Supporting Information

Abdala et al. 10.1073/pnas.1012104107

SI Materials and Methods

Animals. All animal procedures conformed to the United Kingdom Animals (Home Office Scientific Procedures) Act of 1986 and were approved by the University of Bristol ethical review committee and were in agreement with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (1) and were approved by the Oregon Health and Sciences University Institutional Animal Care and Use Committee. Both heterozygous female and null male mice and their WT li.ttermates were of the B6.129P2(C)-Mecp2$^{tm1.1Bird}$ strain. Mice were genotyped using a published method (2).

In Situ Arterially Perfused Brainstem–Spinal Cord Preparation. Studies were performed using the in situ arterially perfused brainstem–spinal cord preparation of mice (WT and heterozygotes) as described previously (3). Mice (18–25 g) were heparinized (5,000 U i.p.) and subsequently anesthetized deeply with halothane until loss of their paw and tail withdrawal reflex. Mice were decerebrated precollicularly, anesthesia withdrawn, and bisected subdiaphragmatically. The head and thorax were immersed in ice-chilled carbogenated Ringer's solution. The left thoracic phrenic (PN), right (mostly) cervical vagus (cVN), right hypoglossal (HN), and right ventral root spinal (T_{13} or L_1) abdominal (AbN) nerves were isolated and cut distally. In a recording chamber a double lumen catheter (Sherwood Davis & Geck; 3.5Fr, 23/20ga) was inserted into the descending aorta for retrograde perfusion. Perfusion was driven by a peristaltic roller pump (Watson Marlow 505D) and consisted of carbogenated Ringer's solution at 32 °C (for constituents, see below). The second lumen of the catheter was used to monitor aortic perfusion pressure. The baseline perfusate flow was preset between 15 and 19 $mL \cdot min^{-1}$ according to the size of the mouse.

Electrophysiological Recordings. Simultaneous recordings of PN, cVN, HN, and AbN motor activities were obtained with bipolar suction electrodes mounted on individual 3D micromanipulators. Nerve activities were AC amplified (20k), band-pass filtered (60 Hz–3 kHz) rectified, and integrated (50 ms time constant) online (Spike 2 software; Cambridge Electronic Design). All electrophysiological data were digitized (3–10 kHz; Cambridge Electronic Design A-D converter) with Spike 2 software and analyzed offline.

Solutions and Pharmacological Agents. The composition of the Ringer's solution was (in mM): NaCl (125); $NaHCO_3$ (24); KCl (3); $CaCl_2$ (2.5); $MgSO_4$ (1.25); KH_2PO_4 (1.25); glucose (10); pH 7.35–7.4 after carbogenation (5–6% CO_2 and 95–94% O_2). Osmolality was 290 ± 5 $mOsm \cdot kg\ H_2O^{-1}$. Ficoll (1.25%; type 70) was added as an oncotic agent. Unless stated, all chemicals were from Sigma-Aldrich. Vecuronium bromide (4 $\mu g \cdot mL^{-1}$; Organon Teknica) was added to the perfusion solution to block neuromuscular transmission.

Pleural Pressure Recordings in Freely Moving Mice. Under general inhalation anesthesia (1.5% isoflurane in oxygen) a midline abdominal incision was made and the liver gently retracted. A ligature was passed around the esophagus at its entrance to the stomach and the stomach retracted to allow visualization of the lower esophagus. A 25-gauge hypodermic needle was used to make an incision in the longitudinal muscle of the esophagus, and with a blunt probe a tunnel was made between the outer and inner layers of the muscle. This tunnel extended beyond the diaphragm into the thoracic cavity. The tip of a catheter attached to a pressure transducer (model PA-C10; Data Sciences International) was advanced into the thoracic cavity and secured by gluing it to the entry point in the longitudinal muscle. The suture rib of the pressure transducer was incorporated into the abdominal musculature closure and the skin approximated with a subcuticular suture. This surgical procedure was adapted from published reports (4, 5). Mice were allowed 5–7 d to recover before any studies were performed. Studies were performed with the mouse freely moving in its home cage. Data were obtained at 2 KHz and analyzed offline using custom-written functions in Igor Pro (WaveMetrics).

