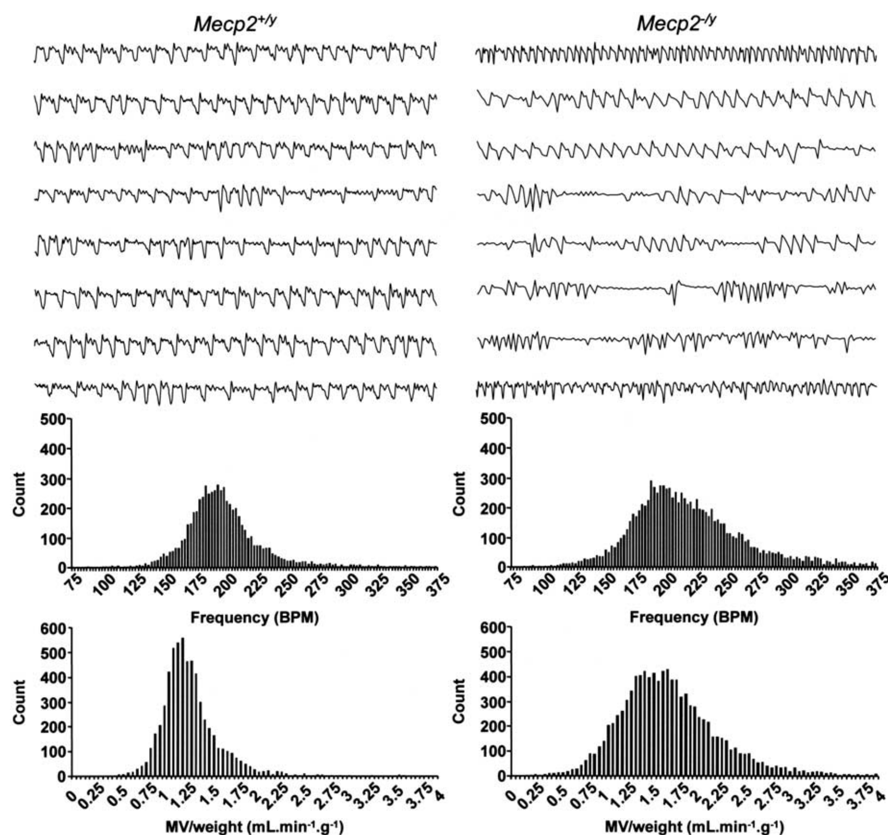

Figure 4. *Mecp2* null mice exhibit a Rett-like respiratory phenotype at 5 weeks of age (P35). Representative plethysmographic recordings from wild-type (*Mecp2*$^{+/y}$) and *Mecp2* null (*Mecp2*$^{-/y}$) mice are shown. Each trace is 10 s quiet breathing in room air. The bottom graphs are frequency histograms from control (compilation of 9776 breath cycles) and mutant (compilation of 6065 breath cycles) mice showing the higher incidence of fast breaths in mutant mice compared with controls, along with a shift to higher values of minute volume/weight. BPM, Breaths per minute; MV, minute volume.

Discussion

Our results demonstrate that MeCP2 is required for normal levels of BDNF expression in nodose sensory neurons under both resting and depolarizing conditions *in vitro*. Moreover, chronic depolarization *in vitro*, or ampakine treatment *in vivo*, can elevate BDNF levels in *Mecp2* null cells. Furthermore, ampakine treatment results in a restoration of wild-type breathing frequency and minute volume/weight in *Mecp2* null mice.

