ORIGINAL RESEARCH

Effect of Sarizotan, a 5-HT$_{1a}$ and D2-Like Receptor Agonist, on Respiration in Three Mouse Models of Rett Syndrome

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Abstract

Disturbances in respiration are common and debilitating features of Rett syndrome (RTT). A previous study showed that the 5-HT$_{1a}$ receptor agonist (R)-(+)-8-hydroxy-dipropyl-2-aminotetralin hydrobromide (8-OH-DPAT) significantly reduced the incidence of apnea and the irregular breathing pattern in a mouse model of the disorder. 8-OH-DPAT, however, is not available for clinical practice. Sarizotan, a full 5-HT$_{1a}$ agonist and a dopamine D2–like agonist/partial agonist, has been used in clinical trials for the treatment of L-dopa–induced dyskinesia. The purpose of this study was to evaluate the effects of sarizotan on respiration and locomotion in mouse models of RTT. Studies were performed in Bird and Jaenisch strains of methyl-CpG–binding protein 2-–deficient heterozygous female and Jaenisch strain $Mecp2$ null male mice and in knock-in heterozygous female mice of a common nonsense mutation (R168X). Respiratory pattern was determined with body plethysmography, and locomotion was determined with open-field recording. Sarizotan or vehicle was administered 20 minutes before a 30-minute recording of respiratory pattern or motor behavior. In separate studies, a crossover design was used to administer the drug for 7 and for 14 days. Sarizotan reduced the incidence of apnea in all three RTT mouse models to approximately 15% of their pretreatment levels. The irregular breathing pattern was corrected to that of wild-type littermates. When administered for 7 or 14 days, apnea decreased to 25 to 33% of the incidence seen with vehicle. This study indicates that the clinically approved drug sarizotan is an effective treatment for respiratory disorders in mouse models of RTT.

Keywords: apnea; locomotion; respiratory arrhythmia

Clinical Relevance

These studies show that sarizotan, a phase III 5-HT1a receptor agonist, decreases the incidence of apnea and corrects irregular breathing in mouse models of Rett syndrome. The results may lead to clinical trials of 5-HT1a receptor agonists.

Rett syndrome (RTT) is an autism spectrum disorder caused by mutations in the gene that encodes the nuclear protein methyl-CpG-binding protein 2 (MeCP2). Because $MeCP2$ is X-linked, the syndrome is seen primarily in female subjects (1, 2). In RTT there are disturbances in respiration characterized by an irregular breathing pattern and frequent apnea, which are common and debilitating (3–5). Various studies in mouse models of the disorder have demonstrated that restoration of MeCP2 in symptomatic animals markedly reverses the phenotype (6, 7). These observations indicate that the syndrome does not result in degeneration of neurons or irreversible developmental impairment of neuronal networks. Therefore, pharmacological treatment of symptoms is a valid approach.

In a mouse model, recent studies demonstrated that excess excitation in expiratory neurons characterizes the respiratory abnormality (8). Building on

earlier reports showing that 5-HT$_{1a}$ agonists inhibit expiratory neurons (9, 10), we showed that (R)-(+)-8-hydroxy-dipropyl-2-aminotetralin hydrobromide (8-OH-DPAT) significantly reduced the incidence of apnea and restored regularity to the breath cycle (8). 8-OH-DPAT, however, is not clinically available. Sarizotan, a full 5-HT$_{1a}$ agonist and a dopamine D2-like agonist/partial agonist (11), has been used in clinical trials for the treatment of L-dopa–induced dyskinesias (12, 13). The goal of the present study was to evaluate the effects of sarizotan on respiration in mouse models of RTT. To determine possible side effects, locomotion was also studied. Because the Kölliker-Fuse (KF) is a key region for control of expiratory neuron activity and expiratory cycle regularity (14) in this study, we also explored it as a potential 5-HT1a agonist site of action for rescuing breathing in RTT.

Materials and Methods

The protocols were approved by the Oregon Health and Science University Animal Care and Use Committee, were in agreement with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals," and were performed according to the UK Home Office (Scientific Procedures) Act (1986). Studies were performed in heterozygous female mice with deletion of the third *Mecp2* exon (15), designated *Mecp2$^{Jae/+}$*, and wild-type (WT) littermates at 6.7 to 12.3 months of age; in null male mice of the same strain designated *Mecp2$^{Jae/y}$* at 1.6 to 2.9 months of age; in heterozygous female mice with deletion of the third and fourth exons of *Mecp2* (16), designated *Mecp2$^{Bird/+}$*, at

6.8 to 14.2 months of age; and in heterozygous female mice carrying a common nonsense mutation seen in patients with RTT (R168X) (17, 18), designated *Mecp2$^{R168X/+}$*, at 8.5 to 14 months of age.

Respiratory pattern was determined with body plethysmography as previously described (19, 20). Briefly, individual unanesthetized animals were placed in a 65-ml chamber with their heads exposed through a close fitting hole in Parafilm (Pechiney, Chicago, IL). A pneumotachograph (19) was connected to the chamber and a differential pressure transducer (Model PT5A; Grass Instrument Co., West Warick, RI). Each study was 30 minutes in duration, and the traces were analyzed in 5-minute blocks. Apnea was defined as an expiratory time of 1.0 second or longer. At the frequency of Mecp2-deficient mice, approximately 140 breaths per minute (bpm) (Table 1), 1.0 seconds is equal to 2.4 breath cycles. The irregularity score was calculated from: absolute (TTOTn-TTOTn+1)/(TTOTn+1) (21) and reported as the variance.

