Methyl CpG Binding Protein 2 Gene Disruption Augments Tonic Currents of γ-Aminobutyric Acid Receptors in Locus Coeruleus Neurons

BACKGROUND: Mecp2 disruption causes hyperexcitability of locus coeruleus (LC) neurons with autonomic dysfunction.

RESULTS: Agonists for extrasynaptic GABA receptors produced large tonic currents, lowered neuronal excitability, and alleviated breathing abnormalities in Mecp2<sup>−/−</sup> mice.

CONCLUSION: Mecp2 disruption augments extrasynaptic GABAergic inhibition in locus coeruleus neurons.

SIGNIFICANCE: These receptors may be targeted to improve neuronal excitability and breathing abnormalities in Rett syndrome.

People with Rett syndrome and mouse models show autonomic dysfunction involving the brain stem locus coeruleus (LC). Neurons in the LC of Mecp2-null mice are overly excited, likely resulting from a defect in neuronal intrinsic membrane properties and a deficiency in GABA synaptic inhibition. In addition to the synaptic GABA receptors, there is a group of GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) that is located extrasynaptically and mediates tonic inhibition. Here we show evidence for augmentation of the extrasynaptic GABA<sub>A</sub>Rs in Mecp2-null mice. In brain slices, exposure of LC neurons to GABA<sub>A</sub>R agonists increased tonic currents that were blocked by GABA<sub>A</sub>R antagonists. With 10 μM GABA, the bicuculline-sensitive tonic currents were ∼4-fold larger in Mecp2-null LC neurons than in the WT. Single-cell PCR analysis showed that the δ subunit, the principal subunit of extrasynaptic GABA<sub>A</sub>Rs, was present in LC neurons. Expression levels of the δ subunit were ∼50% higher in Mecp2-null neurons than in the WT. Also increased in expression in Mecp2-null mice was another extrasynaptic GABA<sub>A</sub>R subunit, α6, by ∼4-fold. The δ subunit-selective agonists 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol hydrochloride and 4-chloro-N-[2-(2-thienyl)imidazo[1,2-a]pyridin-3-yl]benzamide activated the tonic GABA<sub>A</sub> currents in LC neurons and reduced neuronal excitability to a greater degree in Mecp2-null mice than in the WT. Consistent with these findings, in vivo application of 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol hydrochloride alleviated breathing abnormalities of conscious Mecp2-null mice. These results suggest that extrasynaptic GABA<sub>A</sub>Rs seem to be augmented with Mecp2 disruption, which may be a compensatory response to the deficiency in GABAAergic synaptic inhibition and allows control of neuronal excitability and breathing abnormalities.

Rett syndrome (RTT) is a neurodevelopmental disease with ∼0.01% morbidity rate in live-born females worldwide. Over 90% of RTT cases are caused by mutations of the X-linked MECP2 gene encoding methyl CpG binding protein 2 (MeCP2), a transcription regulator. People with RTT usually develop autism-like symptoms 6–18 months after birth, which include stereotypical repetitive hand movements, social anxiety, and seizures. Dysfunctions in the autonomic nervous system, such as breathing instability, gastrointestinal disorders, and cardiac arrhythmia, are common.

The norepinephrine (NE) system in the brain stem is involved in autonomic function, especially NEergic neurons in the locus coeruleus (LC). Recent studies by us and other laboratories have shown that the LC neurons in Mecp2-null mice are abnormal or defective. The defect manifests itself as reduced expression of NE synthetic enzymes, hyperexcitability, and impaired CO<sub>2</sub> chemosensitivity. The hyperexcitability of LC neurons is attributable to the intrinsic membrane properties of the cells and a decrease in synaptic inhibition mediated by GABA.

Mecp2<sup>−/−</sup> mice recapitulate most RTT phenotypes in vivo. Selective deletion of the Mecp2 gene in GABAAergic neurons recapitulates most RTT phenotypes in mice. These findings indicate that the GABA system plays an important role in the development of RTT.

GABA is the most prominent inhibitory neurotransmitter in the brain, acting on both synaptic and extrasynaptic GABAARs. The synaptic GABA<sub>A</sub>Rs are found in postsynaptic membranes, while the extrasynaptic GABA<sub>A</sub>Rs are found in presynaptic terminals.
of neurons. In adult neurons, activation of the synaptic GABA\(\alpha\)Rs produces fast inhibitory postsynaptic currents and hyperpolarization of the postsynaptic cells. The extrasynaptic GABA\(\alpha\)Rs known as tonic receptors are characterized by their extrasynaptic location, high sensitivity to GABA, capability to produce tonic currents with long-lasting hyperpolarization, and availability for modulation by conventional GABA\(\alpha\)R ligands as well as more selective extrasynaptic GABA\(\alpha\)R modulators (16, 17).

Both of the synaptic and extrasynaptic GABA\(\alpha\)Rs are pentamers, usually composed of two to three heteromeric subunits with a total of 19 (\(\alpha1–6, \beta1–3, \gamma1–3, \delta, \theta, \epsilon, \pi, \) and \(\rho1–3\)) (16). GABA\(\alpha\)Rs with different combinations of subunits are found in different neurons. \(\gamma2\)-containing receptors are mainly localized at the synapse, playing a key role in GABA synaptic transmission (18, 19). The \(\delta\) subunit, usually assembled with two \(\alpha\) and two \(\beta\) subunits, is the major contributor of the extrasynaptic GABA\(\alpha\)Rs (17, 19). These receptors are responsible for tonic GABA inhibition without interfering with synaptic transmission, which is due to their high affinity to GABA and weak desensitization.

