Supporting Online Material for

Impaired Respiratory and Body Temperature Control Upon Acute Serotonergic Neuron Inhibition

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MATERIALS AND METHODS

VECTOR CONSTRUCTION AND MOUSE DERIVATION
The $RC::FPDi$ targeting vector was derived from our $pRC::FPmodular$ plasmid which contains $CAG$ promoter/enhancer sequence (44), an $FRT$-flanked cassette consisting of $PKG-neo$ sequence (for positive selection of homologous recombinants) and the $lox^+$ transcriptional stop cassette derived from pBS302 (45), a $loxP$-flanked cassette containing mCherry-encoding sequence followed by a concatemer of SV40pA stop sequences (46), and followed by sequence encoding HA-tagged Di (Di template plasmid (47) provided by Dr. Bryan Roth). Once validated in cell culture for recombination efficacy, this entire transgene was inserted into the p15a_Kan_R26 plasmid containing $R26$ sequence as targeting homology. The completed targeting vector, $pRC::FPDi$, was linearized and electroporated into ES (Tc-1) cells and resulting gancyclovir resistant colonies screened by PCR for homologous recombination at the $R26$ locus; 3' recombination detection: primer RR712 – GGGCGTACTTGGCATATGAT and primer RR754 – CGCCTAAAGAAGAGGCTGTG amplifying a 1472 bp product; 5' recombination detection: primer RR751 – CCAGATTGTGACGAAGCAGA and primer RR763 – TCTCCCCTCAGAGAAATGGA, amplifying a 4844 bp product. Using standard methods, ES cells from a single recombinant ES clone were used to derive $RC::FPDi$ chimeric mice, and germline derivation achieved by crossing to C57BL/6J mice. $RC::PDi$, $RC::FDi$, and $RC::Di$ were derived by crossing $RC::FPDi$ mice to either 129S4/SvJaeSor-Gt(ROSA)26Sor$^{tm1(FLP1)Dym}$ mice (48) to remove the $FRT$-flanked stop cassette, or to Tg($ACTB$-cre)2Mrt (49) to remove the $loxP$-flanked stop cassette.

IMMUNOHISTOCHEMISTRY
Anesthetized $RC::PDi$; $RC::rePe$; Slc6a4-cre mice (N = 3) were perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were extracted, soaked in 4% paraformaldehyde at 4°C for 2 h, cryoprotected in 30% sucrose/PBS and then cryosectioned (40 µm) and processed as floating sections. Sections were bleached in 1% H2O2/PBS for 30 min, blocked with 5% donkey serum/0.1% TritonX-100/PBS and then reacted with an anti-HA antibody for 72 h at 4°C. Biotin-conjugated donkey anti-rabbit secondary antibody was reacted with avidin-biotin complexing reagents and developed with tyramide conjugated Alexafluor 647 (far red channel) for visualization. Similarly, GFP was detected using a chick anti-GFP antibody and visualized with a FITC-conjugated donkey anti-chick antibody (green channel). Tph2 (rate limiting enzyme in serotonin synthesis in the CNS, thus a key serotonergic neuron identity marker) was detected using a rabbit polyclonal antibody and visualized by donkey anti-rabbit antibody conjugated to Cy5 (far red channel). Cell nuclei were visualized with DAPI, 4',6-diamidino-2-phenylindole.

