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Defective GABAergic neurotransmission in the nucleus tractus solitarius in *Mecp2*-null mice, a model of Rett syndrome

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ABSTRACT

Rett syndrome (RTT) is a devastating neurodevelopmental disorder caused by loss-of-function mutations in the X-linked methyl-CpG binding protein 2 (*Mecp2*) gene. GABAergic dysfunction has been implicated contributing to the respiratory dysfunction, one major clinical feature of RTT. The nucleus tractus solitarius (NTS) is the first central site integrating respiratory sensory information that can change the nature of the reflex output. We hypothesized that deficiency in *Mecp2* gene reduces GABAergic neurotransmission in the NTS. Using whole-cell patch-clamp recordings in NTS slices, we measured spontaneous inhibitory postsynaptic currents (sIPSCs), miniature IPSCs (mIPSCs), NTS-evoked IPSCs (eIPSCs), and $GABA_A$ receptor ($GABA_A$-R) agonist-induced responses. Compared to those from wild-type mice, NTS neurons from *Mecp2*-null mice had significantly ($p < 0.05$) reduced sIPSC amplitude, sIPSC frequency, and mIPSC amplitude but not mIPSC frequency. *Mecp2*-null mice also had decreased eIPSC amplitude with no change in paired-pulse ratio. The data suggest reduced synaptic receptor-mediated phasic GABA transmission in *Mecp2*-null mice. In contrast, muscimol ($GABA_A$-R agonist, 0.3–100 μM) and THIP (selective extrasynaptic $GABA_A$-R agonist, 5 μM) induced significantly greater current response in *Mecp2*-null mice, suggesting increased extrasynaptic receptors. Using qPCR, we found a 2.5 fold increase in the delta subunit of the $GABA_A$-Rs in the NTS in *Mecp2*-null mice, consistent with increased extrasynaptic receptors. As the NTS was recently found required for respiratory pathology in RTT, our results provide a mechanism for NTS dysfunction which involves shifting the balance of synaptic/extrasynaptic receptors in favor of extrasynaptic site, providing a target for boosting GABAergic inhibition in RTT.

1. Introduction

Rett syndrome (RTT), caused by loss-of-function mutations in the X-linked gene encoding the epigenetic regulator *Mecp2* (methyl-CpG-binding protein 2), is a devastating neurodevelopmental disorder that primarily affects young girls (Ellaway and Christodoulou, 1999; Chahrour and Zoghbi, 2007). Although it is a rare disorder, RTT has recently become a prototypical model for studying synaptic dysfunction in neurological disorders (Chahrour and Zoghbi, 2007). RTT is also categorized as a syndromic autism spectrum disorder and shares important pathogenic pathways with autism (Levitt and Campbell, 2009; Gonzales and LaSalle, 2010). A major cause of morbidity and mortality in RTT is dysfunctional respiratory control due to *Mecp2* deficiency (Katz et al., 2009). Conclusions from genetic, neurochemical, and pharmacological studies support that depressed GABAergic neurotransmission in the brainstem contributes to RTT breathing abnormalities (Chao et al., 2010; Ure et al., 2016). Augmenting GABAergic neurotransmission has been shown to improve the respiratory phenotype in RTT mouse models and to prolong survival (Abdala et al., 2010; Voituron and Hilaire, 2011; Bittolo et al., 2016).

Respiratory neurons reside mainly in two regions of the brainstem: the dorsal respiratory group in the nucleus tractus solitaries (NTS) and the ventral respiratory group in the ventrolateral medulla (Bonham, 1995). Glutamate is the main excitatory neurotransmitter involved in the central generation of respiratory rhythm while GABA acting on $GABA_A$ receptors ($GABA_A$-Rs) provides phasic waves of inhibition to