The findings of defects in synaptic GABA\(\alpha\)-mediated synaptic inhibition in \(\text{Mecp2}^{-/-}\)null mice are encouraging because therapeutic GABA\(\alpha\)-R activators are widely available. These drugs may be used to correct the defects in the GABA system and relieve RTT-like symptoms. Indeed, several recent studies have shown that the breathing disorders of \(\text{Mecp2}^{-/-}\)null mice can be alleviated by augmenting GABA synaptic inhibition (20, 21). In contrast to the rich information of the synaptic GABA\(\alpha\)Rs in RTT research (10, 11, 13, 22–25), how the extrasynaptic GABA\(\alpha\)Rs are affected by \(\text{Mecp2}\) disruption remains unknown. The capability of these extrasynaptic GABA\(\alpha\)Rs to reduce neuronal excitability without interrupting synaptic transmission suggests that these receptors may allow an alternative therapeutic intervention to RTT. Therefore, we studied the extrasynaptic GABA\(\alpha\) currents in LC neurons in the WT and mouse model of RTT.

**Experimental Procedures**

**Animals**—Female heterozygous mice (genotype, \(\text{Mecp2}^{+/+}\); strain name, B6.129P2(C)-Mecp2tm1.1Bird/J (The Jackson Laboratory stock no. 003890) were cross-bred with WT males to produce RTT model mice with the genotype \(\text{Mecp2}^{-/-}\). The PCR protocol from The Jackson Laboratory was used to identify the genotype. Only \(\text{Mecp2}^{-/-}\) male mice aged 3 weeks were used in the experiments, and their littermates with the genotype \(\text{Mecp2}^{+/+}\) were used as a control. All experimental procedures in mice were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Georgia State University Institutional Animal Care and Use Committee.

**Brain Slice Preparation**—Brain slices were prepared as described previously (4, 26). In brief, a mouse was decapitated after deep anesthesia with inhalation of saturated isoflurane. The brain stem was obtained and immediately placed into ice-cold sucrose-rich artificial CSF (aCSF) containing 220 mm sucrose, 1.9 mM KCl, 0.5 mM CaCl\(_2\), 6 mM MgCl\(_2\), 33 mM NaHCO\(_3\), 1.2 mM Na\(_2\)HPO\(_4\), and 10 mM d-glucose. The solution was bubbled with 95% O\(_2\) balanced with 5% CO\(_2\) (pH 7.40). Transverse pontine sections (250–300 \(\mu\)m) containing the LC area were obtained using a vibratome sectioning system and then recovered at 33 °C for 30 min in normal aCSF containing 124 mM NaCl, 3 mM KCl, 2 mM CaCl\(_2\), 2 mM MgCl\(_2\), 26 mM NaHCO\(_3\), 1.3 mM Na\(_2\)HPO\(_4\), and 10 mM d-glucose. The brain slices were kept at room temperature before use. At recording, the slices were perfused with oxygenated aCSF at a rate of 2 ml/min and maintained at 34–35 °C in a recording chamber.

**Electrophysiology**—LC neurons were identified as described previously (22). Whole-cell voltage clamping and whole-cell current clamping were performed with patch pipettes. A pipette puller (model P-97, Sutter, Novato, CA) was used to pull the patch pipettes with a resistance of 3–5 MΩ. Only neurons with a membrane potential of less than −40 mV and an action potential of more than 65 mV were accepted for further experiments. In voltage clamping, the pipettes were filled with solution containing 50 mM KCl, 85 mM CsCl, 2 mM MgCl\(_2\), 2 mM magnesium-ATP, 1 mM sodium-GTP, 10 mM HEPES, and 0.5 mM EGTA (pH 7.30). The brain slice were perfused with oxygenated aCSF containing 130 mM NaCl, 3.5 mM KCl, 1.25 mM NaH\(_2\)PO\(_4\), 1.5 mM MgSO\(_4\), 10 mM d-glucose, 24 mM NaHCO\(_3\), and 2 mM CaCl\(_2\) (pH 7.40). GABA\(\alpha\)-mediated inhibitory postsynaptic currents (IPSCs) and tonic currents were isolated with the following agents in bath solution: 6-cyano-7-nitroquinoxaline-2,3-dione (10 \(\mu\)M, Tocris, Minneapolis, MN, disodium salt), an AMPA receptor antagonist; DL-2-amino-5-phosphonopentanoic acid (10 \(\mu\)M, Tocris, sodium salt), an NMDA receptor antagonist; and strychnine (1 \(\mu\)M, Sigma-Aldrich, St. Louis, MO), a glycine receptor antagonist. All recordings were performed at a holding potential of −70 mV. 4,5,6,7-Tetrahydro-2-oxazolo[5,4-c]pyridin-3-ol hydrochloride (THIP, also known as gaboxadol, Tocris, hydrochloride), 4-chloro-N-[2-(2-thienyl)imidazo[1,2-a]pyridin-3-yl]benzamide (DS2, Tocris), bicuculline (Tocris), and picrotoxin (Sigma-Aldrich) were used to measure the tonic current. In current clamping, the pipette solution contained 130 mM potassium gluconate, 10 mM KCl, 10 mM HEPES, 2 mM magnesium-ATP, 0.3 mM sodium-GTP, and 0.4 mM EGTA (pH 7.3). The bath solution was normal aCSF bubbled with 95% O\(_2\) and 5% CO\(_2\) (pH 7.40). The current-voltage relationship was recorded by injecting a series of step currents, typically starting from −0.16 nA, with an step increment of 0.016 nA. Recorded signals were amplified with an Axopatch 200B amplifier (Molecular Devices, Union City, CA), digitized at 10 kHz, filtered at 1 kHz, and collected with Clampex 8.2 data acquisition software (Molecular Devices). The temperature was maintained at 33 °C during recording by a dual automatic temperature control (Warner Instruments, New Haven, CT).