PLETHYSMOGRAPHY AND EKG
Awake, freely moving mice were assayed for respiratory parameters and heart rate using continuously recorded whole body plethysmography and EKG. For double transgenic $RC::PDi$; Slc6a4-cre mice and sibling controls, mice were placed in a room-air plethysmograph chamber held at 34°C, acclimatized for 20 min of which recordings from the last 5 min (window “a” in Fig. 3A) were used later for data analyses. Chamber gases were then shifted to 5%CO2 (in air) for 15 min, of which the last 5 min of recordings (window “b” in Fig. 3A) were used for data analyses. Chamber gas was then switched back to room air for 15 min, mice were removed briefly for an intraperitoneal injection of CNO dissolved in saline (1 mg/ml) to an effective concentration of 10mg/kg CNO and then returned immediately to the room-air chamber for an additional 10 min of recordings, of which the last 5 min (window “c” in Fig. 3A) were used for data analyses. Mice were then exposed to a 5%CO2 gas mixture for 15 min, of which the last 5 min of recordings (window “d” in Fig. 3A) were used for data analyses.
In these plethysmography experiments, respiratory airflow was assayed in a 140 ml water-jacketed temperature controlled glass chamber attached to a differential pressure transducer and reference chamber. Water temperature was warmed to 35.1°C, resulting in a chamber temperature of 34°C (thermoneutral zone for mouse) as required to maintain constancy in respiratory and metabolic parameters from trial to trial as well as to maintain linearity of ventilatory pressure changes due to humidification, rarefaction, and thermal expansion and contraction during inspiration and expiration. Gas flow through the chamber was regulated by inline pre- and post-gas flow meters. Flow rate was ~ 325 ml/min. Volume calibrations were performed by repeated injections of a known volume from a 1 ml syringe. Oxygen consumption was determined by measuring the difference between oxygen concentration of gas into the chamber and gas exiting the chamber with an oxygen sensor and oxygen analyzer. Humidified gas flow into the chamber was either room air or a 5% CO₂ mix, balanced with air (medical grade) as needed for each experiment. For EKG, the ventral thoracic region was prepped with a depilatory cream. A vest made from stretchable bandage material was used to hold the EKG electrodes to the chest with EKG conductive paste. EKG was measured by a telemetry amplifier and transmitted to a base station for recording. Mice were assayed for core body temperature both before and after recording in the chamber. Readings from the pressure transducer, oxygen sensor and EKG were acquired at 1kHz and analyzed for peak amplitude, peak frequency, and average voltage off-line. Respiratory volume was determined by the following formula

\[
\frac{\{A/B\}C\times [(D+273.15)\times (F-H)]}{[(D+273.15)\times (F-H)]\times [(E+273.15)\times (F-G)]}
\]

Where:
A = peak of breath signal in volts
B = peak of signal for injection volume
C = volume injection (in ml)
D = mouse body temperature (in °C)
E = chamber temperature (in °C)
F = barometric pressure (in mmHg)
G = pressure of water vapor of mouse = 1.142 + (0.8017*D) - (0.012*D²) + (0.0006468*D³)
H = pressure of water vapor of chamber = 1.142 + (0.8017*E) - (0.012*E²) + (0.0006468*E³)

Results were compared using two-way RM ANOVA followed by Tukey post-hoc analysis for RC::PDi; Slc6a4-cre ventilation experiments, and paired t-test was used for comparison of oxygen consumption. RC::FDi; Pet1::Flpe minute ventilation experiments were analyzed in the same way as RC::PDi; Slc6a4-cre ventilation experiments. Standard error of the mean (SEM) is shown for all plethysmographic data.

For RC::Di genotypes and sibling controls, mice were placed in a room-air plethysmograph chamber held at 34°C, again as required to maintain constancy in respiratory and metabolic parameters from trial to trial as well as to maintain linearity of ventilatory pressure changes due to humidification, rarefaction, and thermal expansion and contraction during inspiration and expiration. Mice were acclimatized for 15 min, and then assayed for respiratory and heart rate values for 5 min. Mice were then briefly removed from the chamber for intraperitoneal injection of CNO dissolved in saline (1mg/ml) to an effective concentration of 10mg/kg. Mice were immediately returned to the chamber and respiratory and heart rate assessments resumed.