Previous studies in cultured newborn cortical neurons indicated that MeCP2 represses *Bdnf* expression at rest (Chen et al., 2003) and that release from MeCP2-mediated repression is required for normal levels of activity-dependent expression of BDNF (Martinowich et al., 2003; Zhou et al., 2006). However, *Mecp2* null mice exhibit deficits in BDNF protein (Chang et al., 2006; Wang et al., 2006) and mRNA (present study) *in vivo*. Moreover, reduced *Bdnf* gene expression has recently been reported in the frontal cortex of RTT patients (Deng et al., 2007). A proposed explanation for these discrepancies between *in vivo* and *in vitro* studies is that *Mecp2* null cortical neurons are less active *in vivo* than wild-type cells (Dani et al., 2005), leading to a reduction in activity-dependent BDNF expression that masks any effects of BDNF derepression (Chen et al., 2003; Chang et al., 2006; Sun and Wu, 2006). However, our data indicate that, as

in vivo, *Mecp2* null NG neurons grown in dissociated cell culture express significantly less BDNF than wild-type cells and that this deficit persists under both nondepolarizing and depolarizing conditions in culture. These observations indicate that reduced activity alone may be insufficient to explain the BDNF deficit in *Mecp2* null neurons. This apparent difference in BDNF regulation in *Mecp2* null mouse cortical and NG neurons, respectively, may indicate a role for cell context, including cell type and age, in determining the interaction between these two genes. For example, it is possible that in P35 NG neurons, unlike newborn cortical neurons (Chen et al., 2003), MeCP2 indirectly regulates BDNF expression, perhaps by repressing a gene or genes that, in turn, repress BDNF. There are other differences between the present study and that of Chen et al. (2003) that may also be important, including the fact that Chen et al. (2003) only looked at regulation of the exon 4-containing *Bdnf* transcript and stimulated their cultures with KCl for 6 h, compared with 3 d in the present study.

The fact that BDNF expression remains plastic in *Mecp2* null NG neurons and can be increased by depolarizing stimuli *in vitro* led us to test whether or not BDNF levels could be increased in *Mecp2* null mice *in vivo* by the ampakine drug CX546. Ampakines are a family of small molecules that trigger short-term increases in the duration of AMPA-mediated inward currents (Nagarajan et al., 2001). In addition, repeated treatment with ampakines can increase the efficiency of long-term potentiation in the hippocampus and facilitate memory processes (Ingvar et al., 1997; Rex et al., 2006; Wezenberg et al., 2006). These long-term effects of ampakine treatment result from their ability to increase *Bdnf* mRNA and protein expression (Lauterborn et al., 2000, 2003; Rex et al., 2006).

