Chemogenetic Inhibition of Serotonergic Neurons Reveals Their Key Role in Physiological Homeostasis

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Introduction: Serotonergic Involvement in Homeostasis

Normal mammalian cell and organism function requires relative constancy around optimal internal physiological conditions. Maintaining this dynamic equilibrium involves neuronal networks affecting numerous brain and body systems. One such homeostatic reflex, the respiratory chemoreflex, controls ventilation in response to deviations in arterial and brainstem pH/PCO_2 (partial pressure of CO2) (Haldane and Priestley, 1905; Leusen, 1954; Kety and Forster, 2001; Smith et al., 2006; Guyenet et al., 2010; Nattie, 2011). Various classes of brainstem cells (Mitchell et al., 1963; Elam et al., 1981; Williams et al., 2007; Li et al., 2008; Gourine et al., 2010; Nattie, 2011) have been implicated, and integration and redundancy among components have been found to be likely essential. Serotonergic neurons of the lower brainstem have been proposed as one critical constituent (Richerson, 1995; Wang and Richerson, 1999; Corcoran et al., 2009; Hodges and Richerson, 2010; Depuy et al., 2011; Nattie, 2011). These neurons also have been implicated in other homeostatic circuitry, such as the thermoregulatory network for maintaining body temperature (Cano et al., 2003; Hodges et al., 2008; Morrison et al., 2008). Fatal or life-threatening clinical disorders of homeostatic dysfunction also point to serotonergic involvement, as in the sudden infant death syndrome (SIDS) (Kinney et al., 2009; Duncan et al., 2010) and serotonin syndrome (Sternbach, 1991). Direct evidence demonstrating a requirement for serotonergic neurons in homeostasis, however, is only now emerging (Hodges et al., 2008; Depuy et al., 2011) as tools with sufficient resolving power become available.

A Genetic Tool for Suppressing Neuronal Excitability

To test whether serotonergic neuron activity is critical to homeostatic control, we engineered a genetic tool, allele *RC::FPDi* (Fig. 1A), for suppressing neuronal excitability in conscious mice inducibly, reversibly, with cell-subtype precision, and with minimal invasiveness. We used conditional intersectional genetics (Awatramani et al., 2003; Jensen et al., 2008; Kim et al., 2009) to switch on the expression of the synthetic receptor Di (DREADD, hM_4D) (Armbruster et al., 2007, in which DREADD stands for "designer receptors exclusively activated by designer drug"). This $G_{i/o}$-protein coupled receptor (GPCR) offers engineered selectivity for the biologically inert synthetic ligand clozapine-*N*-oxide (CNO) while being refractive to

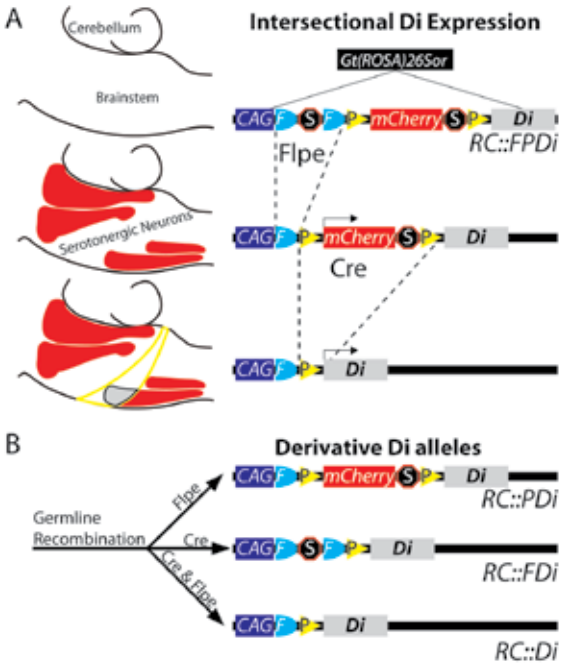

Figure 1. Cell-selective Di expression via *RC::FPDi*. *A,* This *Gt(ROSA)26Sor* knock-in allele consists of *CAG* regulatory elements, an *FRT*-flanked transcriptional Stop, a *lox*P-flanked mCherry-Stop, and HA-tagged-Di-encoding sequence. A hypothetical example (left) illustrates intersectional restriction of Di to a serotonergic neuron subset (gray area, lower schema) after Flpe and Cre recombination; mCherry marks Flpe-only cells (red areas represent serotonergic nuclei); neither Di nor mCherry are expressed in Cre-only cells (yellow circumscribed area). ***B,*** Derivative Di alleles.