**TEMPERATURE ASSAYS:**
For RC::PDi; Slc6a4-cre experiments, temperature was taken rectally with a lubricated thermocouple probe. Mice were weighed and temperature was taken before 10mg/kg CNO administration. After CNO intraperitoneal injection, temperature was taken every 10 min for the
first half hour and every half hour thereafter until all mice recovered to a temperature of at least 36°C or higher. Because of the extremely long duration of the temperature assays to examine recovery, mice were held in standard cages at room temperature 21-23°C with water and food ad libitum and no 4°C cold challenge was used. For initial RC::FDi; PetI::Flpe; experiments, mice were weighed and temperature taken before CNO administration (10mg/kg). After CNO administration, using a protocol modeled after Hodges et al. (50) mice were kept at room temperature for 30 min, their temperature taken, and they were then transferred to pre-chilled cages in a 4°C cold room. Body temperature was then assessed every 10 min for the first hour and every half hour for the second hour. The experiment was concluded after two hours of 4°C exposure.

The results between RC::PDi; Slc6a4-cre and control siblings were compared using an unpaired t-test for each temperature assessment, similarly for RC::FDi; PetI::Flpe and control mice. For comparing recovery times and lowest achieved temperature across trials in RC::PDi; Slc6a4-cre experiments, a one-way repeated measures ANOVA analysis was used. SEM is shown on the graphs.

PRIMARY NEURONAL CULTURES
For primary mouse neuronal cultures, a ventral wedge shaped block of tissue (approximately 1.5-2mm per side with the apex oriented dorsally and the base spanning the ventral medullary surface) running the length of the medulla and caudal pons was dissected from postnatal (P0-P3) mice into ice-cold HEPES buffer (in mM: NaCl 130, KCl 4, MgCl$_2$ 1, CaCl$_2$ 1.5, HEPES 10, Dextrose 10, NaOH 3), digested for 30 minutes at 37°C in HEPES buffer with 1.3% papain, washed with complete MEM (MEM with 10% FBS, 1% Pen/Strep) with 0.15% trypsin inhibitor and 0.15% bovine serum albumin, triturated with a fire polished Pasteur pipette, and plated on 12mm round, poly L-ornithine pretreated coverslips in 12-well plates. After allowing cells to settle onto the coverslips for 45-60 min, complete MEM conditioned by mouse glial cultures was added. After 24 h, half of the medium for each well was exchanged with Neurobasal medium with B27 (1:50 Neurobasal), FGF-5 (10ng/ml) and BFGF (1ng/ml). After a week of culturing, supportive glial beds had formed and Ara-C (0.5-1µM) was added to attenuate glial growth. Cells were maintained at 37°C and 5% CO$_2$ with weekly half-medium changes with Neurobasal/B27 medium for 4-12 weeks.

ELECTROPHYSIOLOGY
Neuron cultures were placed in a recording chamber mounted on a fixed-stage upright microscope equipped with 5x and 40x water immersion objectives, Nomarski optics, epi-fluorescence illumination and filter sets for detection of GFP. The recording chamber was continuously superfused (2.5-3ml/min) at room temperature with one of the solutions described below. Epi-fluorescence microscopy was used to identify GFP+ neurons. Patch electrodes were fabricated from capillary glass on a horizontal pipette puller to a DC resistance of 5-10MΩ after filling with intracellular solutions and connected to the headstage of an amplifier.

A CNO dose of 30µM was initially calculated as comparable to the 10mg/kg dose given to the mice during in vivo assays assuming ~70% of body weight is soluble to CNO and CNO is evenly distributed. As in vitro assays progressed, we found that 1µM CNO elicited similar responses. Except where otherwise noted, all electrophysiological assays used 1µM CNO concentrations.

For experiments measuring the current-voltage relationship, patch pipettes filled with intracellular solution (in mM: KOH 135, methanesulfonic acid 135, KCl 10, Hepes 5, EGTA 1, ATP 3, GTP 0.5, pH7.2) were used to form a gigaseal. The membrane was then ruptured to allow whole-cell access. Experiments were carried out in voltage clamp mode using pCLAMP 10 software. A
A subset of neurons were first spontaneously active, depolarizing current was injected such that baseline firing rate was 1 µM CNO. However, 1 µM CNO elicited similar responses in this assay. No differences were seen, thus data using 30 µM CNO, neurons were perfused with standard Ringer’s supplemented with BaCl2 and the firing rate during the second CNO application (with BaCl2) were normalized to the firing rate in standard Ringer’s supplemented with BaCl2. These ratios were represented as percentages and graphed with standard error of the mean. Data was compared with a Friedman test.

