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The antitussive cloperastine improves breathing abnormalities in a Rett Syndrome mouse model by blocking presynaptic GIRK channels and enhancing GABA release

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Abstract

Rett Syndrome (RTT) is an X-linked neurodevelopmental disorder caused mainly by mutations in the *MECP2* gene. One of the major RTT features is breathing dysfunction characterized by periodic hypo- and hyperventilation. The breathing disorders are associated with increased brainstem neuronal excitability, which can be alleviated with antagonistic agents. Since neuronal hypoexcitability occurs in the forebrain of RTT models, it is necessary to find pharmacological agents with a relative preference to brainstem neurons. Here we show evidence for the improvement of breathing disorders of *Mecp2*-null mice with the brainstem-acting drug cloperastine (CPS) and its likely neuronal targets. CPS is an over-the-counter cough medicine that has an inhibitory effect on brainstem neuronal networks. In *Mecp2*-null mice, CPS (30 mg/kg, i.p.) decreased the occurrence of apneas/h and breath frequency variation. GIRK currents expressed in HEK cells were inhibited by CPS with IC₅₀ 1 μ M. Whole-cell patch clamp recordings in locus coeruleus (LC) and dorsal tegmental nucleus (DTN) neurons revealed an overall inhibitory effect of CPS (10 μ M) on neuronal firing activity. Such an effect was reversed by the GABA_A receptor antagonist bicuculline (20 μ M). Voltage clamp studies showed that CPS increased GABAergic sIPSCs in LC cells, which was blocked by the GABA_B receptor antagonist phaclofen. Functional GABAergic connections of DTN neurons with LC cells were shown. These results suggest that CPS improves breathing dysfunction in *Mecp2*-null mice by blocking GIRK channels in synaptic terminals and enhancing GABA release.

Keywords

Mecp2; GIRK channels; antitussive; electrophysiology; respiratory dysfunction; locus coeruleus

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Christopher M. Johnson: Investigation, Conceptualization, Formal analysis, Methodology, Writing - original draft. **Ningren Cui:** Investigation, Methodology. **Hao Xing:** Investigation, Methodology. **Yang Wu:** Investigation, Methodology. **Chun Jiang:** Supervision, Conceptualization, Writing - review & editing

Conflicts of interest

The authors declare no conflicts of interest.

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1. Introduction

Rett Syndrome (RTT) is an X-linked neurodevelopmental disorder seen in 1 in 10,000 females. Most cases of RTT are caused by mutations in the *MECP2* gene encoding Methyl-CpG-binding protein 2. People with RTT show periodic hypoventilation attacks that contribute to the high incidence of sudden death. Although the RTT-characteristic hypoventilation is known to originate from the central nervous system (CNS), the underlying cellular mechanisms are still unclear (Julu et al., 2001). Several potential defects have been suggested, including impaired higher brain control, abnormal CO₂ chemoreception, and inadequate termination of inspiratory phase (Howell et al., 2017; Stettner et al., 2007; Weese-Mayer et al., 2008; Wu et al., 2019; Zhang et al., 2011). A common event of these defects is the disruption in neuronal communication, which is likely to play a major role in RTT-type breathing disorders. Indeed, several neurotransmitter systems have been reported to be impaired in people with the disease as well as in animal models, including norepinephrine (NE), serotonin (5-HT), GABA and glutamate (Calfa et al., 2015; Chao et al., 2010; Ide et al., 2005; Mokler et al., 1998; Samaco et al., 2009; Viemari et al., 2005). Consistent with these findings, manipulations of these neurotransmitters have been shown to be beneficial for the symptom (Patrizi et al., 2016).

An important consequence of the defects in these neurotransmitter systems is the shift in the excitation and inhibition balance that may play a role in the manifestation and development of RTT symptoms (Banerjee et al., 2016; Dani et al., 2005). Neuronal hyperexcitability is caused by both dysfunctions in neuronal networks (Banerjee et al., 2016; Calfa et al., 2015; El-Khoury et al., 2014; Kline et al., 2010; Zhang et al., 2008) and intrinsic properties of neurons (Ma et al., 2014; Taneja et al., 2009; Zhang et al., 2010a). Although hypoexcitability is seen in some regions of the brain (Dani et al., 2005; Dani and Nelson, 2009) increased neuronal hyperexcitability, especially in the brainstem, is considered to be a significant contributor to the disease (Calfa et al., 2011; Taneja et al., 2009). In fact, drugs reducing neuronal hyperexcitability have been proven effective in treating RTT symptoms (Abdala et al., 2010; Abdala et al., 2016; El-Khoury et al., 2014; Voituron and Hilaire, 2011; Zhong et al., 2015).

Breathing activity is controlled by the brainstem that generates rhythmic breathing and regulates it by integration of multiple sensory inputs from the lung and airways. Such a specialized brainstem function has been a major focus of pharmacological approaches for a number of respiration-related diseases. Several therapeutic agents are currently available that act on the brainstem neuronal networks with relative specificity. Some of them may be useful in the studies of cellular mechanisms for the RTT-type breathing, and helpful for treatment of the RTT hypoventilation.

Cloperastine (CPS) is a central-acting antitussive working on brainstem neuronal networks (Gregori-Puigjane et al., 2012). The drug has several characteristics. 1) It affects the brainstem integration of multiple sensory inputs via multiple sites including K⁺ channels, histamine and sigma receptors. 2) Its overall effect is inhibitory, suppressing cough and reactive airway signals. 3) With a large safety margin, it has been approved as an over-the-counter medicine in several Asian and European countries. 4) As a non-opioid antitussive,

CPS has a potency nearly twice as high as codeine (Soeda et al., 2016; Takahama et al., 2009). These suggest that CPS may be of potential benefit to the understanding and treatment of RTT-type breathing disorders. To test this hypothesis, we performed these studies addressing whether CPS has effects on respiratory abnormalities in *Mecp2* mutant mice, and if so, what are the underlying cellular mechanisms.