The acute effects of sarizotan were examined in Mecp2-deficient heterozygous female mice and in null male mice. For each sarizotan dose, a vehicle (2.5% DMSO in water, intraperitoneally) 30-minute study preceded the drug study. After this, sarizotan was administered intraperitoneally, and 20 minutes later the 30-minute respiratory monitoring was repeated. Sarizotan injections were separated by a minimum of 48 hours.

A crossover design was used for the long-term studies so that half of the *Mecp2$^{Bird/+}$* female mice received vehicle (1.25% DMSO) in their drinking water and half received sarizotan (0.0625 mg/ml). At

the end of 7 days, the treatment was reversed so that the mice that were receiving vehicle were changed to sarizotan and those on sarizotan were changed to water. Vehicle and drug solutions contained 0.1% saccharin. Water intake with sarizotan approached that with vehicle (86 ± 1.9%), and the difference was not significant ($P = 0.37$; paired t test). The dose of sarizotan ingested averaged 13.8 ± 1.9 mg/kg/d. Respiratory pattern was studied on Days 3, 5, and 7 of treatment. An identical design was used for a long-term study in *Mecp2$^{R168X/+}$* mice except that treatment was for 14 days. Water intake and daily dose of sarizotan were similar to those in the 7-day study. The mice were studied with plethysmography on Days 4, 7, 10, and 14 after receiving sarizotan or vehicle.

Motor activity studies were performed at the same time of day (12:00–18:00) and in the same dedicated observation room. Mice were placed singly into a standard open field box with side-viewing and top-viewing cameras (Clever Sys, Reston, VA). Mice were recorded for 20 minutes after an approximate 1-minute delay from initial placement in the center of the open field. Activity traces were acquired in real time using StereoScan Software (Clever Sys).

To determine the location of 5-HT$_{1a}$ receptor agonist effects in the respiratory network, we used an *in situ* decerebrated "working heart-brainstem preparation" of the mouse (22), which is a modified version of cardiopulmonary bypass and allows direct recordings to be made from the phrenic nerve (PN) and the central vagus nerve (cVN) and the ability to manipulate precisely arterial CO_2 concentration. This preparation is not confounded by the depressant action of anesthetic agents or

Table 1: Baseline Respiratory Pattern in Mouse Models of Rett Syndrome

Strain	Frequency (*bpm*)	Irregularity (variance)	Apnea (*no./h*)	Apnea (*range*)
Mecp2$^{Jae/y}$ (*n* = 6)	193 ± 13*	0.62 ± 0.21	215 ± 40	80–382
WT	259 ± 10	0.052 ± 0.008	2.3 ± 1.2	0–9.0
Mecp2$^{Jae/+}$ (*n* = 4)	141 ± 12	0.29 ± 0.05	162 ± 10	140–190
WT	207 ± 16	0.057 ± 0.015	2.1 ± 0.9	0–6.0
Mecp2$^{Bird/+}$ (*n* = 6)	158 ± 20	0.41 ± 0.11	158 ± 52	42–390
WT	224 ± 11	0.096 ± 0.009	5.8 ± 3.1	0–16.8
Mecp2$^{R168X/+}$ (*n* = 8)	132 ± 4	0.18 ± 0.028	168 ± 42	64–421
WT	190 ± 6	0.02 ± 0.003	3.8 ± 1.5	0–9.6

Definition of abbreviations: bpm, breaths per minute; WT, wild type.
*Values are mean ± SEM.

changes in sleep/arousal states. Briefly, female heterozygous $Mecp2^{Bird/+}$ mice ($n = 4$) were heparinized (1,500 U intraperitoneally) and anesthetized deeply with halothane until loss of their paw withdrawal reflex. Mice were decerebrated and exsanguinated and bisected subdiaphragmatically, and the head and thorax were immersed in ice-cold carbogenated artificial cerebrospinal fluid. The thoracic PN and cVN were cut distally. Preparations were moved to a recording chamber. A double-lumen catheter (Braintree Scientific, Braintree, MA) was inserted into the descending aorta for retrograde perfusion by a peristaltic roller pump (505Du; Watson Marlow, Falmouth, UK). The perfusion solution consisted of carbogenated modified Ringer's at 32°C with an oncotic agent replacing serum albumin (Ficoll 1.25%). The second lumen of the catheter was used to monitor aortic perfusion pressure. The baseline perfusate flow was preset between 18 and 20 ml/min. The composition of the Ringer's solution was 125 mM NaCl, 24 mM $NaHCO_3$, 3 mM KCl, 2.5 mM $CaCl_2$, 1.25 mM $MgSO_4$, 1.24 mM KH_2PO_4, and 10 mM glucose; pH ranged from 7.35 to 7.4 after carbogenation (6% CO_2 and 94% O_2). Osmolality was 290 ± 5 mOsm per kg H_2O. Ficoll (1.25%; type 70) was added as an oncotic agent. Unless stated, all chemicals were from Sigma-Aldrich (St. Louis, MO). Vecuronium bromide (4 μg/ml) (Organon Teknica, Durham, NC) was added to the perfusion solution to block neuromuscular transmission.

Simultaneous recordings of the left PN and cVN motor activities were obtained with bipolar suction electrodes mounted on individual 3D micromanipulators. Nerve activities were AC amplified (10 k), band-pass filter (100 Hz to 5 kHz) rectified, and integrated (50 ms time constant) online (Spike 2 software; Cambridge Electronic Design, Cambridge, UK). All electrophysiological data were digitized (10 kHz) with an A-D converter (Cambridge Electronic Design) with Spike 2 software and analyzed offline.