Plethysmography. Respiratory pattern in null males was determined in a body plethysmograph. Individual unanesthetized animals were placed in a 65-mL chamber with their head exposed through a close-fitting hole in Parafilm. A pneumotachograph (6) was connected to the chamber and a differential pressure transducer (model PT5A; Grass Instrument). The chamber was calibrated by injecting known volumes of air. The analog signal from the transducer was amplified, converted to digital, displayed on a monitor, and stored to disk by computer for later analysis. Data were sampled at 200 Hz and analyzed offline using custom-written functions in Igor Pro.

Chronic Pharmacological Treatment. Respiratory pattern was examined at weekly intervals in Mecp2$^{-/y}$ males until they developed an abnormal pattern (approximately postnatal day 40). Under general anesthesia (1.5% isoflurane in oxygen) a 100-μL volume osmotic pump (Alzet model 1004) was placed in the peritoneal cavity through a midline abdominal incision. The pump contained 8-OH-DPAT (20 mM or 6.25 μg/μL) and delivered 0.11 μL/h for 28 d.

Protocols. ***In situ experiments.*** Initial observations in both WT and Mecp2$^{-/+}$ female mice showed that the incidence of apneas between 30 and 60 min after initiating perfusion exceeded those in the first 30 min. Therefore, baseline data were taken beginning after 30 min of arterial perfusion. After baseline a GABA uptake blocker, 1-[2-[[(diphenylmethylene)imino]oxy] ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride (NO-711) (5.2 μM), was added to the perfusate and data obtained for the following 60 min. In separate studies, 10 min after adding NO-711, 8-hydroxy-dipropyl-aminotetralin (8-OH-DPAT) (0.1 μM), a 5-HT_{1a} agonist, was added to the perfusate and data obtained for the next 60 min. In the NO-711 plus 8-OH-DPAT experiments, after both drugs the 200-mL recirculating perfusate was washed out with 400 mL of fresh Ringer solution and a further 30–45 min of data obtained.

Telemetry experiments. Mecp2$^{-/+}$ animals were on a 12/12-h light/dark cycle with lights out at 1000 hours. Respiratory pattern was obtained from 1000 to 1300 hours. On separate days either vehicle or NO-711 (1.0 mg/kg), or 7-chloro-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2(^{1}H)-one (diazepam) (0.5 mg/kg), or L-838,417 (20 mg/kg) was given i.p. at 1000 hours. A minimum of 48 h separated administration of drugs.

Plethysmography studies. Mecp2 null males, postnatal days 40–75, were used to determine the dose–response effect of 8-OH-DPAT (75–300 μg/kg, i.p.) on respiratory patterns. Baseline or drug data were obtained over 30 min after accommodating to the chamber. Baseline and drug studies were separated by either 2 or 24 h.

Analysis. The in situ experiments were analyzed using Spike 2 (Cambridge Electronic Design) with custom-written scripts for measurements of nerve activity: PN apnea, defined as expiratory duration of 1.0 s or longer, duration of PN apnea, and area of tonic HN discharge during PN apnea. These parameters were determined for the entire baseline and postdrug periods. The irregularity score was determined from absolute $((TTOT_N - TTOT_{n+1})/TTOT_{n+1})$ (7). The telemetry and plethysmography studies were analyzed in 5-min blocks and averaged using custom functions in Igor Pro. Apnea was defined as above and periodic breathing as an episode of three or more cycles of 3–30 breaths separated by respiratory pauses. The latter was defined as an interval equal to or greater than twice the normal intervals. Individual comparisons, for example WT compared with Mecp2-deficient, were made with unpaired Student's t test. Multiple comparisons, for example effects of two or more drugs, were made with two-way repeated-measures ANOVA with strain and treatments as the two factors, followed by Newman–Keuls post hoc test. $P < 0.05$ was considered significant. Results are given as means ± SEM.

1. Guide for the Care and Use of Laboratory Animals, National Academy Press, Washington, D.C. 1996.
2. Miralvès J, Magdeleine E, Joly E (2007) Design of an improved set of oligonucleotide primers for genotyping MeCP2tm1.1Bird KO mice by PCR. *Mol Neurodegener* 2:16.
3. Paton JF (1996) A working heart-brainstem preparation of the mouse. *J Neurosci Methods* 65:63–68.
4. Murphy DJ, Renninger JP, Gossett KA (1998) A novel method for chronic measurement of pleural pressure in conscious rats. *J Pharmacol Toxicol Methods* 39:137–141.
5. Bissonnette JM, Knopp SJ (2008) Effect of inspired oxygen on periodic breathing in methy-CpG-binding protein 2 (Mecp2) deficient mice. *J Appl Physiol* 104:198–204.
6. Mortola JP, Noworaj A (1983) Two-sidearm tracheal cannula for respiratory airflow measurements in small animals. *J Appl Physiol* 55:250–253.
7. Viemari JC, et al. (2005) Mecp2 deficiency disrupts norepinephrine and respiratory systems in mice. *J Neurosci* 25:11521–11530.
8. Guy J, Hendrich B, Holmes M, Martin JE, Bird A (2001) A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. *Nature genetics* 27:322–326.