**Quantitative PCR**—Brain slices were obtained from 3- to 4-week-old mice. Transcripts were obtained from micro-punches (∼1.5 mm in diameter) from the LC area, and cDNAs were synthesized with the high-capacity cDNA reverse transcription kit (Life Technologies). PCR primers for GAPDH (forward, CCAGCCTCGTCCCGTAGA; reverse, TGCCGTGATGTTGACCTA) and \(\alpha4\) subunit (forward, GGCTTCTTCTTGTTTTCTC; reverse, CACCCCCACTGGTTTTTC) were used.
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AATGCAGGGCAGGTGGAG; α5 subunit (forward, CAA- AAGAGCCAGCTCCAG; reverse, GAAAGTGCCAAACAA- GATGG), α6 subunit (forward, GACTTTGCCCCATCGTCTC; reverse, TGCAAAAGCTACTGGGAAGGAG), β1 subunit (forward, TGTTGTTTGATCTTGTTGCAG; reverse, AGGCC- ACCTCTCTTTTTGTGTGTT), β2 subunit (forward, TTGC- CACTGCTTCTCTCATAAC; reverse, ATCTAACC- ACTCTCTCTTTTTTTC), and β3 subunit (forward, GTTGA- TTGTTGTGTTCGGCAATG; reverse: ATGTCCCGGTGT- GGCATC) were designed with Primer Express software and synthesized from Sigma Genesis (Sigma-Aldrich). Quantitative PCR (qPCR) was performed with Fast SYBR Green Master Mix (Applied Biosystems, Life Technologies), following the instruc- tions of the manufacturer, in a fast real-time PCR system (Applied Biosystems 7500) for 40 cycles. GAPDH was used as the internal control for the quantification of subunit expression.

**Single-cell PCR**—Transcripts were obtained from single neu- rons that were studied in whole-cell recording, with which cDNAs were synthesized as described above. Three microliters of reverse transcription product were used to perform PCR with TaqDNA polymerase (Promega, Madison, WI) for 35 cycles following the instructions of the manufacturer. Three microliters of the PCR reaction were performed with the same PCR cycling protocol as before. The second PCR reaction product was run on 2% agarose gels and was then imaged using an Alphalanger 3400 multi-function gel imager (Alpha Innotech, Santa Clara, CA). GAPDH was used as the positive control, and green fluorescent protein was used as the negative control.

**Western Blot Analysis**—Ponitne slices (300 μm thick) containing the LC area were obtained from 3- to 4-week-old mice with a vibratome sectioning system, and the ponis was processed in radioimmune precipitation assay buffer (Sigma-Aldrich) with 1% protease inhibitor. BCA protein assay reagent (Pierce) was used to estimate the protein concentrations, and 30 μg of proteins was used to detect δ subunit signals in 10% SDS-PAGE gels and electrophoretically transferred to nitrocellulose membranes. The membranes were then blocked for 2 h in 5% nonfat milk and incubated overnight at 4 °C with rabbit GDPH primary antibody (1:10,000, Sigma-Aldrich) and rabbit δ subunit primary antibody (1:1000, EMD Millipore, Billerica, MA) (27). After washing in PBS Tween, the membranes were incubated by HRP-conjugated goat anti-rabbit secondary antibody (1:10,000, Life Technologies) for 1 h at room temperature. The chemiluminescent detection system (Pierce) was used to expose the membrane to films (Hy Blot CL, Denville, Metuchen, NJ), and the photographs were scanned. The immuno- blotting signals were quantified using ImageJ software (National Institutes of Health). The δ subunit signals were normalized to the internal GAPDH controls.

**Plethysmograph Recording**—Mecp2-null mice 25–33 days of age were randomly separated into two groups (five mice in each group). One group of mice was injected with drug (10 mg/kg intraperitoneally), and the other group was injected with saline as a control. The breathing activities of unanesthetized mice were recorded by the plethysmograph system with an ~40-ml plethysmograph chamber and a connected reference chamber. The individual animal was kept in the plethysmograph cham- ber with airflow at a rate of 60 ml/min for at least 20 min for adaptation, followed by a 20-min recording. The breathing activities were recorded continuously with a signal transducer as the barometrical changes between the plethysmograph chamber and the reference chamber. The signal was amplified and collected with Pclamp 9 software. The data analysis was done blinded to the treatment. Apnea was considered only when the breathing cycle lasted twice or longer than the previous cycle. Breathing frequency variation was calculated as the division of standard deviation of the frequency by their arith- metic mean. All standard deviations and arithmetic means were measured from three to four stretches of recordings with at least 50 breaths in each.

**Data Analysis**—The electrophysiological data and the plethysmograph data were analyzed with Clampfit 10.3 software. The sample sizes in all the experiments were examined with G-Power Analysis (28) to yield sufficient statistical power. Data are presented as mean ± S.E. Two-tailed Student’s t test, one-way ANOVA, two-way ANOVA and Tukey’s post hoc test were used to perform the statistical analyses. The difference was con- sidered significant when p ≤ 0.05.

**Results**

**GABA<sub>ergic Tonic Currents in WT Neurons**—To determine the GABA<sub>A</sub> tonic currents in LC neurons, whole-cell voltage clamping was performed in brain slices of WT mice. Inward Cl<sup>−</sup> currents were studied, with 135 mM Cl<sup>−</sup> in both the pipette and bath solutions at a holding potential of −70 mV, and glutamatergic and glycinergic currents were blocked (see “Experi- mental Procedures”). Under these conditions, the LC neurons showed spontaneous GABAergic IPSCs that were blocked by bicuculline (50 μM) or picrotoxin (20 μM). Meanwhile, we found that these GABA<sub>AR</sub> blockers also suppressed tonic inward currents. Therefore, we studied the GABA<sub>A</sub>ergic tonic currents. The current amplitude histograms were generated under stable conditions before and after GABA<sub>AR</sub> blockade and were then fit with Gaussian distribution. The opening of the ionotropic receptors also increases the current noise levels, which were measured as the standard variation of the Gaussian distribution. We analyzed the ratio of noise levels before versus after a treatment with GABA<sub>AR</sub> blockers.