endogenous ligands. Like endogenous $G_{i/o}$-GPCRs, the binding of CNO by Di has been shown capable of triggering cell-autonomous hyperpolarization and diminished cell excitability (Mark and Herlitze, 2000; Armbruster et al., 2007; Ferguson et al., 2011). Our tool, *RC::FPDi*, a knock-in *ROSA26* allele utilizing the CAG promoter (Dymecki and Kim, 2007), offers nonviral means of Di expression. It exploits the dual-recombinase methodology that endows Cre and Flpe transgenics with the potential to switch on Di expression in combinatorially defined neuron subtypes, as previously demonstrated for various intersectional alleles (Awatramani et al., 2003; Jensen et al., 2008; Kim et al., 2009).

To meet the needs for single recombinase-mediated Di expression (versus the dual-recombinase strategy), we generated derivatives of *RC::FPDi* (Fig. 1*B*): *RC::PDi* requires only Cre to switch on Di expression because the *FRT*-cassette was excised in the ancestral germline; reciprocally, *RC::FDi*

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requires only Flpe to switch on Di expression. By partnering *RC::PDi* with *Slc6a4-cre*, we expressed Di in virtually all serotonergic neurons and a small subset of thalamic neurons. We also incorporated a Cre-responsive GFP allele, *RC::rePe*, to fluorescently visualize the *Slc6a4-cre* lineage, thereby facilitating validation of lineage-specific Di expression as well as the electrophysiological study of Di-expressing neurons (Fig. 2).

Modulating Serotonergic Neuron Activity

To test Di in modulating serotonergic neuron activity, as driven by *RC::PDi*, we made current–clamp membrane potential recordings (Fig. 2) from lower brainstem serotonergic neurons cultured from *RC::PDi; Slc6a4-cre* mice. Once again, these were coupled with *RC:rePe* to fluorescently identify Cre-expressing, and thus, Di-expressing neurons. CNO reduced the firing rate of Di-expressing neurons by ~40% on average (Fig. 2*D*). This reduction was similar to that successively observed in the same neurons following application of 8-OH-DPAT (Figs. 2*C, D*). 8-OH-DPAT is an agonist for the endogenous inhibitory serotonin autoreceptor $5HT_{1A}$, which is a GPCR known to act through $G_{i/o}$ and Kir3 channels to inhibit serotonergic neuron excitability (Penington et al., 1993; Mark and Herlitze, 2000).

The response onset to CNO occurred on average 1.3 ± 0.1 (SEM) min after switching the perfusion port from artificial cerebrospinal fluid (aCSF) to CNO, with bath superfusate exchange taking ~1 min, suggesting response onset within seconds. Peak responses to CNO occurred 2.8 ± 0.3 (SEM) min after port switching, thus, within ~2 min of CNO exposure. The CNO-induced suppression of action potential firing reversed on return to control superfusate (Figs. 2*A–D*). This reversal reached full recovery on average in 12.4 ± 2.9 (SEM) min (though with notable variation from 0.9 to 25.4 min), loosely correlating with duration of CNO exposure. The CNO-induced suppression could be blocked by the potassium-channel inhibitor barium chloride (300 μM) (Figs. 2*E, F*), consistent with a Kir3 mechanism of neuron inhibition. Neuron-to-neuron variation in firing-rate suppression occurred not only in response to CNO (0.58 ± 0.07 [SEM], normalized to baseline values pre-CNO) (Fig. 2*D*) but also to 8-OH-DPAT (0.37 ± 0.09 [SEM]), likely reflecting endogenous heterogeneity among 5-HT neuron subtypes. That is, not all serotonergic neurons express $5HT_{1A}$ autoreceptors (Bonnavion et al., 2010), nor may they all express Kir3-type channels.