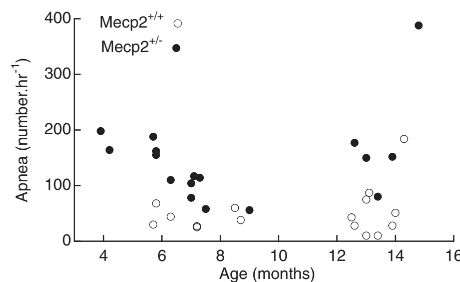

Fig. S1. Incidence of apnea in Mecp2 female mice studied in situ as a function of age.

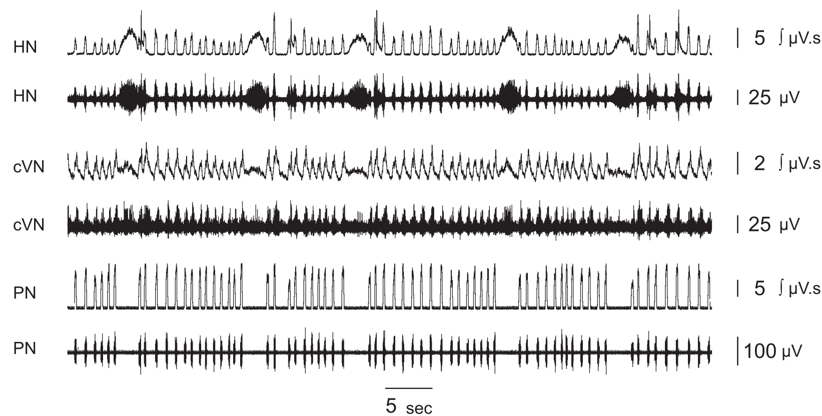

Fig. S2. Periodic breathing in a $Mecp2^{-/+}$ mouse studied in situ. The respiratory pauses, defined as absence of PN activity for an expiratory time ≥2× that of normal PN burst interval, are characterized by prolonged postinspiration in cVN and tonic activity in HN.

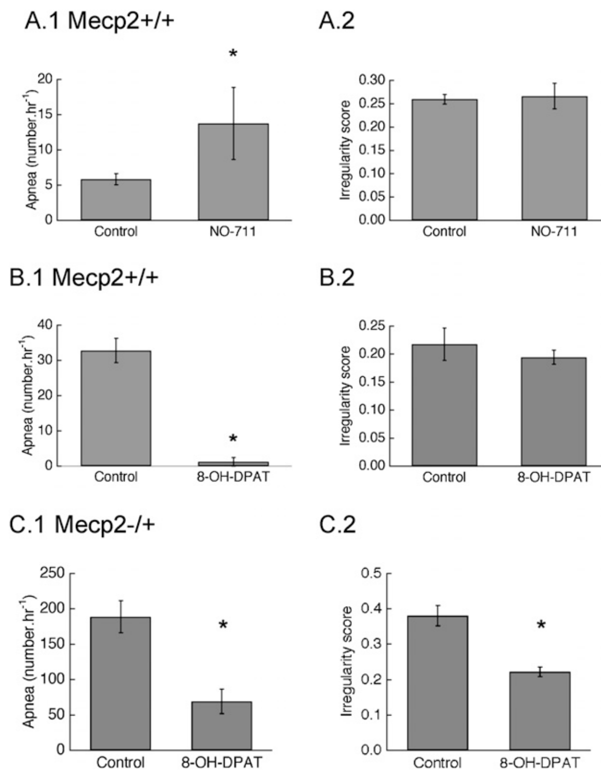

Fig. S3. Effect of GABA reuptake block and of serotonin 1a agonist on respiratory pattern in Mecp2 female mice. (*A*) Effect of NO-711 (1.0 mg/kg) in Mecp2$^{+/+}$ mice. (*A.1*) Effect on apnea. *Greater than control ($P = 0.02$) ($n = 5$, paired *t* test). (*A.2*) Effect on irregularity score. (*B*) Effect of 8-OH-DPAT (0.5 μM in perfusate) in Mecp2$^{+/+}$ mice studied in situ. (*B.1*) Effect on apnea. *Less than control ($P = 0.0017$) ($n = 5$, paired *t* test). (*B.2*) Effect on irregularity score ($P = 0.54$). (*C*) Effect of 8-OH-DPAT (50 μg/kg i.p.) in Mecp2$^{-/+}$ mice. (*C.1*) Effect on apnea. *Less than control ($P = 0.002$) ($n = 4$, paired *t* test). (*C.2*) Effect on irregularity score. *$P = 0.053$.