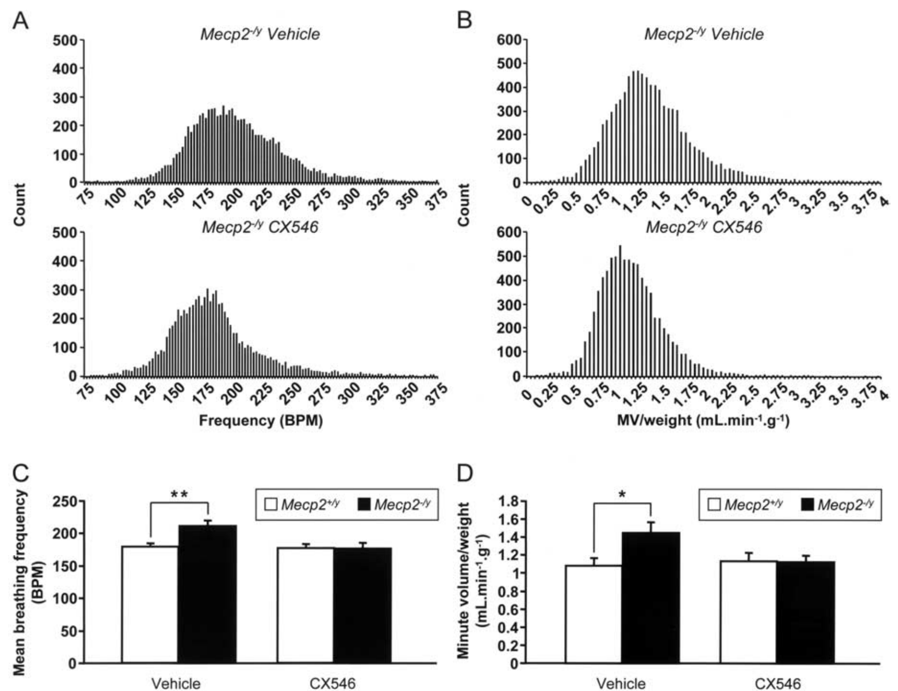

Figure 5. Chronic treatment with CX546 restores normal breathing frequency and minute volume/weight in P35 *Mecp2* null mice. ***A***, ***B***, Representative histograms of breathing frequency (***A***) and minute volume/weight (***B***) from two mutant mice, one treated with vehicle (9227 breath cycles) and one treated with CX546 (8393 breath cycles), showing that drug treatment (40 mg/kg, b.i.d for 3 d) decreases episodes of high breathing frequency and minute volume/weight. ***C***, ***D***, Summary data for breathing frequency (***C***) and minute volume/weight (***D***) for all animals. Ampakine treatment completely restores wild-type frequency and minute volume/weight in mutant animals and has no effect in wild types. Results are the mean ± SEM ($n = 8$ for vehicle-treated wild types; $n = 7$ for CX546-treated wild types; $n = 8$ for vehicle-treated mutants; $n = 9$ for CX546-treated mutants). $^*p < 0.05$; $^{**}p < 0.01$, ANOVA I with *post hoc* Tukey's test. BPM, Breaths per minute.

Our study reveals that chronic treatment with CX546 significantly improves respiratory behavior in adult symptomatic *Mecp2* null mice. Indeed, drug treatment significantly decreased breathing frequency and minute volume/weight, two parameters that are markedly increased in RTT patients and may contribute to severe hypocapnic alkalemia and hypoxemia (Southall et al., 1988). The respiratory improvement was not an acute effect of ampakine treatment, because CX546 has an extremely short half-life (<1 h) (Hampson et al., 1998; Wezenberg et al., 2006) and breathing was analyzed 18–24 h after the last drug injection. Thus, improved breathing is attributable to long-term effects of the ampakine treatment. Although mechanisms that underlie improved respiration in ampakine-treated *Mecp2* null mice remain to be defined, our data are consistent with a role for increased BDNF expression in the NG. NG neurons project centrally to the brainstem nTS, the primary site for afferent input to the brainstem respiratory rhythm generating network, where BDNF inhibits glutamatergic excitation of second-order vagal sensory relay neurons (Balkowiec et al., 2000). In *Mecp2* null mice, BDNF is severely depleted in NG afferents and their projections to nTS (Wang et al., 2006), and activity of postsynaptic neurons is increased (D. D. Kline, personal communication) compared with wild-type controls. Thus, we suspect that elevated respiratory frequency in *Mecp2* null mice may result, in part, from increased excitability in nTS and that ampakine treatment restores wild-type respiratory frequency by enhancing BDNF modulation of primary afferent transmission. This possibility is supported by recent findings that breathing dysfunction in *Mecp2* null mice results from enhanced excitatory (or decreased inhibitory) neurotransmission affecting both vagal sensory and brainstem respiratory cell groups. In particular, Stettner et al. (2007) described hyperexcitability of pontine cell groups involved in the regulation of postinspiratory discharge (Kolliker-Fuse and lateral parabrachial nuclei) and a loss of desensitization in vagal afferent control of breathing in *Mecp2* null mice. It is also possible that ampakine treatment has direct effects in the brainstem as well.

Neuropathological studies in RTT patients and *Mecp2* null mice indicate relatively subtle structural abnormalities, such as decreased dendritic arbor complexity (Chen et al., 2001; Armstrong, 2002; Kishi and Macklis, 2004), that likely reflect disruptions in transynaptic signaling rather than overt neuronal degeneration, raising the possibility that functional deficits in RTT may be reversible. This possibility has recently been strengthened by the demonstration that postnatal re-expression of *Mecp2* in severely symptomatic *Mecp2* null mice is associated with symptom reversal (Guy et al., 2007). Our findings demonstrate that ampakine treatment of symptomatic *Mecp2* null mice can significantly improve respiratory function, raising the possibility that this class of compounds may be of therapeutic value in the treatment of RTT patients.

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