Bicuculline reduced the tonic currents by 2.9 ± 0.6 pA (n = 5), and the noise ratio was 1.31 ± 0.06 (n = 5) (Fig. 1B). Similar results were obtained with picrotoxin (Fig. 1A). The effects of bicuculline on the tonic currents and the noise ratio were more obvious in the presence of GABA in the perfusion solution. A pretreatment with 1 μM GABA augmented the tonic currents to 5.0 ± 1.0 pA and the noise ratio to 1.37 ± 0.08 (n = 5) (Fig. 1C). With 10 μM GABA, the tonic currents were raised to 13.6 ± 1.4 pA (n = 6) and the noise ratio to 3.97 ± 0.76 (n = 6) (Fig. 1D). The bicuculline-sensitive tonic currents and noise augmentation increased dose-dependently with increased GABA concentrations (p < 0.001, one-way ANOVA) (Fig. 1, E–G).

**Enhancement of GABA<sub>A</sub>ergic Tonic Currents in Mecp2-null Mice**—At baseline, the bicuculline-sensitive tonic currents were significantly larger in Mecp2-null mice than in WT mice (6.4 ± 0.8 pA, n = 5 versus 2.9 ± 0.6 pA, n = 5; p < 0.01, Student’s t test; Fig. 2, A, D, and E). These tonic currents in
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**FIGURE 1. GABA\textsubscript{A}R antagonists reduce the tonic currents of LC neurons in WT mice.** Tonic GABA\textsubscript{A} currents were recorded by whole-cell voltage clamping with ion substitution and selective receptor blockers. A and B, in the absence of exogenous GABA in the bath solution (baseline), tonic currents were measured as the difference before and after treatment of GABA\textsubscript{A}Rs antagonists. Picrotoxin (20 \( \mu \)M) and bicuculline (50 \( \mu \)M) reduced both the synaptic GABA\textsubscript{A} currents and the tonic GABA\textsubscript{A} currents of LC neurons in WT mice. Noise was measured as standard deviation of the currents and is shown as a ratio with ion substitution and selective receptor blockers.

*Effects of Specific Agonists for Extrasynaptic GABA\textsubscript{A}Rs—The GABA\textsubscript{A}ergic tonic currents are likely to be mediated by extrasynaptic GABA\textsubscript{A}Rs expressed in LC neurons. Because the molecular compositions of extrasynaptic GABA\textsubscript{A}Rs are different from those of synaptic GABA\textsubscript{A}Rs, these extrasynaptic receptors can be activated with selective agonists, such as THIP and DS2, that do not affect the synaptic GABA\textsubscript{A}Rs (29, 30). In the presence of 1 \( \mu \)M THIP in the bath solution, the GABA\textsubscript{A}ergic tonic currents (18.6 \( \pm \) 2.9 pA, \( n = 5 \)) and noise ratio (1.94 \( \pm \) 0.07, \( n = 5 \)) were both augmented in WT neurons (Fig. 3A). In Mecp2-null neurons, the same concentration of THIP raised the tonic currents (55.3 \( \pm \) 6.6 pA, \( n = 5 \)) and the noise ratio (2.91 \( \pm \) 0.29, \( n = 5 \)) to significantly greater degrees than in the WT neurons (\( p < 0.001 \) and \( p < 0.01 \), respectively, Student’s \( t \) test; Fig. 3, B–E). THIP did not affect the frequency and amplitude of the GABAergic IPSCs in both WT and Mecp2-null LC neurons (Student’s \( t \) test; Fig. 3, F–I).

Similarly, application of DS2 (20 \( \mu \)M), a positive allosteric modulator of extrasynaptic GABA\textsubscript{A}Rs, augmented the tonic currents and noise ratio. This effect was larger in Mecp2-null mice (19.5 \( \pm \) 2.3 pA, 2.32 \( \pm \) 0.21, respectively; \( n = 5 \)) than in WT mice (8.5 \( \pm \) 1.2 pA, 1.53 \( \pm \) 0.07, respectively; \( n = 5 \); \( p < 0.01 \) and \( p < 0.01 \), respectively, Student’s \( t \) test; Fig. 4, A–E).

Unlike THIP, DS2 augmented the frequency and amplitude of the GABAergic IPSCs in LC neurons, which may be attributed to their affinity for \( \alpha 3\beta 2 \)-type GABA\textsubscript{A}Rs. Despite this, we did not find significant differences between WT and Mecp2-null mice (Student’s \( t \) test; Fig. 4, F–I). These results indicate that the extrasynaptic GABA\textsubscript{A}Rs existing in LC neurons seem to have a greater effect on LC neuronal activity in Mecp2-null mice.

*Differential Expression of GABA\textsubscript{A}R Subunits in WT and Mecp2-null Mice—The \( \delta \) subunit is the principal component of extrasynaptic GABA\textsubscript{A}Rs, and is localized exclusively outside of the synaptic cleft, mediating GABA\textsubscript{A}ergic tonic currents (5, 45). If Mecp2-null LC neurons have more extrasynaptic GABA\textsubscript{A}Rs, then the \( \delta \) subunit should be expressed in these cells at a higher level than in WT neurons. To test this possibility, we studied \( \delta \) subunit expression at the mRNA and protein levels. Single-cell PCR analysis showed that the \( \delta \) subunit was expressed in most LC neurons in both WT and Mecp2-null mice (14 of 14 WT
FIGURE 2. Bicuculline-sensitive tonic currents are increased in \textit{Mecp2}^{−/−} mice. \textbf{A}, in comparison with WT mice, bicuculline (50 \textmu M) reduced more tonic currents and noise in \textit{Mecp2}-null mice in the absence of exogenous GABA (baseline). \textbf{B} and \textbf{C}, in the presence of 1 \textmu M or 10 \textmu M GABA, the bicuculline effects on the tonic currents and noise ratio were significantly larger in \textit{Mecp2}-null neurons than in the WT. \textbf{D–F}, both tonic currents and the noise ratio increased dose-dependently with an increase in GABA concentrations. Such effects were more obvious in \textit{Mecp2}-null mice. \textit{pF}, picofarad. *, \textit{p} < 0.05; **, \textit{p} < 0.01; ***, \textit{p} < 0.001; Student’s \textit{t} test.