Custom–built, three-barreled micropipettes ($\sim$ 20 μm ø) were used for brainstem microinjections. For that, three glass-calibrated micropipettes (0.4 mm inner diameter) (Drummond Scientific, Broomall, PA) were glued together, twisted to form a single tip, and pulled on a Narishige Pipette Puller (Model PE 2;

Narishige, East Meadow, NY). Micropipettes contained 10 mM L-glutamate (Sigma-Aldrich), 100 μM 8-OH-DPAT (Tocris Bioscience, Minneapolis, MN), and 2% Evans Blue dye (Sigma-Aldrich), all dissolved in aCSF (Harvard Apparatus, Holliston, MA) at pH 7.4.

Micropipettes were placed just caudally to the left inferior colliculus, 1.7 to 1.8 mm lateral of the midline and 1 to 1.5 mm ventral of the dorsal surface. After functional identification of an effective KF injection site (i.e., a prolonged PN apnea with prolonged postinspiratory activity on cVN) with L-glutamate (30 nl), 8-OH-DPAT was microinjected (60 nl). At the end of the experiment, Evans blue (30 nl) was injected to mark the injection site. The injected volumes were measured through a microscope fitted with a calibrated ocular graticule. Repeated microinjections into the same site, performed at least 15 minutes apart (for washout and recovery), yielded reproducible responses. In all four mice, microinjections were delivered on the left side; in two mice, after an appropriate washout interval of at least 15 minutes, an additional contralateral microinjection was performed. There was no difference between right and left side injections, so they were grouped. At the end of each experiment, the brainstem was removed and fixed in 4% paraformaldehyde for 48 hours (Sigma-Aldrich) and cryoprotected in 20% sucrose in PBS for 24 hours (Sigma-Aldrich). Brainstems were then cut in a series of 30-μm-thick coronal sections through the pons with a cryostat. The sections were counterstained with DAPI and examined under and fluorescence microscope (Evans blue emission peak = 680 nm; DAPI emission peak = 461 nm).

Data are reported as mean ± SEM. Comparisons between WT and Mecp2-deficient groups were made with unpaired t tests. The effect of a single dose of sarizotan was determined with a paired t test. The effects of long-term treatment in Mecp2-deficient female mice were determined with two-way repeated measures ANOVA with treatment and days as the two factors. Post hoc testing used the Tukey method. The effects of KF microinjections were compared using one-way repeated measures ANOVA followed by a comparison versus baseline using the Dunnett method. $P < 0.05$ was taken as significant. Sigma Stat 3.1 (Systat, Chicago, IL) was used for statistical evaluation.
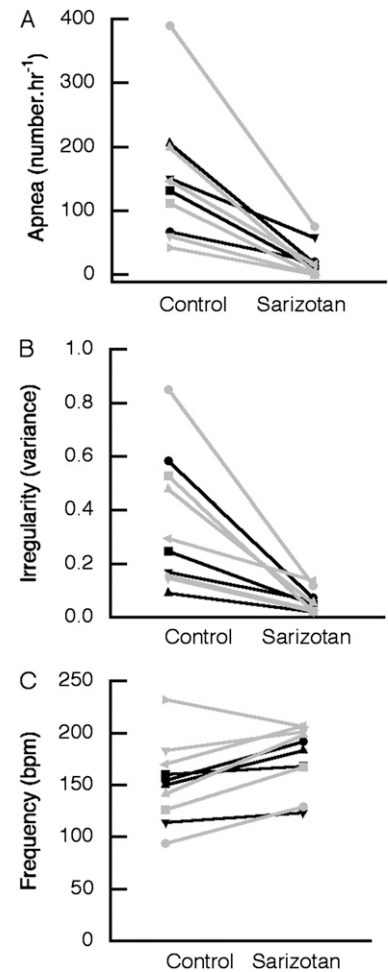

Figure 1. Acute effect of sarizotan (5 mg/kg) on respiratory pattern in Mecp2-deficient heterozygous female mice. Data shown for individual mice (*black lines* = Mecp2$^{Jae/+}$ mice; *gray lines* = MeCP2$^{Bird/+}$ mice). (*A*) Number of apneas per hour. (*B*) Respiratory irregularity given as the variance in absolute (TTOTn − TTOTn + 1)/(TTOTn + 1). (*C*) Respiratory frequency in breaths per minute (bpm).

Results

Baseline Respiratory Patterns
Respiratory frequency was decreased by approximately 25 to 30% in Mecp2-deficient mice compared with WT in all four strains (Table 1). The interbreath period was very irregular and the incidence of apnea (Table 1) much greater in the Mecp2-deficient animals.

Acute Effect of Sarizotan on Respiration
Preliminary studies were performed to estimate the effective dose of sarizotan. A

Figure 2. Acute effect of sarizotan (10 mg/kg) on respiratory pattern in Mecp2$^{Jae/y}$ null male mice. Data are shown for individual mice. (*A*) Number of apneas per hour. (*B*) Respiratory irregularity given as the variance in absolute (TTOTn − TTOTn + 1)/(TTOTn + 1). (*C*) Respiratory frequency in breaths per minute (bpm).