The current–voltage relationships characterizing Di-expressing serotonergic neurons revealed activation, by CNO/Di, of an inwardly rectifying conductance reversing near the predicted reversal potential for potassium (predicted E_K = –81 mV; measured E_K = –78 ± 1.9 [SEM] mV) (Fig. 2G). Responsive neurons (8 of 17 in this assay) demonstrated an increase in slope conductance averaging 2.7 ± 1.1 (SEM) pS between –110 and –90 mV, and a hyperpolarizing current of 16 ± 3.6 (SEM) pA at –60 mV (Fig. 2*H*), ranging between 5 and 45 pA across potentials of –60 to –50 mV. This resting-potential range included most serotonergic neurons (Wang, Pizzonia et al., 1998; Wang and Richerson, 1999). The response to 8-OH-DPAT in the same neurons was similar: hyperpolarizing currents of 17.7 ± 4.7 (SEM) pA at –60 mV ranged between 5 and 85 pA across –50 to –60 mV, and slope conductance increased by 4.7 ± 1.3 (SEM) pS between –110 and –90 mV. Control serotonergic neurons (Figs. 2*D, H*) exhibited a similarly robust response to 8-OH-DPAT but not to CNO; this difference indicates that CNO in the absence of Di does not measurably affect membrane conductance or action potential firing rate. Collectively, these findings point to CNO/Di, like 8-OH-DPAT and the $5HT_{1A}$ autoreceptor, evoking hyperpolarizing outward potassium currents via Kir3-type channels.

Assessing serotonergic neuron activity in the respiratory chemoreflex

Hypercapnic acidosis (a decrease in tissue pH caused by PCO_2 elevation from ventilatory dysfunction or cellular metabolism) is a powerful respiratory stimulus. To assess the requirement for serotonergic neuron activity in the CO_2-driven respiratory chemoreflex, we measured the ventilatory response of adult *RC::PDi; Slc6a4-cre* mice and sibling controls to an increase in inspired CO_2 from 0% (room air) to 5% (a modest rise) before and after CNO injection (Figs. 3A–C). The typical increase in ventilation for restoring normal arterial pH/PCO_2 levels was reduced by ~50% in *RC::PDi; Slc6a4-cre* mice within minutes following CNO administration (Fig. 3*B*). Prior to CNO, CO_2 exposure had evoked a ventilatory response comparable with that seen in control siblings, indicating that Di expression alone does not affect the chemoreflex. Nor is CNO alone inhibitory, given the normal ventilatory response of CNO-treated control siblings (Fig. 3C). We obtained similar results when the *Slc6a4-cre* driver was replaced with *Pet1::Flpe* (Jensen et al., 2008), an alternative serotonergic neuron driver, and partnered with *RC::FDi*. In this way, we independently confirmed attribution of function to serotonergic neurons.