2. Material and methods

2.1. Animals

Female heterozygous *Mecp2*^{R168X} mice (B6J;129S6.MeCP2^{R168X}, stock no. 024990; Bar Harbor, ME) from Jackson Lab were crossbred with wild-type (WT) male mice to produce *Mecp2*^{R168X/Y} mice. Offspring were genotyped using the PCR protocol from Jackson Laboratory. GAD-ChR mice were generated by cross-breeding the strain of GAD2-Cre mice (*Gad2*^{tm2(cre)Zjh}/J, Jackson Laboratory SN 010802) with the ChR2-eYFP-LoxP strain (*B6;129S-Gt(ROSA)26Sor*^{tm32(CAG-COP4*H134R/EYFP)Hze}/J, Jackson Laboratory SN 12569). The offspring genotypes were identified with a PCR protocol provided by Jackson Laboratory. All experimental procedures in the animals 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.

2.2. Plethysmography

As *Mecp2*^{-Y} mice became available, breathing activity was recorded from unrestrained conscious mice of 30–90 days of age without anesthesia in an ~50 ml plethysmograph chamber attached to a force electricity transducer (Validyne Engineering, Northridge, CA). The mice were given normal room air at a flow rate of 20 ml/min and allowed to acclimate to the chamber for 15 min before recording. Breathing activity of the mice was recorded for 20 min, while the mouse was monitored using a video camera. Mice were randomly injected with a single dose of CPS (30 mg/kg, i.p.) or saline 30 min before experiments. Experimenters were not blinded to treatment. Frequency variation was calculated by the standard deviation divided by the mean frequency. At least 200 breaths were measured which were composed of three or four randomly sampled stretches of at least 50 consecutive breaths. Apneas were counted manually during the entire duration of the 20 min recording. A breath was considered an apnea if the breath lasted more than twice the duration of the previous breath. Periods of movement and potential sleep were recognized with video records and excluded from analysis. Data were stored and analyzed offline using Clampfit 10.3 software (Molecular Devices, Sunnyvale, CA).

2.3. Channel expression

Rat GIRK1 (Kir3.1) cDNA (GenBank accession no. [U01071](#)), rat GIRK4 (Kir3.4) cDNA (GenBank accession no. [X83584](#)), rat GIRK2 (Kir3.2) cDNA, and GFP cDNA constructs were expressed in human embryonic kidney (HEK) cells as detailed in our previous reports (Jin et al., 2012; Mao et al., 2004). The HEK cells were cultured as a monolayer Dulbecco's modified Eagle's medium (DMEM)–F12 with 10% fetal bovine serum and penicillin–streptomycin added at 37°C with 5% CO₂. The HEK cells were split twice weekly and

transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) with 2 μ g GIRK channel cDNA. 0.5 μ g green fluorescent protein (GFP) cDNA (pEGFP-N2, Clontech, Palo Alto, CA, USA) was added to the cDNA mixture to identify transfected cells. Cells were disassociated from the monolayer using 0.25% trypsin ~24 h post-transfection. The re-suspended cells were added on to 5 $\times$ 5 mm cover slips in a 35 mm Petri dish. The cells were then incubated at 37°C for an additional 24–48 h before experiments.

2.4. HEK Cell Recordings

Single-electrode whole-cell voltage clamp was performed on HEK cells at room temperature. Patch pipettes were pulled with a Sutter pipette puller (model P-97, Novato) and had an open tip resistance of 3–6 M Ω . Seals >1 G Ω were obtained from GFP expressing cells before breaking into the whole-cell mode. Membrane currents were recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Recordings were lowpass filtered (2 kHz, Bessel, 4-pole filter, –3 dB), digitized (10 kHz, 16-bit resolution), and stored on a computer for later data analysis using pCLAMP 10 software (Molecular Devices). Bath solution contained (in mM): KCl 20, NaCl 140, CaCl₂ 0.5, EGTA 5, MgCl₂ 5.46, HEPES 10. Pipette solution contained (in mM): KCl 130, NaCl 20, CaCl₂ 0.5, MgCl₂ 2, K₂ATP 2.56, Li₂GTP 0.3, HEPES 10.

2.5. Brain Slice

Brain slices were prepared as described previously (Zhang et al., 2010a). In brief, mice were anesthetized with isoflurane and decapitated. The brainstem was obtained rapidly and placed in an ice-cold, sucrose-rich artificial cerebrospinal fluid (sucrose aCSF) containing (in mM) 220 sucrose, 1.9 KCl, 0.5 CaCl₂, 6 MgCl₂, 33 NaHCO₃, 1.2 NaH₂PO₄ and 10 D-glucose. The solution was bubbled with 95% O₂ balanced with 5% CO₂ (pH 7.40). Coronal pontine sections (160–200 μ m) containing the LC area and DTN were obtained using a vibratome sectioning system (1000 Plus, Vibratome, St. Louis, MO). The slices recovered in normal aCSF (in mM) containing 124 NaCl, 3 KCl, 2 CaCl₂, 2 MgCl₂, 26 NaHCO₃, 1.3 NaH₂PO₄ and 10 D-glucose at 33°C for 30 min. The slices were then kept at room temperature before recording. During recordings, slices perfused with oxygenated aCSF at a rate of 2 ml/min and maintained at 32–35°C (Warner Instruments, TC-344B) in a recording chamber.