10 studies, apnea was decreased to levels that were within the range seen in WT mice (Figure 1A; Table 1). Sarizotan resulted in a decrease in the irregularity score from 0.34 ± 0.07 to 0.06 ± 0.01 (*P* < 0.0001). In 8 of 10 experiments, the irregularity score was corrected to that of WT littermates (Figure 1B). Sarizotan in heterozygous female mice significantly increased respiratory frequency from 153 ± 12 bpm to 177 ± 10 bpm (*P* = 0.012) (Figure 1C). After a dose of 5.0 mg/kg, the drug did not affect core body temperature in MeCP2-deficient female mice (36.2 ± 0.3 versus 35.2 ± 0.4; *P* = 0.093; *n* = 6). The effects of sarizotan on improving respiration did not depend on the age of female mice at the time of study. The heterozygous female mice were grouped into younger (6.7–10.7 mo; *n* = 5) and older animals (12.0–14.2 mo; *n* = 5). Apnea was reduced to 5.4 ± 2.3% of baseline in the younger mice and to 15.6 ± 7% in the older animals (*P* = 0.248; unpaired *t* test). Irregularity fell to 24.3 ± 5.8% of baseline in younger female mice and to 12.7 ± 5.7% in older female mice (*P* = 0.319). Respiratory frequency increased to 109.9 ± 6.4% of baseline in younger mice and to 128.0 ± 5.9% in older mice (*P* = 0.071).

Sarizotan (10.0 mg/kg) was equally effective in improving respiration in null male mice. The incidence of apnea fell from 200 ± 42 to 30 ± 16/h (*n* = 6) (*P* = 0.003; paired test). In three of six male mice, apnea was reduced to that seen in WT animals (Figure 2A; Table 1). Irregularity declined from 0.55 ± 0.21 to 0.11 ± 0.05 (*P* = 0.048) (Figure 2B). Sarizotan did not affect frequency in null male mice (220 ± 13 versus 192 ± 12; *P* = 0.094) (Figure 2C).

Long-Term Effect of Sarizotan on Respiration

When added to drinking water, sarizotan reduced the incidence of apnea in *Mecp2*$^{Bird/+}$ mice by 63.3% on Day 5 of treatment (*P* = 0.009) and by 66.1% on Day 7 (*n* = 8) (*P* = <0.001; two-way repeated measures ANOVA) (Figure 3A). In addition to its effects in reducing the incidence of apnea, sarizotan corrected the irregular breathing pattern that is characteristic of RTT. On Days 5 and 7 of treatment, the irregularity score reverted to a level similar to that of WT mice (0.096 ± 0.009) (Figure 3B). Sarizotan slightly increased respiratory frequency from 156 ± 10 while receiving
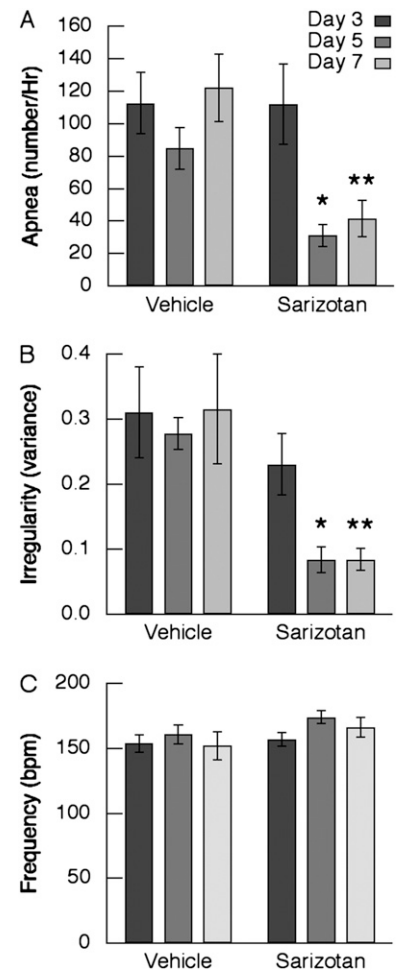

Figure 3. Effect of 7-day treatment with sarizotan on respiration in Mecp2$^{Bird/+}$ female mice (*n* = 8). Thirty-minute monitoring of respiratory pattern with plethysmography was performed on the Days 3, 5, and 7 of vehicle or sarizotan administered in drinking water. (*A*) Incidence of apnea. (*B*) Irregularity score expressed as the variance. (*C*) Respiratory frequency. **P* < 0.05 versus corresponding day receiving vehicle. ***P* < 0.01.

vehicle to 174 ± 5 on Day 5 of drug treatment and to 166 ± 8 on the Day 7, but not to a significant level (*P* = 0.093 and 0.087).

Sarizotan was also effective in improving respiration in *Mecp2*$^{R168X/+}$ mice (*n* = 8) in a 2-week trial. Treatment with this drug reduced the incidence of apnea by 73.9, 75, and 75.6% compared with vehicle on Days 7, 10, and 14 of treatment, respectively (*P* = 0.008–0.022) (Figure 4A). On Days 7 (*P* = 0.006), 10 (*P* = 0.049), and 14 (*P* = 0.05), irregularity was significantly below that of animals receiving vehicle but