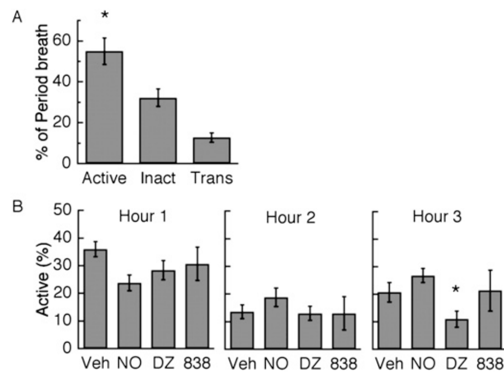

Fig. S4. Effect of blocking GABA reuptake and of allosteric potentiation of $GABA_A$ receptors on activity in freely moving Mecp2$^{-/+}$ mice. (*A*) Percentage of periodic breathing episodes that occurred while animals were active, inactive (Inact), or transitioned between the two behavioral states (Trans). *Greater than inactive ($P = 0.019$) and transition ($P = 0.0004$) ($n = 5$, one-way ANOVA and Tukey all pairs comparison); **greater than transition ($P = 0.041$). (*B*) Percentage time active in the 3-h study as a function of vehicle (Veh) or drug administration (NO, NO-711; DZ, diazepam; 838, L-838,417). *Less than NO-711 ($P = 0.018$) and trend less than vehicle ($P = 0.059$) (two-way repeated-measures ANOVA with treatment and time as the two factors). Activity was determined from the signal strength of the telemetry transmitter.

Fig. S5. Representative traces from male mice obtained with body plythsmography. Respiratory patterns are similar to those seen in female mice obtained from telemetry recordings of pleural pressure in freely moving animals (Fig. 2*A*, main text).

Fig. S6. Effect of combined GABA reuptake block and serotonin 1a agonist on activity in WT and Mecp2-deficient female mice. Activity and number of vertical rears determined as previously described (8). (*A*) Activity. Active minutes per 5-min observation. C1, first control, observation; N+D, NO-711 (1.0 mg/kg), 8-OH-DPAT (50 μg/kg); C2, second observation period; Veh, vehicle. *Significantly different from $Mecp2^{+/+}$ ($P = 0.044$ and 0.007) (two-way repeated-measures ANOVA with strain and treatment as the two factors); **significantly different from drugs and vehicle ($P = 0.049$ and 0.006); ***significantly different from vehicle ($P = 0.002$). (*B*) Vertical rears. Number of vertical rearing movements per 5-min observation period. Abbreviations as in *A*. *Significantly different from drugs, control 2, and vehicle (*P* values between 0.022 and <0.001); **significantly different from drugs and vehicle ($P = 0.02$ and 0.015).

Fig. S7. Effect of combined GABA reuptake block and serotonin 1a agonist on activity in WT and Mecp2 null male mice. Activity and number of vertical rears determined as previously described (8). (*A*) Activity. Active minutes per 5-min observation. C1, first control, observation; N+D, NO-711 (1.0 mg/kg), 8-OH-DPAT (150 μg/kg); C2, second observation period; Veh, vehicle. The order of drugs and vehicle were varied. *Significantly different from $Mecp2^{+/y}$ (*P* values between 0.010 and 0.014) (two-way repeated-measures ANOVA with strain and treatment as the two factors). There was no effect of treatment for either $Mecp2^{+/y}$ or $Mecp2^{-/y}$. (*B*) Vertical rears. Number of vertical rearing movements per 5-min observation period. Abbreviations as in A. *Significantly different from $Mecp2^{+/y}$ (*P* values between 0.001 and 0.03); **different from C1 and C2 ($P < 0.001$). For $Mecp2^{-/y}$ there was no effect of drug ($P = 0.109$) or vehicle ($P = 0.161$).