2.6. Electrophysiology

Whole cell current-clamp and voltage clamp studies were performed on cells visualized using a near-infrared charge-coupled device camera. Cells with resting membrane potentials (V_m) more negative than –40 mV and action potential (AP) amplitudes >65 mV were considered healthy and used for further analysis. Patch pipettes were pulled with a Sutter pipette puller (model P-97, Novato) with a resistance of 3–6 M Ω . The slice was perfused with normal aCSF and superfused with 95% O₂ and 5% CO₂ at 34°C. The pipette solution for current-clamp experiments contained (in mM) 130 potassium-gluconate, 10 KCl, 10 HEPES, 2 Mg-ATP, 0.3 Na-GTP, and 0.4 EGTA (pH 7.30). For voltage clamp experiments, the pipette solution contained 50 mM KCl, 85 mM CsCl, 2 mM MgCl₂, 2 mM magnesium-ATP, 1 mM sodium-GTP, 10 mM HEPES, and 0.5 mM EGTA (pH 7.30). Recorded signals were amplified with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA), digitized at 10 kHz, filtered at 1 kHz, and collected with the Clampex software. The

temperature was maintained at 33°C during recording by a dual automatic temperature control (Warner Instruments, Hamden, CT).

In voltage-clamp and current clamp studies synaptic blockade was achieved by addition of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, 10 μ M), the N-methyl-D-aspartate (NMDA) receptor antagonist DL-2-Amino-5-phosphonopentanoic acid (DL-APV, 10 μ M), the glycine receptor antagonist strychnine (1 μ M) and in some experiments the GABA_A receptor antagonist, bicuculline (20 μ M), was added to the external solution. Recorded signals were amplified with an Axopatch 700B amplifier (Molecular Devices, Union City, CA), digitized at 10 kHz, filtered at 2 kHz using the low-pass filter, and collected with the Clampex 10.3 data acquisition software (Molecular Devices). Additional drugs used include 20 μ M Phaclofen, and 300 μ M BaCl₂.

2.7. Field Potential Recordings

Field potentials were recorded with glass-coated tungsten microelectrodes with 20–30 μ m tip exposure. The signals were amplified (Electro 705 Electrometer, World Precision Instruments; differential AC amplifier model 1700, AM Systems), digitized (Digidata 1440A, Axon Instruments, Inc.), recorded (Clampex 10.3 software, Molecular Devices), and analyzed offline (Clampfit 10.3 software, Molecular Devices). The field potential latency was measured between the onset of light stimulus and the onset of response. According to our previous study in the same neurons (Jin et al., 2016), it takes approximately 9 ms for the photo current to start. Thus, this delay was subtracted from the measured latency. Data analysis was done with an average of at least 50 traces of signals synchronized to the onset of stimulating light pulses.

2.8. Optogenetics

GABAergic neurons were detected in brain slices with YFP expression using fluorescence microscopy in excitation at 480 nm and emission at 520 nm (green). Optostimulation was performed by using a xenon arc light source with a high-speed switcher (Lambda DG-4, Sutter Instruments, Novato, CA). The light source was connected to the incident-light illuminator port of the microscope, and delivered blue light through a 470 nm bandpass filter. The 2–100 ms pulse trains were triggered by a Grass Stimulator S88 Dual Output Square Pulse Stimulator (Natus Medical Inc, Can).

2.9. Data Analysis

The electrophysiological and plethysmograph data were analyzed with Clampfit 10.3 software (Molecular Devices) and voltage clamp (or IPSC) data were analyzed using Mini Analysis Program 6.0.7 software (Synaptosoft). Data are presented as means $\pm$ SE. Statistical analysis was performed using two-way repeated measures ANOVA for evoked firing frequency data, Two-Way Mixed-Model ANOVA for breathing data plotted against time, with Bonferroni post hoc, and two-tailed Student's *t*-test. Greenhouse-Geisser correction was applied when the assumption of sphericity was violated. Differences were considered significant when *P* < 0.05.

3. Results

3.1. CPS Inhibited GIRK1-GIRK2 Channels Expressed Heterologically in HEK-293 Cells

Previous reports suggest that CPS inhibits GIRK channels (Soeda et al., 2016; Soeda et al., 2014; Takahama et al., 2009). However, its potency still remains unknown. Thus, we overexpressed the heteromeric GIRK1-GIRK2, the common isoforms of GIRK channels in the CNS, in HEK cells followed by studying GIRK currents in whole cell voltage clamp as we did previously (Jin et al., 2012; Mao et al., 2004). In the transfected cells with positive GFP expression, inward rectifier currents were recorded with ramp command potentials from -100 to 100 mV (Fig 1A, B). These currents were strongly inhibited by CPS in a dose dependent manner with the concentration for 50% current inhibition (IC_{50}) of approximately $1 \mu\text{M}$ ($n=12$ cells from 3 batches) (Fig. 1A, C). Similar current inhibition was seen in HEK cells transfected with GIRK1-GIRK4 ($n=11$ cells from 4 batches) (Sup. Fig. 1).

3.2. CPS Decreased Apnea and Breathing Irregularity in *Mecp2*-Null Mice

The capability of CPS to suppress cough suggests that it works on brainstem neuronal networks that have been known to work abnormally in RTT. Thus, we tested the effectiveness of CPS in improving respiratory symptoms in *Mecp2*-null mice. Breathing activity was studied in *Mecp2*-null mice using plethysmography before and after a peritoneal injection of saline or 30 mg/kg CPS. The *Mecp2*-null mice exhibited breathing abnormalities characterized by frequent apneas and breathing frequency variation (f variation) (Fig. 2A). Both were suppressed after CPS injection. Quantitative analysis of apnea counts as apneas/h and f variation as standard deviation of breathing activity divided by arithmetic mean indicated that significant reductions of both occurred 30 minutes after CPS injection, and the effects lasted up to 4 hours (significant interaction between time and treatment on apneas/h, $F_{(3,60)} = 4.05$, $P = 0.01$ and significant interaction between time and treatment on f variation, $F_{(3,60)} = 7.69$, $P < 0.001$). These changes were not seen in the saline group (Saline, $n=9$; CPS, $n=13$) (Fig. 2B–F).