dose of 1.0 mg/kg reduced the incidence of apnea in *Mecp2*$^{Jae/+}$ mice to 47.5 ± 10.4% (*P* = 0.089) of that seen after vehicle (*n* = 4). This dose reduced apnea to 36.9 ± 14.5% (*P* = 0.091) in *Mecp2*$^{Jae/y}$ male mice (*n* = 4). Subsequently, 5.0 mg/kg was used in heterozygous female mice, and 10.0 mg/kg was used in null male mice. The effects of sarizotan (5.0 mg/kg) in MeCP2$^{Jae/+}$ and *Mecp2*$^{Bird/+}$ mice were similar, so the results have been combined (Figure 1). This compound significantly reduced the incidence of apnea in MeCP2-deficient heterozygous female mice (*n* = 10) from 143 ± 31/h to 20 ± 8/h (*P* = 0.001; paired *t* test) (Figure 1A). In 5 of
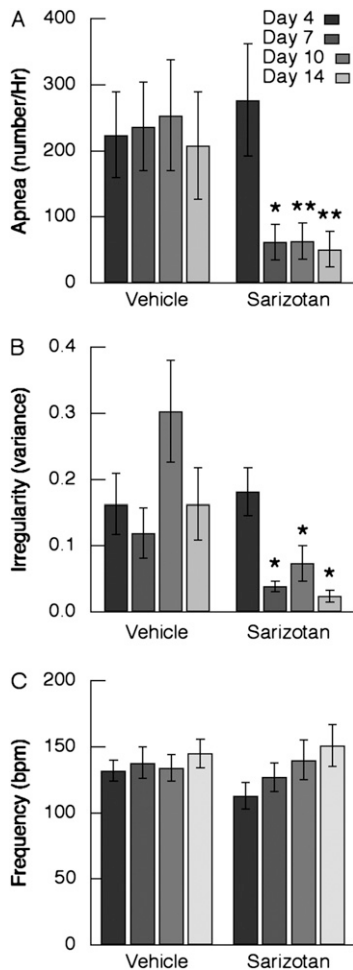
Figure 4. Effect of 14-day treatment with sarizotan on respiration in Mecp2$^{R168X/+}$ mice ($n = 8$). Monitoring of respiratory pattern with plethysmography was performed at 0 minutes on Days 4, 7, 10, and 14 of vehicle or sarizotan administered in drinking water. (A) Incidence of apnea. (B) Irregularity score expressed as the variance. (C) Respiratory frequency. *$P < 0.05$ versus corresponding day receiving vehicle. **$P < 0.01$.

did not reach WT levels (0.02 ± 0.003) (Figure 4B; Table 1). Respiratory frequency was not affected by sarizotan during the 14-day treatment (Figure 4C).

Effect of Sarizotan on Locomotion

Open-field behavioral studies were performed with $Mecp2^{Jae/+}$ and $Mecp2^{Bird/+}$ heterozygous female mice. There were no differences in the effects of sarizotan, and the data were combined. Acute administration of sarizotan (5.0 mg/kg) significantly reduced the total distance traveled in WT ($P < 0.0001$) and

Mecp2-deficient animals ($P = 0.0004$) (Figure 5B). The drug had no effect on the velocity of movement ($P = 0.135$) or on the percentage of time that mice spent in the center square ($P = 0.091$). In the 7-day long-term study, locomotion was examined on Day 6 of treatment. Total distance traveled (31.3 ± 4.6 m in 20 min) while receiving sarizotan was not different than that with vehicle (32.6 ± 6.8 m) (*see* Figure E1A in the online supplement). In addition, average velocity (58.1 ± 4.1 versus 53.9 ± 4.9 mm/s) was not affected by the drug (Figure E1B). Mecp2$^{-/+}$ mice receiving vehicle spent less time in the center square than WT animals (Figure E1C) ($P = 0.032$). The $Mecp2^{R168X/+}$ mice treated with sarizotan for 14 days were examined in open field on Day 13. Total distance traveled in mice receiving vehicle was less than the total distance traveled by WT mice and animals receiving sarizotan ($P = 0.031$) (Figure 6B).

Effect of 5-HT$_{1a}$ Agonist Injected into the KF Nucleus on Respiratory Pattern

At the concentration needed for intraparenchymal administration, sarizotan could only be solubilized in 5% DMSO, thus precluding its use in direct instillation in the brainstem. Diluted DMSO solutions (5% and lower) applied directly to neuron soma and axon projections were shown to reduce action potential velocity and to block sodium, potassium, and calcium currents (23, 24). This could lead us to under- or overestimate the effect of the sarizotan even if DMSO did not show any acute respiratory effects. Therefore, to localize the site of action of 5-HT$_{1a}$ receptor agonists, we used 8-OH-DPAT for the *in situ* experiments. These studies used $Mecp2^{Bird/+}$ mice that were 4 to 10 months of age. The KF was identified by a prior microinjection of glutamate that evoked PN apnea and prolonged postinspiratory activity in the cVN. Figure 7 shows the
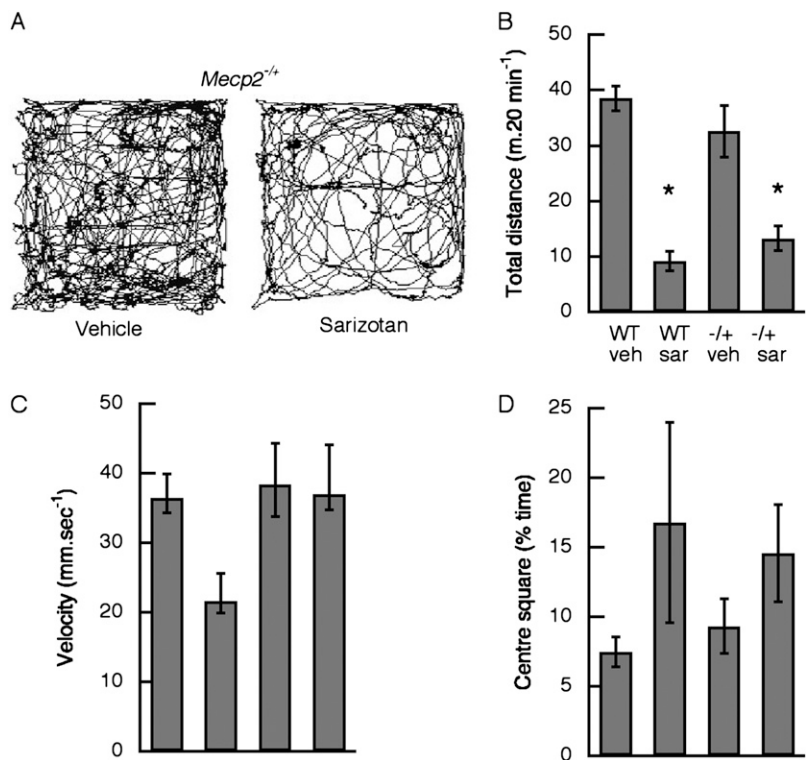