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shape the pattern of the respiratory motor output (Bonham, 1995). Wasserman and colleagues showed that blocking GABA reuptake with nipecotic acid in the NTS increased inspiratory duration that frequently culminates in apneustic breathing (Wasserman et al., 2002). They further showed that $GABA_B$ receptors mediated the increased inspiratory duration and $GABA_A$-Rs mediated the apnea effects. Importantly, in *Mecp2*-null mice, selective recovery of *Mecp2* expression in the HoxA4 domain, which includes caudal parts of NTS and ventral respiratory column, was sufficient to restore normal respiratory rhythm and prevent apnea (Huang et al., 2016), suggesting an important role of the NTS in RTT pathophysiology. Thus, we focused on NTS $GABA_A$-R dysfunction in a *Mecp2-null* mouse model in order to provide a pathophysiological basis for abnormal respiratory regulation in RTT.

2. Materials and methods

2.1. Mouse model of RTT

All experimental protocols were carried out with approval from the Institutional Animal Care and Use Committee of the University of California Davis. *Mecp2*$^{tm1.1Bird/+}$ mice, which originated from Dr. Adrian Bird's laboratory (Guy et al., 2001), were obtained from Jackson Laboratories (Bar Harbor, Maine). Mice were mated with C57BL/6 J mice (Jackson Laboratories). Mice were genotyped to determine the *Mecp2* deletion according to the protocol provided by the Jackson Laboratory. The *Mecp2*-null males 7 to 10 weeks old were used in the present study as a mouse model of RTT, while their male littermates served as the WT control. The sex of the pups was determined using primers for the *Sry* gene on Y chromosome, which were 5′-TGG GAC TGG TGA CAA TTG TC-3′ and 5′-GAG TAC AGG TGT GCA GCT CT-3′.

2.2. Whole-cell patch-clamp recordings

Brain slices containing the NTS were obtained as previously described (Chen et al., 2009; Sekizawa et al., 2012, 2013). The mice were anesthetized with isoflurane and decapitated. The brain was rapidly removed and submerged in ice-cold (< 4 °C) high-sucrose artificial cerebrospinal fluid (aCSF) that contained (mM): 3 KCl, 2 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 220 sucrose and 2 $CaCl_2$, pH 7.4 when continuously bubbled with 95% O_2/5% CO_2. Brainstem transverse slices (250 μm thick) were cut with the Vibratome 1000 (Technical Products International, St. Louis, MO). After incubation for 45 min at 35 °C in high-sucrose aCSF, the slices were placed in normal aCSF that contained (mM): 125 NaCl, 2.5 KCl, 1 $MgCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 10 glucose and 2 $CaCl_2$, pH 7.4 when continuously bubbled with 95% O_2/5% CO_2. During the experiments a single slice was transferred to the recording chamber, held in place with a nylon mesh, and continuously perfused with oxygenated aCSF at a rate of ~3 ml/min. Borosilicate glass electrodes (BF150-86-10, Sutter Instrumnt, Novato, CA) were filled with a CsCl solution containing (in mM): 140 CsCl, 5 NaCl, 1 $MgCl_2$, 3 K-ATP, 0.2 Na-GTP, 10 EGTA, 10 HEPES, and 5 QX314. The pH was adjusted to 7.3 with CsOH. The seal resistance was > 1 GΩ. The series resistance was no > 15 MΩ and not different (*t*-test, $p > 0.05$) between wild type (13.2 ± 0.5 MΩ, mean ± SEM) and *Mecp2*-null (13.6 ± 0.6 MΩ, mean ± SEM) mice. Recordings were made with a MultiClamp 700B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA). Whole-cell currents were filtered at 2 kHz and digitized at 10 kHz with a DigiData 1440A interface (Molecular Devices).

The neurons were voltage clamped at − 60 mV. All experiments were performed in the presence of ionotropic glutamate receptor antagonists, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[*f*]quinoxaline-7-sulfonamide disodium salt (NBQX, 10 μM) and DL-2-amino-5-phosphonopentanoic acid (AP-5, 50 μM) to isolate the inhibitory currents from excitatory currents.