3.3. CPS Suppressed LC Excitability by Presynaptic Mechanisms

One group of cells that modulate central respiratory activity in the brainstem neuronal networks is the locus coeruleus (LC), which plays an important role in central CO_2 chemosensitivity and norepinephrinergic modulation (Elam et al., 1981; Oyamada et al., 1998). Hyperexcitability has been demonstrated in these neurons in *Mecp2*-null mice, which likely contributes to breathing abnormalities in the mice (Roux et al., 2007; Viemari et al., 2005; Zanella et al., 2008). Thus, we studied how CPS affects the LC neurons in the experiments.

LC neuronal activity was studied in whole-cell current clamp in brain slices. With step depolarizing current injections, firing activity was measured together with membrane potentials in the LC neurons. In the presence of CPS, the firing activity was significantly reduced without obvious changes in membrane potentials in 12 cells from WT mice (significant main effect of treatment, $F_{(1,11)} = 18.58$, $P = 0.001$ and group $\times$ current injection interaction: $F_{(3,06,33.69)} = 16.865$, $P < 0.001$; two-way repeated measures ANOVA) (Fig 3A–

C). This result suggests that CPS has effects on respiration-related brainstem neuronal networks.

Such an effect does not seem to be a result of inhibition of somata GIRK channels in the LC cells alone, because GIRK channel inhibition leads to depolarization and an increase in neuronal excitability, which suggests that the decreased firing activity with CPS may be mediated by presynaptic inhibitory neurons. To test this possibility, we recorded from the LC neurons in the presence of the GABA_A receptor blocker bicuculline. Under this condition, the inhibitory effect of CPS was abolished. Instead, CPS increased firing activity of LC neurons, which appears to be due to inhibition of postsynaptic GIRK channels (significant main effect of treatment, $F_{(1,5)} = 24.98$, $P < 0.01$ and treatment $\times$ current injection interaction: $F_{(1.71,8.55)} = 5.06$, $P < 0.05$; two-way repeated measures ANOVA) ($n=6$) (Fig. 3D). The effects of CPS on LC neuronal excitability is likely to be mediated by modulation of GIRK channels, as CPS failed to produce any significant changes in firing activity in the presence of 300 μ M BaCl₂ ($F_{(1,11)} = 0.149$) ($n=12$) (Sup. Fig. 2). These results suggest that CPS inhibits GIRK channels by both pre- and postsynaptic mechanisms, while the former appears more prominent.

3.4. CPS augmented GABA synaptic drive to LC Cells

The elimination of the inhibitory effect of CPS with bicuculline indicates that the presynaptic cells are GABAergic. Thus, we studied how CPS affected GABA_A IPSCs in LC neurons. In voltage clamp, CPS significantly increased GABAergic IPSC frequency with no significant effect on IPSC amplitude ($n=23$) (Fig. 4A–E). The IPSCs are GABA_A receptor-dependent as they were totally suppressed with bicuculline (Sup. Fig. 3A–D).

In addition to GABA_A receptors, GABA_B receptors also play a role in regulating excitability of postsynaptic cells through the feedback control of GABA release on presynaptic terminals. Using phaclofen, a selective GABA_B receptor antagonist, to block GABA_B receptor-dependent feedback control, we found that CPS no longer augmented GABAergic IPSCs ($n=10$) (Sup. Fig. 4A–D). Therefore, it is very likely that CPS enhanced GABA transmission by blocking GIRK channels in the synaptic terminals including those coupled to GABA_B receptors.

3.5. GABAergic neurons in the DTN Projected to LC

A large group of GABAergic neurons, the dorsal tegmental nucleus (DTN), is located in close vicinity of the LC (Allen and Hopkins, 1989; Wirtshafter and Stratford, 1993), which largely remained in our coronal brainstem slice preparations, and may function to inhibit LC neurons in the slices. To demonstrate such a network connection, we firstly generated a strain of transgenic mice with Channelrhodopsin (ChR) expression in GABAergic cells using GAD-Cre and ChR-flox systems. Secondly, DTN neuronal responses to optostimulation were studied in whole-cell voltage clamp. We found that light pulses evoked a large inward current with the reversal potential near 0 mV ($n=13$) (Fig. 5B), indicating that the current is produced by activation of ChR. When the DTN cells were patched randomly without verification of their GFP expression, we found that 93% of DTN cells showed the opto-current indicating that the nucleus is highly homogenous ($n=65$).

After confirming ChR expression in DTN cells, we recorded field potentials in the LC nucleus while stimulating the DTN nucleus. The blue light stimulation to the ipsilateral DTN evoked field potentials in the LC ($n=13$, Fig. 5 C₁). Latency between onset of light and field potentials for 2 ms pulses was 4.0 ms (Fig. 5C₂). The effect was dose-dependent as well, as longer pulses (5 ms) produced larger responses ($n=12$) (Fig. 5C₃). Because it takes at least 2 ms for neurons to initiate an activation potential with optostimulation (Boyden et al., 2005), these results indicate that DTN neurons have monosynaptic connections to LC neurons. The LC also responded to contralateral optostimulation with much smaller field potentials than ipsilateral stimulation (Sup Fig. 5A). The opto-responses in the LC were specific, which were not seen in the ventrolateral reticular formation in the same slices (Sup. Fig. 5B).