Figure 5. Acute effect of sarizotan (5 mg/kg) on motor activity in Mecp2-deficient heterozygous and wild-type (WT) female mice. (A) Representative trace of locomotor activity in a Mecp2$^{-/+}$ mouse after receiving intraperitoneal injection of vehicle (*left panel*) and after sarizotan (*right panel*). (B) Total distance traveled in a 20 minute period. (C) Average velocity for all movements that exceed 10 mm/second^{-1}. (D) Percent time mouse spent in the centre square of the nine square grid. Total distance traveled was significantly decreased after sarizotan compared with after vehicle injections. *$P < 0.0001$ for WT; $P = 0.0004$ for Mecp2$^{-/+}$. Effects on velocity and time spent in center square were not significant. −/+, heterozygous Mecp2 deficient; sar, sarizotan; veh, vehicle.
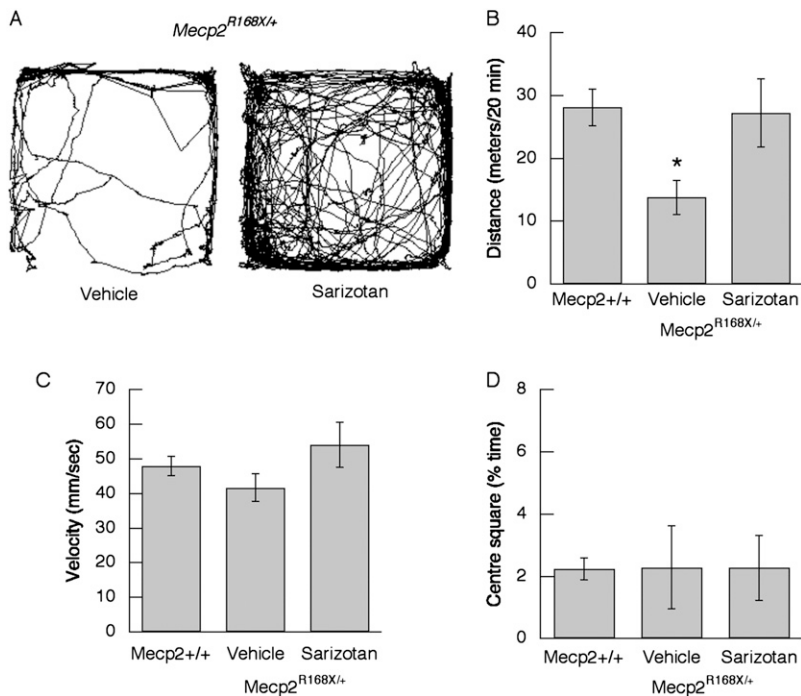
Figure 6. Effect of 13-day treatment with sarizotan on motor activity in Mecp2$^{R168/X}$ mice (*n* = 8). (*A*) Representative trace of locomotor activity in a Mecp2$^{R168X/+}$ mouse on Day 13 of vehicle in drinking water (*left panel*) and on Day 13 of sarizotan (*right panel*). (*B*) Total distance traveled in a 20 minute period. (*C*) Average velocity for all movements that exceed 10 mm/second^{-1}. (*D*) Percent time mouse spent in the centre square of the nine square grid. *P = 0.031.

effect of a unilateral 8-OH-DPAT microinjection into the KF nucleus. Unilateral injections of dipropyl-2-aminotetralin in the KF reduced apnea in *Mecp2*$^{Bird/+}$ heterozygote mice studied *in situ* from 188.6 ± 21 to 102.9 ± 30/h (*n* = 6; *P* < 0.01). In addition, the 5-HT$_{1a}$ agonist lowered the coefficient of variation for breath intervals from 32 ± 5 to 20 ± 2 (*P* < 0.01). When the agonist was washed out, apnea and irregularity returned to their preinjection levels. Ineffective 8-OH-DPAT injections proved to be outside the KF region on *post hoc* histological analysis (Figure E2). Vehicle and glutamate injections had no effect on the number of apneas or on breath interval variation (*P* > 0.05).

Discussion

This study shows, to the best of our knowledge, for the first time, that the class III drug sarizotan reduces the incidence of apnea and corrects the irregular interbreath pattern in three mouse models of RTT. Sarizotan is formulated into an oral medication (25) and has been used in two clinical trials in adults (12, 13). The similar results in *Mecp2*$^{Jae/+}$, *Mecp2*$^{Bird/+}$, and *Mecp2*$^{R168X/+}$ heterozygous

female mice indicate that the effects are not strain specific or confined to animal models with null mutations of the DNA binding protein. R168X retains the methyl-DNA binding domain but lacks the large C-terminus of the protein including the transcription repression domain (17, 18). This mutation is seen in approximately 10 to 15% of patients with RTT (26, 27). The results extend the initial observation by demonstrating that sarizotan, a phase III drug, is equally as effective as 8-OH-DPAT (8). In addition, the beneficial effects on respiratory disturbances occurred without a significant alteration in breathing frequency. As with 5-HT$_{1a}$ receptor agonists, augmenting GABA inhibition in heterozygous Mecp2-deficient female mice ameliorated respiratory symptoms (8). These treatments reduced respiratory rate by approximately 35%. In a separate study, the GABA-A allosteric modulator midazolam was shown to correct posthypoxic apnea and irregularity in *Mecp2*$^{Bird/y}$ null male mice (28). This compound caused a 28% reduction in frequency.