FIGURE 3. THIP boosts larger tonic currents in \textit{Mecp2}^{−/−} mice. \textbf{A}–\textbf{E}, THIP (1 \textmu M) applied to the bath solution triggered bicuculline-sensitive tonic currents, measuring 18.6 ± 2.9 pA, and noise ratio, measuring 1.94 ± 0.07, in WT neurons. In \textit{Mecp2}-null neurons, THIP-activated tonic currents were augmented 3-fold compared with WT cells, and the noise ratio was also increased significantly. \textit{pF}, picofarad. \textbf{F} and \textbf{G}, the effects of THIP on IPSC frequency and amplitude were not significantly different between WT and \textit{Mecp2}-null LC neurons. \textbf{H} and \textbf{I}, analysis of the cumulative fraction of IPSCs showed that 1 \textmu M THIP treatment did not alter the interevent interval and amplitude of GABAergic IPSCs in WT neurons. *, \textit{p} < 0.05; **, \textit{p} < 0.01; ***, \textit{p} < 0.001; Student’s \textit{t} test.
null mice were unlikely to be produced by increased
expression in Mecp2-null mice over the WT (31, 32). In qPCR, the expression level of the subunit was found to be expressed in WT LC neurons. There was a significant reduction of Mecp2-null neurons compared with the WT (n = 6, p < 0.01; Student’s t test). These results suggest that the δ subunit is expressed in LC neurons and that its increased expression in Mecp2-null mice may contribute to the abundant tonic GABA\textsubscript{A} currents.

The δ subunit is another important contributor to extrasynaptic GABA\textsubscript{A}Rs (24). In single-cell PCR, the δ transcript was barely detected in LC neurons (0 of 14 in WT and 2 of 17 in Mecp2-null mice). qPCR analysis showed that δ subunit protein expression was increased 1.6-±0.1-fold in Mecp2-null mice over the WT (n = 6, p < 0.01; Student’s t test, Fig. 5, E and F). Western blot analysis showed a significant increase of δ subunit in WT mice. In Mecp2-null mice, the essential subunit for extrasynaptic GABA\textsubscript{A}-Rs, was reduced (3–4-fold and raised the noise ratio to a significantly greater degree in Mecp2-null neurons compared with the WT. pF, picofarad. F and G, the effects of DS2 on IPSC frequency and amplitude were not significantly different between WT and Mecp2-1 null LC neurons. H and I, cumulative analysis of IPSCs showed that DS2 shifted the interevent interval to the higher frequency range without altering the amplitude of IPSCs in WT LC neurons. **, p < 0.01; Student’s t test.

The δ-containing extrasynaptic GABA\textsubscript{A}Rs are usually composed of one δ, two α, and two β subunits. Previous studies have reported that all three β subunits (β1–3) and α4 and α6 subunits contribute to the assembly of extrasynaptic GABA\textsubscript{A}Rs (17, 31, 32). In qPCR, α6 expression was increased ~4-fold in Mecp2-null mice over WT levels, whereas α5 expression was reduced (n = 4, p < 0.05; Student’s t test; Fig. 5D). Transcript levels of β1 and β2 subunits were both reduced (n = 5, p < 0.001 and 0.01, respectively, Student’s t test), whereas the β3 transcript did not change (Fig. 5D).

Modulation of LC Neuronal Firing Activity by Extrasynaptic GABA\textsubscript{A}-R Agonists—LC neuronal electrophysiological activity was studied with current clamping. In WT mice, THIP reduced LC neuronal electrophysiological activity was studied with current clamping. In WT mice, THIP reduced neurons and 15 of 17 Mecp2-null cells, Fig. 5A), consistent with the presence of GABA\textsubscript{A}ergic tonic currents in LC neurons.

In qPCR, the δ subunit was found to be expressed in WT LC neurons with the 2$^{\Delta\text{Ct}}$ method (41). With the 2$^{-\Delta\Delta\text{Ct}}$ method, the expression level of the δ subunit increased 1.8-±0.5-fold in LC tissue micropunches obtained from Mecp2-null mice in comparison with the WT (n = 9, p < 0.01, Student’s t test; Fig. 5, B–D). Western blot analysis showed that δ subunit protein expression was increased 1.6-±0.1-fold in Mecp2-null mice over the WT (n = 6, p < 0.01; Student’s t test, Fig. 5, E and F). These results suggest that the δ subunit is expressed in LC neurons and that its increased expression in Mecp2-null mice may contribute to the abundant tonic GABA\textsubscript{A} currents.

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FIGURE 4. DS2 raises tonic currents in Mecp2$^{-/-}$ mice. A–E, application of DS2 (20 μM) enhanced tonic currents ~3-fold and raised the noise ratio to a significantly greater degree in Mecp2-null neurons compared with the WT. In F, the effects of DS2 on IPSC frequency and amplitude were not significantly different between WT and Mecp2-1 null LC neurons. H and I, cumulative analysis of IPSCs showed that DS2 shifted the interevent interval to the higher frequency range without altering the amplitude of IPSCs in WT LC neurons. **, p < 0.01; Student’s t test.