3.6. CPS enhanced presynaptic GABAergic inhibition to DTN neurons

The projection of DTN GABAergic neurons to the LC strongly suggests that these GABAergic neurons contribute to the synaptic modulation of LC neurons, which may be affected by CPS. To test this scenario, we tested how CPS affects the excitability of these GABAergic cells. In whole-cell current clamp with step depolarizing currents before and during CPS administration, we found that CPS decreased excitability of DTN cells ($F_{(1,9)} = 11.8$, $P < 0.01$; two-way repeated measures ANOVA) ($n=10$) (Fig. 6A–D).

Because CPS acts on both GIRK channels in the soma and synaptic terminals (see above), the mechanisms causing the DTN neuronal inhibition may be similar to those seen in LC neurons. If that is true, there should be previously unidentified recurrent inhibition in the DTN cells. We found that optostimulation also produced outward currents resembling IPSCs in some DTN cells (Fig. 7A–D). With a series of command potentials, these IPSCs reversed polarity in hyperpolarizing potentials, and a reversal potential occurred around -60 mV to -70 mV (Fig. 7A), which is consistent with Cl^- reversal potential and indicates that the DTN GABAergic neurons also receive GABAergic inhibition. Thus, in conjunction with our other results (Sup. Fig. 4), this data suggest that CPS may augment GABA release from presynaptic terminals in DTN cells enhancing GABAergic inhibition to the DTN cells themselves by inhibiting GIRK channels coupled to GABA_B receptors.

With the evidence that DTN cells receive GABAergic recurrent inhibition, we tested whether the inhibitory effect of CPS was caused by enhanced GABAergic transmission. Thus, we recorded the evoked firing activity of DTN cells before and during bath application of CPS in the presence of $20 \mu\text{M}$ bicuculline. Under this condition, CPS failed to decrease the excitability of DTN neurons ($F_{(1,9)} = 0.41$, $P > 0.05$; two-way repeated measures ANOVA) ($n=9$) (Fig. 8), indicating that the inhibitory effect relies on GABA_A synaptic input.

4. Discussion

This is the first demonstration of the use of a GIRK channel blocker to improve respiratory symptoms in an animal model of Rett Syndrome. We have found that a single dose of CPS decreases the occurrence of apnea and suppresses breathing frequency variation in RTT mice up to four hours. This effect seems to be mediated by blocking GIRK channels in synaptic

terminals and enhancing GABA release, as seen in multiple brain areas including the LC and the DTN.

4.1. CPS and GIRK

According to previous studies, CPS has GIRK channel antagonistic properties (Soeda et al., 2016; Soeda et al., 2014), which was based on two abstracts showing that CPS increases tissue 5-HT and dopamine levels in rats (Inoue et al., 2009; Inoue et al., 2008). In addition, another abstract reported that IC_{50} for GIRK1–2 was 5–25 μ M (Kinoshita et al., 2008). These concentrations are higher than that (IC_{50} 1 μ M) showed in this study. CPS was first discovered (Takagi et al., 1961) as a derivative of the antihistamine diphenhydramine, and like diphenhydramine, CPS has an antitussive effect. Subsequent studies confirmed CPS binding to histamine receptors (Gregori-Puigjane et al., 2012) as well as sigma receptors, (Gregori-Puigjane et al., 2012), inhibition of hERG channels (Takahara et al., 2012), and suppression of sodium-dependent glucose transporters (Oranje et al., 2019). Our current studies support that CPS also acts as a potent inhibitor of GIRK channels.

4.2. GIRK Channels and Breathing

Previous studies indicate that GIRK channels are involved in breathing regulation in WT animals (Levitt et al., 2015; Montandon et al., 2016). Activation of opioid receptors and GIRK channels in the pre-Bötzinger complex (PBC) as well as the Kölliker-Fuse (KF) depress respiration in mice (Levitt et al., 2015; Montandon et al., 2016). However, in GIRK2 knockout mice, the opioid receptor and GIRK channel agonists have only a modest or no effect on respiratory rate (Montandon et al., 2016). Interestingly, administration of fentanyl to WT mice induced apneas similar to the natural occurrence of apneas in *Mecp2*-null mice, which is less severe in GIRK2 knockout mice (Montandon et al., 2016). These results demonstrating respiratory depression by direct and indirect activation of GIRK channels suggest that GIRK channels contribute to the regulation of respiratory-related neuronal activity and the maintenance of respiratory rhythms.

4.3. Treatment of Breathing Abnormalities

Periodic hypoventilation with apneas and breathing irregularity is a hallmark of Rett Syndrome, which is attributable to ~26% of sudden death cases in these patients (Kerr et al., 1997). This dysfunction is likely to involve multiple neurotransmitter systems including monoamine systems. For example, enhancing the amount of NE available in the CNS of RTT mice improves respiratory and other symptoms (Roux et al., 2007; Zanella et al., 2008; Zhang et al., 2011). Restoring the excitation and inhibition balance by targeting the glutamate and GABA systems improves RTT symptoms as well. The NMDA receptor antagonist and antitussive dextromethorphan has been tested for its effectiveness on RTT symptoms in humans. The clinical trial in RTT patients over a six month period showed an overall improvement in condition with a decrease in seizure events, increase in cognitive function, and improvement in behavioral functioning (Smith-Hicks et al., 2017). CPS also has the antitussive effect, although its targets are different from dextromethorphan's. Our results indicate that GIRK channel inhibition with CPS improves respiratory symptoms in *Mecp2* mutant mice. This finding suggests that agents such as CPS acting on brainstem

GIRK channels appear useful as therapeutic drugs for treatment of RTT-type breathing disorders (Catania and Cuzzocrea, 2011).