The baseline respiratory pattern for mice lacking the third *Mecp2* exon in the present studies differed from some previous

reports. *Mecp2*$^{Jae/y}$ null male mice on a mixed (129v, C57BL/6, BALB/c) background studied at postnatal day (P)35 were found to have a respiratory frequency that was greater than that seen in WT mice (29). In contrast, null male mice of this same strain on a C57BL/6 at P42 had a longer total breath cycle (30). *Mecp2*$^{Jae/y}$ mice on C57BL/6 or BALB/c studied between P48 and P88, reported herein, had a respiratory frequency that was less than WT mice (Table 1). The differences may be due to background and/or age at which the studies were conducted. A recent study found that respiratory frequency in *Mecp2*$^{Jae/y}$ male mice declined in older animals (31). Fifty percent of *Mecp2*$^{Jae/+}$ female mice on a mixed background studied under urethane anesthesia at 18 to 29 months of age showed a respiratory frequency that exceeded that of WT mice (32). These mice did not exhibit frequent apnea, in contrast to those reported here. The differences may be due to background and/or the effects of anesthesia. Recently *Mecp2*$^{Jae/+}$ female mice on a mixed background have been found to have an increased respiratory frequency at 10 and 12 weeks of age (33). Age at time of observation and/or background strain may account for the differences from the results found in the present studies, as might anesthetic agents, which is why we chose to study conscious animals.

The serotonin metabolite 5-hydroxyindoleacetic acid is reduced in cerebrospinal fluid of individuals with RTT (34). 5-HT concentrations are reduced in pons and brainstem of mature (P42–P60) *Mecp2* null male mice (21, 34–36). 5-Hydroxyindoleacetic acid was found to be reduced in one of these studies (36) but not in the other two (21, 35). Selective removal of *Mecp2* from serotonergic neurons caused a marked reduction in brain 5-HT and its rate-limiting enzyme tryptophan hydroxylase (34). The respiratory pattern in these conditional Mecp2-deficient mice was not different from controls that carried a floxed *Mecp2*. The latter are hypomorphic for Mecp2, and their brain levels of the protein are only one half that of WT (37). In addition, mice that had deletion of LIM homeobox transcription factor 1β confined to pheochromocytoma 12 ETS factor-1–expressing 5-HT neurons displayed normal baseline breathing (38). More recently, a genetic strategy was used to
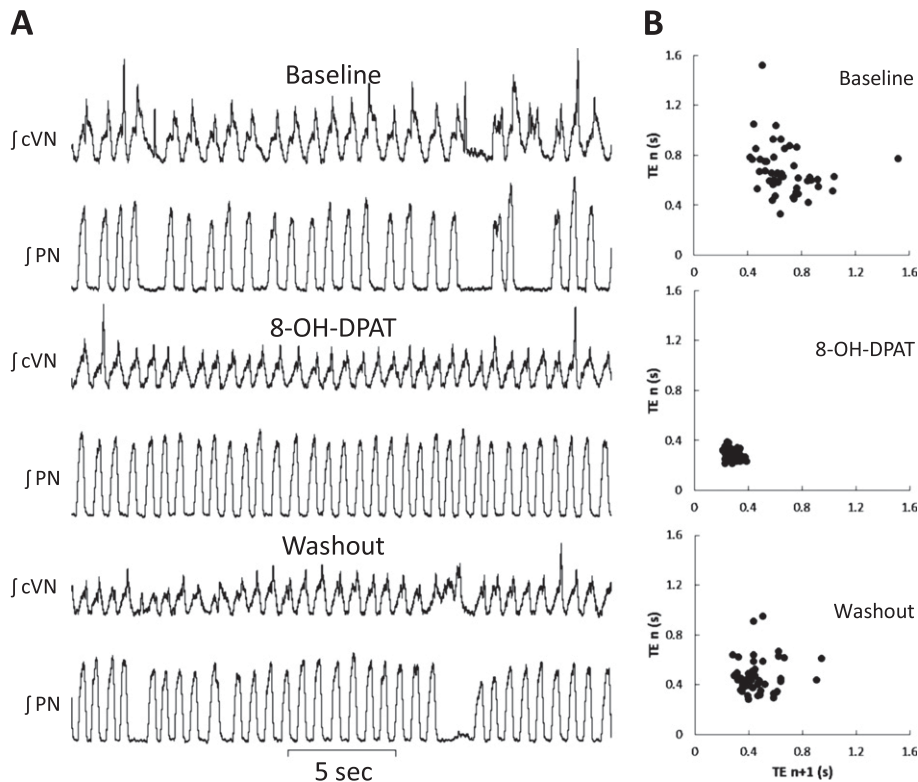
A