FIGURE 5. GABA\textsubscript{A}-R subunit expression in the LC region. A, single-cell PCR showed that the δ subunit, the essential subunit for extrasynaptic GABA\textsubscript{A}-Rs, was found in most LC neurons with negative GFAP and positive GAPDH expression in both groups of mice. B, RT-PCR showed expression of δ and α5 subunits. C, qPCR analysis with 2$^{\Delta\Delta\text{Ct}}$ indicated high relative quantity (RQ) of α6, β1, β2, and δ subunits in WT mice. D, in comparison with WT levels in the 2$^{-\Delta\Delta\text{Ct}}$ measure, the δ subunit level was significantly increased, and α5 subunit level was reduced in Mecp2-null mice. Transcript level of the α6 subunit was also significantly higher in Mecp2-null mice. Note that the seeming increase in β3 expression was insignificant as the expression ratio was based on barely detectable WT β3 in C (means ± SE; *, p < 0.05; **, p < 0.01; ***, p < 0.001). E and F, Western analysis showed a significant increase of δ subunit protein expression in Mecp2-null mice.
the input resistance from 480.5 ± 7.1 mΩ to 458.7 ± 7.6 mΩ. Meanwhile, THIP hyperpolarized the cells by 1.1 ± 0.4 mV and decreased the firing rate by 17.3 ± 3.3% (n = 14, Fig. 6A and C–E). In Mecp2-null mice, the same THIP treatment reduced the input resistance from 500.5 ± 8.7 mΩ to 424.9 ± 17.7 mΩ, hyperpolarized the cells by 3.0 ± 0.5 mV, and lowered the firing rate from 5.1 ± 1.3 Hz to 3.6 ± 1.6 Hz (n = 9; p < 0.05, p < 0.05 and p < 0.05, respectively, Student’s t test; Fig. 6, B–E), which is approximate to the baseline level in WT LC neurons (2.9 ± 0.2 Hz in WT baseline, n = 14, Fig. 6E). All of these percentile changes were significantly greater than in the WT neurons (p < 0.01, p < 0.01, and p < 0.05, respectively; Student’s t test; Fig. 6, F–H).

In either WT or Mecp2-null mice, 1 μM THIP did not affect the superthreshold and repetitive firing properties, including action potential morphology, after hyperpolarization (Fig. 7), spike frequency adaptation (Fig. 8), and delayed excitation (Fig. 9), when synaptic transmission was deliberately blocked. Post-inhibitory rebound and bursting activity were not found in LC neurons before and after THIP treatment in WT mice or Mecp2-null mice. Taken together, these results indicate that activation of δ subunit-containing GABAA Rs leads to an inhibition of LC neurons, an effect that is greater in Mecp2-null mice than in the WT, which brings the neuronal firing from hyperexcitable status to the level of WT neurons.

**The Extrasynaptic GABAA Rs Agonist Improved Breathing Abnormalities**—Previous studies have indicated that LC neurons are sensitive to high CO2 and low pH and that they play an important role in regulating breathing (9). In Mecp2-null mice, several groups of neurons, including LC neurons, are hyperexcitable (4, 20, 23, 33), which appears to contribute to the breathing disorders in Mecp2-null mice. To test whether activation of the extrasynaptic GABAA Rs can alleviate the breathing abnormalities, we studied breathing activity using plethysmography in conscious Mecp2-null mice. The mice were divided into two groups, with one receiving THIP injections (10 mg/kg intraperitoneally) and the other receiving a saline injection. At 3 weeks of age, Mecp2-null mice started to develop breathing disorders with obvious breathing frequency variation and frequent apneas. Therefore, we monitored the breathing activity...
Therefore, the results suggest that the extrasynaptic GABAARs reduced breathing disorders to 46% and C, increase (p < 0.05 and p < 0.05, respectively; Student’s t test). Figure 7. THIP does not affect the morphology of action potential (AP) and afterhyperpolarization (AHP) in either WT or Mecp2-null neurons. A, spontaneous APs recorded from an LC neuron. No obvious changes in AP morphology were found after exposure to THIP. B–E, in the presence of ionotropic receptor blockers (AP5, 6-cyano-7-nitroquinoxaline-2,3-dione, and strychnine) in the bath solution, THIP did not change the AP threshold (the potential at the AP initiation point), AP amplitude (the amplitude from threshold to peak), rise time, and half-width (D1⁄2, measured at 50% amplitude) of APs in either WT or Mecp2-null neurons (n = 7 and 12; p > 0.05 and p > 0.05, respectively; Student’s t test). F and G, in the presence of ionotropic receptor blockers, AHP was also not affected by THIP in WT and Mecp2-null neurons. AHP amplitude was measured from the AP threshold to the lowest hyperpolarization point, and the time constant of AHP was described with a single exponential in the period from 10% to 90% of the AHP amplitude (n = 7 and 12; p > 0.05 and p > 0.05, respectively; Student’s t test).

of Mecp2-null mice with THIP/saline injection once a day for 7 consecutive days starting at 26 days of age. After the 7-day treatment, Mecp2-null mice with the saline injection continued to develop severe breathing disorders with 89 ± 16 apneas/hour, an ∼50% increase compared with day 0 (n = 5, Fig. 10, A and C), and 0.29 ± 0.03 breathing frequency variation, an ∼20% increase (n = 5; Fig. 10, A and D). THIP treatment markedly reduced breathing disorders to 46 ± 6 apneas/hour, an 18% reduction compared with day 0 (n = 5; Fig. 10, B and C), and 0.17 ± 0.04 breathing frequency variation, a 32% reduction (n = 5; Fig. 10, B and D). Both morphological parameters improved significantly in the THIP group over their saline counterparts (Fig. 10, C and D; two-way ANOVA and Tukey’s post hoc test). Therefore, the results suggest that the extrasynaptic GABAAR agonist THIP alleviates the breathing disorders of Mecp2-null mice.