To test CO2/pH sensitivity, changing the bath solution to 9% CO2 / 91% O2 resulted in a change in extracellular pH from 7.4 to approximately 7.16 while bath pH was continuously measured with a pH electrode at the inflow to the recording chamber. Recordings were made from cultures 20-35 days in vitro, a range when serotonergic neurons demonstrate the maximum chemosensitivity index (51). Peak firing rates during 9% CO2 were determined for 1) challenges pre-CNO (baseline aCSF), 2) challenges during CNO application plus 3 challenges after CNO application (aCSF + CNO) and 3) the last 2 challenges of the recording (aCSF WO). For each neuron, the averages for condition 2 (aCSF + CNO) and 3 (aCSF WO) were normalized to condition 1 (baseline aCSF). The first half of the data was collected using 30 µM CNO, however, as stated above, we found that 1 µM CNO elicited similar responses in this assay. No differences were seen, thus data using 30 µM and 1 µM CNO was grouped for this assay. Data was compared using a Friedman test. A Mann-Whitney test was used for comparison of RC::FPDi; RC::rePe; Slc6a4-cre and RC::rePe; Slc6a4-cre responses to CNO.

In all cases, data are shown as mean ± SEM

Sup. Fig. 1. CNO/Di-inhibition of Pet1::Flpe-defined serotonergic neurons disrupts the central respiratory CO2 chemoreflex in conscious adult mice. Double transgenic RC::FDi; Pet1::Flpe mice were assessed as in Fig. 3, with plethysmograph chamber temperature held at 34ºC. (A) RC::FDi; Pet1::Flpe showed reduced hypercapnic ventilatory responses after administration of CNO (10 mg/kg), *p = 0.001 (RM ANOVA followed by Tukey post-hoc). (B) Sibling controls. CNO administered trials shifted slightly to the right for clarity.
Sup. Fig. 2. Reduced firing rate response to 9% CO₂ in RC::PDi; RC::rePe; Slc6a4-cre neurons upon 1μM CNO application. Traces of firing rate (Hz) and simultaneous bath pH from cultured medullary serotonergic neurons from control RC::rePe; Slc6a4-cre mice (top) and RC::PDi; RC::rePe; Slc6a4-cre mice (bottom), similar to Fig. 3 (E).
Sup. Fig. 3. CNO/Di-inhibition of Pet1::Flpe-defined serotonergic neurons results in reduced ability to thermo-regulate. Body temperature at room temperature (~23°C) was assessed 5 min before a single CNO intraperitoneal injection (10 mg/kg) and then again after 30 min. Mice were then subject to ambient conditions of 4°C with body temperature measurements taken every 10 min for the next hour and then every 30 min. RC::FDi; Pet1::Flpe male mice showed an inability to regulate body temperature as compared to control siblings, *p < 0.01 (unpaired t-test).
Sup. Fig. 4. Broad constitutive Di expression is compatible with development and triggers respiratory and cardiac arrest upon CNO injection. (A) Example trace showing that pre-CNO, RC::Di adult mice have normal respiratory and heart rates (52) (154 ± 15 (SEM) breaths/min and 478 ± 57 (SEM) beats/min; animals analyzed = 4) that depress severely (55 ± 11 breaths/min, 70 ± 15 beats/min) within 10 min of CNO injection (10mg/kg i.p.). (B) An example full respiratory and heart rate trace showing heart rate reduction within 30 s of CNO injection, with cardiac arrest occurring within 20 minutes (4 minutes after respiratory arrest).