B

Figure 7. Effect of serotonin 1a agonist injection unilateral into a Kölliker-Fuse nucleus. (*A*) Integrated nerve activity of central vagus (cVN) and phrenic (PN) nerves. The top two traces were made during baseline recording. The frequent phrenic apneas are characterized by prolonged cVN activity. The middle two traces were made after injection of (R)-(+)-8-hydroxy-dipropyl-2-aminotetralin hydrobromide (8-OH-DPAT) (60 nl, 500 μM) into a Kölliker-Fuse nucleus that had been identified by an earlier injection of glutamate. The bottom two traces were made after washout of dipropyl-2-aminotetralin. (*B*) Poincaré plots for periods that correspond to the adjacent traces.

acutely inhibit serotonergic neurons (39). These mice did not have significant baseline respiratory disturbances. Thus, it is unlikely that treatment with 5-HT$_{1a}$ agonists modify respiratory disturbances in RTT mice by overcoming a defect in 5-HT content. Given that apneas are associated with excessive expiratory discharges in vagal and abdominal motor outflows (8, 40), the relative inhibition of expiratory neurons secondary to activation of G-protein–coupled inward rectifying potassium channel by the 5-HT$_{1a}$ agonist is most likely the mechanism for sarizotan's effects (9, 10).

Previous studies indicate that 5-HT$_{1a}$ receptors in the KF region play a role in respiratory rhythm regularity. The KF is a key region for controlling postinspiration and expiratory cycle length, it suppresses inspiration, and it can reset the respiratory oscillator phase (41). Furthermore, KF microinjections of a 5-HT$_{1a}$ receptor antagonist (WAY100635) increased

respiratory irregularity in WT mice during normal breathing, suggesting a role for endogenous 5-HT$_{1a}$ activity in rhythm regularity (42). Other work also suggested hyperexcitability of respiratory reflexes mediated in this region in MeCP2-deficient mice (40). In the present study, we showed that local microinjection of the 5-HT$_{1a}$ into the KF reduced the number of apneas and irregularity scores in *MeCP2$^{Bird/+}$* female mice, suggesting this is a key target site for sarizotan's beneficial effects on breathing rhythm in RTT.

A number of regimens have been used to treat the respiratory disorders in mouse models of RTT. Catecholaminergic defects have been demonstrated in the brain of human subjects with RTT (34) and in RTT mouse models (35). They have also been documented in a number of respiratory-related regions, including pons, medulla (21), locus coeruleus (43), carotid body, and petrosal ganglion (44). These observations prompted acute and long-term examination

of the effects of augmenting norepinephrine (NE) systemically. In juvenile Mecp2 null mice (P14–P21), NE eliminated cycle irregularity (21). Chronic treatment with the NE reuptake blocker desipramine given intraperitoneally beginning when Mecp2$^{-/y}$ animals apneas reached 100/h stabilized apnea at approximately 100/h and rose to approximately 300/h in mice that received a placebo (45). Oral treatment beginning at P30 with the reuptake blocker reduced the incidence of apnea and the irregularity score at P51 and P65 (46). Unlike the results shown herein, these long-term treatments did not reduce apnea to levels approaching that in WT no correct irregularity.

Brain-derived neurotrophic factor (BDNF) is depleted in brain (47) and nodose ganglia (48) of *Mecp2$^{Jae/y}$* mice. The respiratory phenotype of these mice on a mixed background was characterized by a rapid frequency and irregular pattern. Ampakine therapy beginning at P35 increased nodose BDNF content and reduced respiratory frequency to that of WT mice (29). The authors suggested and later demonstrated that the increased BDNF inhibits glutamatergic excitation in the nucleus of the solitary tract (49). Ampakine treatment, unlike 5-HT$_{1a}$ receptor agonists, did not restore a regular breathing pattern. Two recent papers have examined the effects of small molecules that activate TrkB, the BDNF receptor. In *Mecp2$^{Jae/y}$* on a C57BL6 background, 7,8-dihydroxyflavone shortened their prolonged breath cycle and reduced irregularity, but it remained above that of WT mice (30). LM22A-4 reduced the elevated frequency seen in *Mecp2$^{Jae/+}$* heterozygous female mice on a mixed background to that of WT at 12 weeks of age (33). This compound at the dose used did not affect apnea. Irregularity was not reported in this study.

More recently, the effects of a tripeptide derivative of insulin-like growth factor 1 have been examined (50). Long-term treatment in null male mice started at P15. There was a modest reduction in breath irregularity when the animals were studied at P56. The incidence of apnea was not mentioned. Motor activity was improved to that of WT mice with this treatment. Another recent study showed in *Mecp2$^{Jae/y}$* male mice that inducing stress using movement restraint or threatening odorant (trimethylthiazoline) increased the

incidence of apnea (31). In older male mice, however, the corticotropin-releasing hormone receptor 1 antagonist antalarmin did not reduce the incidence of apnea.

The effects of sarizotan mediated through dopamine D2–like receptors should not detract from its beneficial modulation on respiration. Indeed, activation of these receptors has been shown to depress expiratory neurons, an effect that would be beneficial (51) given the excessive expiratory motor activity that is associated with the apneas (8, 40). The depression of motor activity seen with the acute intraperitoneal administration of sarizotan (Figure 5) is most likely due to D2 receptor activation of the indirect basal ganglia motor pathway (52, 53), and not 5-HT_{1a} receptor activation.

In summary, the 5-HT_{1a} agonist sarizotan is an effective pharmacologic approach for the respiratory disturbances in mouse models of RTT. When used orally over a 7- or 14-day period, there was no indication of desensitization, and the locomotor inhibitory effects seen with an acute administration were not observed. We support the use of a 5-HT_{1a} receptor agonist in a new trial for treating RTT. ■

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