An early study in human RTT patients has shown that the opioid receptor antagonist Naltrexone improves respiratory disturbances and reduces the occurrence of seizures, though motor impairment progresses faster (Percy et al., 1994). Because opioid receptors are coupled to GIRK channels, this previous study supports the use of CPS for RTT. While Naltrexone's effects are specific to opioid receptors, CPS's effect seems theoretically more widespread, targeting GIRK channels that are coupled to other neurotransmitter systems as well. For instance, studies have shown that agonists of the GPCR 5-HT_{1a} improve respiratory symptoms in human RTT patients and RTT mice (Abdala et al., 2014; Levitt et al., 2013; Ohno et al., 2016). In our study, we used a concentration of CPS that is consistent with *in vivo* use in other studies (Soeda et al., 2016; Soeda et al., 2014), but perhaps, different concentrations of CPS and administration over a longer period of time may further improve RTT symptoms. Before moving to clinical trials, the effect of the drug on other behaviors should be tested also and it should be tested in female models.

4.4. Locus Coeruleus, GIRK and GABA

We found that CPS decreases LC excitability, which apparently cannot be a result of inhibition of somata GIRK channels in these cells alone, because such a GIRK channel inhibition would lead to depolarization increasing excitability of LC neurons. Thus, it is possible that the inhibitory effect of CPS is mediated by presynaptic mechanisms of enhanced GABA inhibition, or pre-presynaptic mechanisms of glutamatergic disfacilitation. Supporting the idea of enhanced GABAergic presynaptic inhibition are our results showing that CPS augments GABAergic IPSCs, and the inhibitory effect of CPS is completely eliminated in the presence of a GABA_A receptor antagonist. Instead, the inhibitory effect of CPS is reversed to excitatory. The LC cells are known to have GIRK channel expression (Kawano et al., 2004). Inhibition of the somata GIRK channels seems to play a role in the excitatory effect of CPS after presynaptic GABA input is blocked.

Although we have suggestive evidence for the mechanisms of CPS's effects, they are still unclear. One possible explanation for CPS's preferential targeting of presynaptic GIRK channels is that CPS could have an affinity for specific isoforms of GIRK channels, which are primarily expressed in synaptic terminals of GABAergic cells. In pyramidal neurons the GIRK2 splice variants GIRK2a and GIRK2c overlap in their subcellular expression locations, but GIRK2a is mostly located on the soma and proximal dendrites while GIRK2c is reportedly located uniformly throughout the entire neuron (Marron Fernandez de Velasco et al., 2017). The GABAergic cells projecting to the LC including the DTN may also differentially express GIRK channel isoforms similar to pyramidal cells with different binding affinities to CPS.

Another potential mechanism is CPS's interactions with other identified target proteins such as histamine receptors and sigma receptors (Gregori-Puigjane et al., 2012). CPS as a histamine 1 receptor agonist could enhance GABA release and CPS as a histamine 3 receptor antagonist could also cause excitation. In addition, sigma-1 receptor activation downregulates voltage-gated Ca²⁺ channels, which play an important role in

neurotransmission (Zhang et al., 2017). Thus, if CPS antagonizes sigma-1 receptors, this could lead to increased Ca^{2+} on the pertaining cells and enhanced neurotransmission, especially GABA. Future studies could test the necessity of GIRK channels for CPS's effects as well as the involvement of other potential proteins to fully understand its mechanisms.

The enhancement of GABA release with CPS treatment is consistent with the improvement of symptoms in *Mecp2* mutant mice as demonstrated in several previous studies (Abdala et al., 2010; Abdala et al., 2016; El-Khoury et al., 2014; Voituren and Hilaire, 2011; Zhong et al., 2015; Zhong et al., 2016). Dysfunction of GABAergic networks, especially within the brainstem contribute to the development of breathing irregularities in *Mecp2*-KO mice. Whole-brain disruption of the GABA system causes abnormal respiration in RTT, but selective deletion of *Mecp2* only in GABAergic cells of the hindbrain and not the forebrain recapitulates the breathing abnormalities seen in whole-body knockouts (Chao et al., 2010). Given that reduced GABAergic modulation of LC neurons contributes to the altered properties of these cells (Jin et al., 2013) and their known role in regulating breathing, it is important to study the local circuitry of these neurons. Reducing hyperexcitability of LC cells in RTT mice may help reverse dysfunctional NE synthesis (Samaco et al., 2009; Zhang et al., 2010b) since increased LC firing activity does not result in greater NEergic neuromodulation (Zhang et al., 2016).

4.5. GABAergic Projections to LC

Our results have shown that the DTN, a highly homogenous group of GABAergic cells also known as the dorsal tegmental nucleus of Gudden (DTg), has direct axonal projection to the LC, which has not been reported previously. This adds to the list of a number of known GABAergic afferents to the LC including the ventrolateral preoptic area, the periaqueductal grey, the central amygdala nucleus of the forebrain, the prepositus hypoglossi, the rostral ventrolateral medulla, and recently demonstrated local GABAergic neurons in the dmLC nucleus (Aston-Jones et al., 2004; Breton-Provencher and Sur, 2019; Dimitrov et al., 2013; Iijima et al., 1987; Jin et al., 2016). The information of the local circuitry of the GABAergic neurons in the brainstem improves our understanding of the LC and its functional impairment in Rett Syndrome.