Discussion

To our knowledge, this is the first demonstration of extrasynaptic GABAAR currents in the mouse model of RTT. Our results have shown that tonic GABAAR currents in LC neurons are significantly larger in Mecp2-null neurons than in the WT, likely because of the overexpression of the GABAAR species containing δ and 6β subunits. Furthermore, activation of the extrasynaptic GABAARs appears to reduce neuronal excitability and alleviate breathing abnormalities of Mecp2-null mice.

Decreased Activity in Synaptic GABAARs in Mecp2-null Mice—Previous studies have shown that the GABAAR system decreases activity in Mecp2-null mice and people with RTT. In mice, this is associated with multiple RTT-like phenotypes, including progressive motor dysfunction and abnormal breathing (15). In GABAergic neurons, impaired GABA synthesis and the consequent reduction in GABA quantum release have been found (10, 15, 25). In postsynaptic cells, there is a marked reduction in GABAAR density in the brain of RTT patients and Mecp2-null mice (34, 35). An epigenetic study in a mouse model of RTT indicates that GABAARβ3 subunit expression is reduced in the cerebellum, and another molecular study confirmed down-regulation of the β3 subunit in the cerebellum (12, 36). The α1 subunit in the frontal cortex and the α2 and α4 subunits in the ventrolateral medulla were also reduced in Mecp2-null mice (25). In LC neurons, our previous studies have shown that the postsynaptic GABAARs and GABAβ currents are both defective in Mecp2-null mice (10). Consistent with these findings, the therapeutic GABAAR activators diazepines and the reuptake blocker NO-711 improve RTT symptoms in animal models, including breathing (20, 21). However, it is still unclear how the extrasynaptic GABAARs are affected in Mecp2-null mice, which makes this study remarkable.

Presence of Extrasynaptic GABAARs in LC Neurons—Extrasynaptic GABAARs were first described in cerebellar cortical gray matter (37) and found later in many other brain areas, such as the cerebral cortex, dentate gyrus granule, thalamus, and neocortex (38–43). Extrasynaptic GABAARs are sensitive to low levels of ambient GABA with little desensitization. They are
likely key targets for neurosteroids and alcohol (44–47) and useful targets for the treatment of some neuronal diseases, such as sleep disorders, epilepsy, stroke, and Parkinson disease (48, 49). The \( \gamma_2 \) subunit is the primary component of extrasynaptic GABA receptors, found exclusively in extrasynaptic locations mediating tonic inhibition (17, 19). Some other GABA receptor subtypes may be also involved in extrasynaptic GABA receptors, such as \( \delta \)-subunit containing receptors (50–52). The results from this study suggest that extrasynaptic GABA receptors are also present in LC neurons. Activation of these receptors results in tonic hyperpolarizing currents that are sensitive to GABA and the GABA receptor blockers bicuculline and picrotoxin. Consistent with these electrophysiological studies, our molecular biological evidence indicates that the \( \gamma_2 \) subunit is expressed in LC neurons.

Accompanying the \( \gamma_2 \) subunit are two \( \alpha \) and three \( \beta \) subunit species forming pentameric extrasynaptic GABA receptor. Our qPCR results suggest that \( \alpha_6 \)-containing extrasynaptic GABA receptor seem to play a major role in MeCP2-null LC neurons, similar to mature cerebellar granule cells (53). The \( \beta_1–3 \) subunits are necessary components in both extrasynaptic and synaptic GABA receptor. Previous studies have reported an ~30% reduction in post-synaptic GABAergic IPSCs in MeCP2-null LC neurons. The reduced \( \alpha_4 \) level found in this study is therefore consistent with the deficiency in synaptic GABA receptor. Regarding the increase in \( \alpha_6 \) transcript level, it is possible that deletion of MeCP2 leads to reorganization of the GABA receptor species in LC neurons by increasing \( \alpha_6 \) subunit expression and reducing the expression of other \( \alpha \) subunits. A similar reorganization has been found in nicotinic ACh receptors in MeCP2-null LC neurons (54). The increased \( \alpha_6 \) subunits may assemble the extrasynaptic receptors together with the \( \delta \) subunit as well as synaptic GABA receptor subunits. Because all \( \beta \) subunits contribute to synaptic GABA receptor and because synaptic GABA receptor is lowered with MeCP2 knockout, it is possible that their overall reduction masks the potential up-regulation of some subunits in the extrasynaptic location. Another possibility is that the extrasynaptic GABA receptor in MeCP2-null mice might be composed of more than two \( \alpha \) subunits, which may explain the reduced expression of \( \beta \) subunits and the unproportionally increased expression of \( \alpha_6 \) subunits, although the two \( \alpha \), two \( \beta \), and one \( \delta \) stoichiometry of GABA receptor has been shown previously in an exogenous expression system (55). Despite the uncertainty of \( \beta \) subunits, it is likely that the overly expressed GABA receptor species in MeCP2-null mice appears to contain \( \delta \) and \( \alpha_6 \) subunits.