To determine the inhibitory synaptic input, spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded for 6 min. The sIPSCs in the last 3 min of the recording were used for data analysis. To isolate the action potential-independent synaptic inputs, miniature IPSCs (mIPSCs) were recorded in the presence of the sodium channel blocker, tetrodotoxin (TTX, 1 μM). To stimulate GABA release from local inhibitory neurons in the NTS for evoked-IPSCs (eIPSCs), a bipolar tungsten electrode (1 μm tips separated by 80 μm) was placed in the intermediate NTS ipsilateral and medial to the recording site. We used the minimal intensity (4–8 V, 0.1 ms duration) required to consistently evoke IPSCs. There was no difference (*t*-test, $p > 0.05$) in the intensities used to evoke IPSCs in WT (6.6 ± 0.3 V, mean ± SEM) and *Mecp2*-null mice (6.7 ± 0.4 V, mean ± SEM). The averaged distance between the stimulating electrode and the recorded neurons was 361 ± 5.71 μm (mean ± SEM; ranging from 300 μm to 420 μm). Pairs of NTS stimuli with an inter-pulse interval of 60 ms were delivered at 0.1 Hz to determine paired-pulse ratios (Chen and Bonham, 2005). In separate neurons, muscimol (30 s perfusion, 0.3–100 μM)- or THIP (5 μM)-induced whole-cell currents were recorded. $GABA_A$R agonist, muscimol, was applied in the bath for 30 s, after a stable baseline was recorded. To evaluate changes in GABAergic tonic current, 10 min were allowed in order for the response to stabilize, and the recorded baseline activity for approximately 3 min follow by the bath perfusion for 4 min of the $GABA_A$R partial agonist THIP (5 μM) to increase GABAergic tonic current. After THIP perfusion, $GABA_A$R antagonist bicuculline (30 μM) was added to block both phasic and tonic currents. To confirm that the recorded currents were IPSCs and agonist-induced whole cell currents were recorded before and after perfusion with the $GABA_A$-R antagonist bicuculline (30 μM) in some neurons.

2.3. Quantitative PCR (qPCR)

Brainstem slices (300 μm) containing the NTS were obtained as described above. Bilateral punches of the NTS were obtained with a 0.5 mm biopsy punch (World Precision Instruments, Sarasota, FL). RNA was extracted using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's protocol. cDNA was synthesized using iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA). RNA purity and concentrations were assessed by measuring the absorbance at 260 nm, and 280 nm using a NanoDrop 2000C Spectrophotometer (Thermo Scientific, Waltham, MA). Quantitative PCR (qPCR) was performed using the SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA) in the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The primer sequences used to quantify $GABA_A$-R subunit mRNAs are listed in Table 1. For β-actin, we used the commercially available primer set Mouse ACTB (Actin, Beta) Endogenous Control FAM Dye/MGB Probe, Non-Primer Limited (Invitrogen, Carlsbad, CA). Gene expression was normalized to an endogenous reference gene, β-actin. Data were analyzed with the 2-ΔCt method. All experiments were performed in duplicate.

2.4. Statistical analysis

The sIPSC and mIPSC events were detected with MiniAnalysis software (Synaptosoft, Fort Lee, NJ). The accuracy of detection was confirmed by visual inspection. Data are expressed as means ± SEM. Statistical analyses were performed using SigmaPlot software (Systat Software, Inc., San Jose, CA). A *t*-test was used to compare between wild type and *Mecp2*-null mice for the following measurements: sIPSC/mIPSC frequency and amplitude, eIPSC amplitude, decay time constant, and paired-pulse ratio. A two-way repeated ANOVA was used to compare THIP- and muscimol-induced responses, followed by Fisher's LSD test for pairwise comparison when appropriate. Relative cDNA levels for the target genes were analyzed by the 2-ΔΔCt method using Actb (β-actin) as the internal control for normalization (Livak and Schmittgen, 2001). A *t*-test was used to compare between WT and *Mecp2*-null mice. A *p* value of < 0.05 was considered statistically significant.