GABAergic cells in the DTN project primarily to lateral mammillary neurons (Allen and Hopkins, 1989; Morest, 1961; Saunders et al., 2012; Wirtshafter and Stratford, 1993). DTN neurons play a role in several behaviors including directional navigation and spatial orientation (Clark et al., 2013). Subpopulations of the DTN neurons respond differently to changes in angular head velocity and head direction (Bassett and Taube, 2001; Bassett et al., 2007; Sharp et al., 2001). With its proximity to the LC, the DTN seems to be positioned as a significant contributor of GABA input to LC cells as well. The DTN neurons also play a role in sleep and wakefulness (Chazalon et al., 2018; Torterolo et al., 2002), functions that rely primarily on the LC neurons. In the present study, we find evidence for functional DTN projections to the LC. Why such a projection has not been found in previous studies is unclear. The close locations of LC to DTN may limit histological resolution with labeling dye injections to either nucleus. This limitation does not seem to exist in optogenetics, as the

optostimulation is rather specific for cells that express ChR. By optostimulating the DTN nucleus, we have shown that the DTN cells not only project to the LC, but also inhibit LC neurons mon-synaptically.

When optostimulating the DTN, we also found that some DTN cells also receive GABAergic inputs. We did not try to determine the origin of such a GABAergic input to the DTN, as such a study would deviate markedly from the objective of our current studies. We speculate that the DTN neurons have recurrent inhibition as a feedback control, as such self-regulatory mechanism is widely seen in GABAergic neurons (Schneider and Fyffe, 1992). Other potential sources include known afferents to the DTN such as the mammillary nucleus, habenular nuclei, the prepositus hypoglossus, supragenualis nucleus, septal nuclei, diagonal band of Broca, preoptic area, and the reticular formation (Hayakawa and Zyo, 1990; Herkenham and Nauta, 1979; Liu et al., 1984), some of which contain GABAergic afferents that express ChR and may be optostimulated as well (Hardy and Corvisier, 1991).

4.6. CPS Potential Side Effects

The occurrence of CPS side effects such as somnolence is rare given its use as an over-the-counter medicine, but they must also be examined in the context of RTT. There are reports of patients developing dystonia when taking CPS, though this occurred in combination with other drugs (Linazasoro et al., 2000; Serrano et al., 2012). It is unclear how or whether CPS contributes to the development of dystonia in these patients.

Also, one study shows that CPS can inhibit hERG channels and prolong the QT interval, which play a role in cardiac function (Snyders, 1999; Takahara et al., 2012). Prolonged QT interval is found in RTT patients as well, and may be a cause of sudden death, which could potentially be exacerbated by CPS (Sekul et al., 1994). The percentage of RTT patients with prolonged QT is not yet clear as reports vary from 7% to 55% (Crosson et al., 2017; Ellaway et al., 1999; Sekul et al., 1994). One explanation for the variability in RTT patients with prolonged QT is that some *MECP2* mutations may contribute to the manifestation of this symptom while others do not. One study supports this idea by demonstrating that RTT patients with the *MECP2* mutations R255 and large deletions were more likely to have prolonged QT while those with R106C, R106W, R133C, R168, R255, R270, R294, R306C, R306H, and R306P mutations did not have the symptom (Crosson et al., 2017). In the same study, only one out of nine patients taking medications that could potentially prolong QT had prolonged QT (Crosson et al., 2017). This evidence suggests that CPS may potentially increase QT in some RTT patients, especially those with preexisting prolonged QT condition. Thus, a precaution needs to be taken before CPS administration.

Conclusion

Our results show that the over-the-counter cough medicine CPS improves respiratory symptoms of *Mecp2* mutant mice. This effect appears to be mediated by blockade of GIRK channels located on GABAergic synaptic terminals. Inhibition of these GIRK channels can produce depolarization of the synaptic terminals enhancing GABA release. Given that neuronal hyperexcitability is attributed to symptom development in Rett Syndrome, blocking K⁺ channels in GABAergic synaptic terminals seems to be a novel therapeutic approach.

Consistently, previous literature and the new evidence from our current studies indicate that CPS has an overall inhibitory effect in the brainstem supporting further investigation of the use of this drug to treat RTT breathing symptoms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

RTT	Rett Syndrome
CPS	cloperastine
DTN	dorsal tegmental nucleus
PBC	pre-Bötzinger complex
KF	Kölliker-Fuse

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Highlights

- Cloperastine inhibited GIRK channel activity in human embryonic kidney cells.
- Cloperastine reduced the severity of breathing abnormalities in *Mecp2*-null mice.
- Cloperastine increased GABA release.
- Cloperastine decreased locus coeruleus and dorsal tegmental nucleus excitability.

Fig. 1. Cloperastine inhibited GIRK1-GIRK2 channels heterologically expressed in HEK Cells. **A.** Whole cell voltage clamp was performed in HEK cells expressing GIRK1 and GIRK 2 channels. Inward rectifier currents were recorded with a ramp command potential from -100mV to 100mV . The horizontal bars indicate the duration of the recording during each drug exposure and the labels indicate the concentrations of Cloperastine (CPS) used. **B.** Individual traces of currents obtained from A and displayed in the voltage vs current system. The baseline (BL) currents showed clear inward rectification, which was reduced in the presence of $10\text{ }\mu\text{M}$ CPS. **C.** Concentration-dependent inhibition of the GIRK1-GIRK2 currents by Cloperastine (CPS). The amplitude of the currents was described as a function of CPS with the Hill equation showing IC_{50} $1.0\text{ }\mu\text{M}$. Data are shown as means $\pm$ SEM (n=12).