FIGURE 9. THIP does not affect delayed excitation (DE) in both WT and MeCP2-null neurons. A, DE was measured as the time delay between the starting point of the depolarization pulse and initiation of the first action potential after a prior hyperpolarization. B, DE was described as the function of the conditioning hyperpolarization, which was fit with a Boltzmann equation as \( D = D_{\text{max}} / (1 + \exp(V - V \text{/}_1) / k) \), where \( D_{\text{max}} \) is the maximum DE period, \( V \) is the hyperpolarizing membrane potential, \( V \text{/}_1 \) is the half-inactivation, and \( k \) is the Boltzmann constant or slope factor. C–E, neither WT nor MeCP2-null neurons showed a significant difference on \( V \text{/}_1 \), slope factor, or DE period before and after THIP treatment (\( n = 8 \) and 7; \( p > 0.05 \) and \( p > 0.05 \), respectively; Student’s \( t \) test).
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The presence of tonic GABA<sub>A</sub> currents in LC neurons prompted us to study extrasynaptic GABA<sub>A</sub>Rs in Mecp2-null mice. We found that bicuculline-sensitive GABA<sub>A</sub>ergic tonic currents not only existed in Mecp2-null mice but were also enhanced markedly because extrasynaptic GABA<sub>A</sub>R agonists also elicited significantly larger tonic GABA<sub>A</sub> currents in Mecp2-null neurons. How the large tonic GABA<sub>A</sub> currents are produced in Mecp2-null neurons is unclear, but it may result from a relief of direct transcription repression by MeCP2 or its indirect effects on transcriptional regulators and second messenger systems. The highly excitable state of some of these neurons seems to contribute to cognitive defects, motor abnormality, and breathing disturbances (9, 20, 56, 57). Clearly, such an overexcitation in central neurons can be alleviated by GABAergic inhibition, in which excessive extrasynaptic GABA<sub>A</sub>Rs are beneficial. Interestingly, this seemingly compensatory up-regulation of GABAergic inhibition has been reported in the synaptic GABA<sub>A</sub> system (24, 59, 60). In neocortical layer 5 neurons of Mecp2-null mice, an increase of spontaneous IPSCs has been recorded that seems to result from the deficit in GABA release from presynaptic terminals (24). Therefore, the large tonic GABA<sub>A</sub>ergic currents in LC neurons of Mecp2-null mice found in this study might be a compensatory response to the deficient GABA synaptic inhibition.

Although the expression level suggests that the large tonic GABA<sub>A</sub> currents in Mecp2-null mice are likely to be due to the overexpression of δ subunit-containing receptors, we cannot rule out the possibilities that other expression patterns of GABA<sub>A</sub>Rs or a change in GABA affinity could also contribute to the enhanced tonic GABA inhibition in Mecp2-null mice. Nevertheless, RTT patients and Mecp2-null mice with insufficient GABAergic inhibition may benefit from these overexpressed extrasynaptic GABA<sub>A</sub>Rs because they may provide alternative targets for pharmaceutical interventions in addition to synaptic GABA<sub>A</sub>Rs.

Modulation of Neuronal Activity and Breathing—Experimental evidence suggests that LC neurons play an important role in brain stem CO<sub>2</sub> chemosensitivity and breathing regulation (9, 33, 61). Several groups of respiratory neurons and motoneurons are modulated by NE because NE augments cellular excitability via α adrenoceptors (62, 63). This NEergic modulation relies on firing activity and NE biosynthesis in LC neurons. It is possible that there is a homeostatic state between LC neuronal excitability and NE biosynthesis, allowing a stable release of NE at synapses. Although high LC neuronal excitability may lead to more NE release, persistent hyperexcitability may have adverse effects. Hyperexcitability often leads to Ca<sup>2+</sup> overload, whereas a persistent elevation of cytosolic Ca<sup>2+</sup> can activate a variety of degradative enzymes, including proteases, lipases, and endonucleases (64, 65). These may trigger a cascade of events leading to abnormal cellular activity and metabolic dysfunction, which may, paradoxically, compromise NE biosynthesis.

Mecp2 disruption in mice also causes hyperexcitability in LC neurons and impaired metabolic activity, which is attributable to the defects in neuronal intrinsic membrane properties and insufficient GABA inputs (4, 9, 33). Consistent with these findings, treatment with GABA or diazepam rebalances the hyperexcitability of respiratory neurons and improves the breathing activity in Mecp2-null mice (20, 21). In this study, we found that administration of THIP activates extrasynaptic GABA<sub>A</sub>Rs and reduces the excitability of LC neurons as well. Like diazepam, THIP treatment alleviates the breathing abnormalities of Mecp2-null mice. Therefore, excitability stabilization appears to be crucial for the reinstallation of brain stem autonomic function. To avoid potential effects of THIP on arousal states, we monitored animal activity with a video camera during plethysmograph recordings and confirmed that they were not in behavioral sleep.

In Mecp2-null mice, overexcitation of LC neurons may contribute to their metabolic dysfunction by disturbing the homeostasis of NE synthesis, NE production, and NE release by presynaptic terminals. A previous study has reported that, in COS-7 cells, chronic overexcitation impaired homeostatic synaptic plasticity by decreasing AMPA receptor expression (66). Indeed, decreased expression of tyrosine hydroxylase and dopamine β hydroxylase is known to occur in LC neurons of Mecp2-null mice, leading to insufficient NE biosynthesis (5, 67). THIP application seems to correct LC neuronal hyperex-
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citability by activation of extrasynaptic GABA<sub>A</sub>Rs, as shown in this study, which we believe may stabilize neuronal activity and metabolism, rebalancing the homeostatic state and improving NE biosynthesis.

In conclusion, bicuculline-sensitive tonic currents were recorded from LC neurons, which were increased dose-dependently with increased GABA concentrations. In comparison with WT mice, these GABA<sub>A</sub>ergic tonic currents were increased significantly in MeCP2-null mice. Agonists specific to extrasynaptic GABA<sub>A</sub>Rs triggered larger tonic GABA<sub>A</sub> currents in MeCP2-null LC neurons. Consistently, the δ subunit, the principal component of extrasynaptic GABA<sub>A</sub>Rs, was expressed in LC neurons, whose expression level, together with α6 expression in the LC area, became higher in MeCP2-null mice than in the WT, which may contribute to the enhanced tonic GABA<sub>A</sub> currents. The presence of extrasynaptic GABA<sub>A</sub>Rs in MeCP2-null mice seems to control of neuronal excitability and breathing abnormalities with GABA<sub>A</sub>R activators.

Note Added in Proof—Fig. 5 in the Papers in Press version of this article that was published on May 15, 2015 has been revised. Specifically, panels C and G have been merged into panel C, and original panels D and H have been merged into panel D to clarify the presentation. These changes do not affect the interpretation of the results or conclusions of this work.

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