Fig. 2. Cloperastine reduced apnea counts and inhibited breathing irregularity in *Mecp2* mice. **A-D.** Breathing activity was studied in plethysmography *Mecp2* mutant mice before, 30min, 4h and 8h after Cloperastine injection. **E, G.** The number of apneas/h and breath frequency variation were decreased after Cloperastine injection (dark circles, n=13). In comparison to mice with saline injection, significant suppressions of both were seen at 0.5h, 1h and 2h after the injection, respectively (open circles, n=9) (significant interaction between time and treatment on apneas/h, $F_{(3,60)} = 4.05$, $P = 0.01$ and significant interaction between time and treatment on f variation, $F_{(3,60)} = 7.69$, $P < 0.001$; Two-Way Mixed-Model ANOVA;

Bonferroni post hoc, Cloperastine treatment compared to baseline,* $P < 0.05$, comparison between treatment groups, #, $p < 0.05$ ##, $p < 0.01$ ###, $p < 0.001$). **F, H.** After data normalization to the baseline levels, the differences in both apnea counts and breath frequency variation remained (significant interaction between time and treatment on apneas/h, $F_{(3,60)} = 7.11$, $P < 0.001$ and significant interaction between time and treatment on f variation, $F_{(1.98,39.54)} = 6.91$, $P < 0.01$; Two-Way Mixed-Model ANOVA; Bonferroni post hoc). **I, J.** With pooling data from 30 min to 2 h, robust inhibitions of apnea counts and f variation by Cloperastine were shown. Data are shown as means $\pm$ SEM. (Student's t test;***, $P < 0.001$).

Fig. 3. Cloperastine decreased LC excitability by presynaptic mechanisms.

Whole-cell current clamp was performed in brain slices *in vitro*. **A.** LC cells increased their firing activity in response to depolarizing current injections (lower panel). **B.** Evoked firing activity was lower in the presence of Cloperastine. **C.** The baseline firing frequency of LC cells (open circles, $n=12$) was compared in the presence of the same level of depolarizing command currents with (dark circles) and without 10 μ M Cloperastine (open circles). Significant reductions in firing activity were seen with CPS. A significant difference of main effect of treatment was found, ($F_{(1,11)} = 18.58$, $P < 0.001$). A significant interaction was found as well (treatment $\times$ current injection interaction: $F_{(11,121)} = 16.87$, $P < 0.001$; Two-way repeated measures ANOVA). **D.** In the presence of a GABA_A blocker, the effect of Cloperastine was reversed to stimulation, which was also statistically significant. A significant difference of main effect of treatment was found, ($F_{(1,5)} = 24.98$, $P < 0.01$). A significant interaction was found as well (treatment $\times$ current injection interaction: $F_{(1.71,8.55)} = 5.06$, $P < 0.05$; Two-way repeated measures ANOVA). Data are shown as means $\pm$ SEM.

Fig. 4. Cloperastine Increased spontaneous IPSCs in LC Cells.

A. Representative recordings of sIPSCs from a WT neuron before and during administration of 10 μ M Cloperastine. These sIPSC are likely GABA_Aergic as glycinergic and glutamatergic receptors were blocked. The bottom traces in each panel are expanded traces from the areas indicated between the two arrows in the upper traces. **B, D.** Comparisons of IPSC frequency and amplitude with and without 10 μ M CPS. Cloperastine selectively enhanced IPSC frequency without any effects on IPSC amplitude ($n=23$). **C, E.** Similar results are seen after normalization of IPSC frequency and amplitude to baseline levels respectively. (paired Student's t test, *, $P<0.05$, NS, no significance).

Fig. 5. Optogenetic identification of GABAergic input to DTN.

A. In whole-cell current clamp of DTN neurons in a GAD-ChR mouse, the optostimulating pulses (470 nm light with 10 ms duration indicated by the blue bar) were delivered to the same DTN cell through the objective lens 500ms after the onset of command current injection. The light evoked larger depolarization with firing activity in the DTN cell. **B.** In voltage clamp from another DTN cell, light evoked inward currents that decreased their amplitude with depolarization and disappeared at 0mV (likely to be the reversal potential). **C₁.** Schematic diagram of a brain slice containing the DTN and LC with a recording electrode in the LC nucleus and optostimulation to the DTN. **C₂, C₃.** Blue light stimulation (2.0 ms and 5.0 ms, respectively) to the DTN evoked field potentials in the LC. The shortest latency between onset of light and the onset of field potentials was 4.0 ms.

Fig. 6. Cloperastine lowered the excitability of DTN neurons.

A, B. DTN cells firing activity increased in response to depolarizing current injections. **B.** DTN cells had lower firing frequencies in the presence of Cloperastine. **C.** Plot of average evoked firing frequency versus current injection ($F_{(1,9)} = 11.8$, $P < 0.01$; Two-way repeated measures ANOVA; *, $P < 0.05$, **, $P < 0.01$, $n=10$). **D.** Firing frequencies at peak current injections compared between baseline and 10 μ M Cloperastine exposure. (paired Student's t test, **, $P < 0.01$)

Fig. 7. DTN cells showed GABAergic IPSCs during and immediately after optostimulation to the same nucleus.

A. Voltage clamp recording of light-induced inward currents in a DTN neuron. IPSC-like outward currents were seen immediately after the blue light pulse (horizontal bar 10 ms). Arrows indicate these IPSCs. **B.** Currents evoked by optostimulation from a DTN cell with a holding potential -30 mV. The inward current is likely to be the opto-current, while the outward current seems to be an average of the IPSCs. **C.** With prolonged optostimulation, the characteristics of IPSCs were revealed: They occurred only during the light exposure and immediately after. **D.** During prolonged optostimulation, the IPSCs reversed their polarity becoming inward current with hyperpolarization with the reversal potential between -60 mV and -70 mV.

Fig. 8. Bicuculline blocked Cloperastine-induced hyperexcitability of DTN neurons.

A, B. DTN firing activity was not affected by Cloperastine administration in the presence of bicuculline compared to baseline activity. **C.** Plot of average evoked firing frequency versus current injection showing no significant difference between baseline and drug treatment ($n=9$) ($F_{(1,9)} = 0.41$; Two-way repeated measures ANOVA; NS, no significance). **D.** Firing frequencies at peak current injections (Student's paired t-test, NS, no significance).