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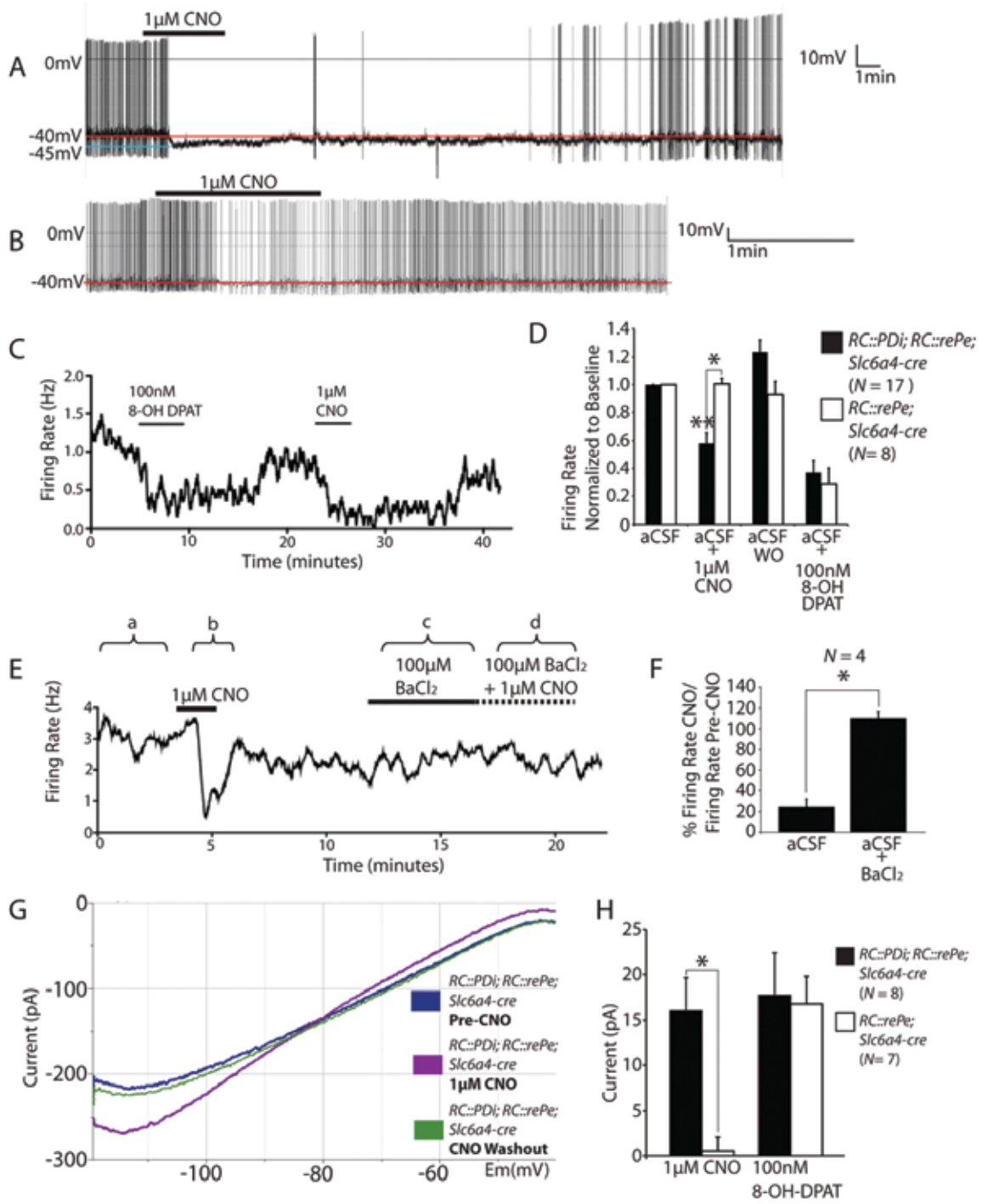

Figure 2. Inducible and reversible suppression of serotonergic neuron excitability using *RC::PDi*. ***A, B,*** Recordings of cultured medullary serotonergic neurons from *RC::PDi; RC::rePe; Slc6a4-cre* mice showing CNO-induced abolishment *(A)* or moderate suppression *(B)* of action potential firing, with recovery following return to aCSF superfusate. ***C,*** Firing rate during sequential applications of 8-OH-DPAT and CNO. ***D,*** Average firing rates (normalized to baseline ± SEM) of Di/green fluorescent protein (GFP)–expressing versus control neurons. Inhibition by CNO was observed for Di neurons compared with pre-CNO, post-CNO aCSF superfusate (aCSF washout [WO] of CNO), and CNO-exposed control neurons; $^{**}p < 0.0001$ (Friedman test); $^{*}p < 0.005$ (Mann–Whitney *U* test). CNO-inhibition was comparable with that of 8-OH-DPAT (*n = 9 RC::PDi; RC::rePe; Slc6a4-cre* neurons; *n = 8 control neurons*). ***E,*** Sample trace of $BaCl_2$-mediated block of CNO-induced suppression. ***F,*** Average ratio of firing rate for CNO versus pre-CNO, expressed as a percentage ± SEM. Neurons were assayed in aCSF (*a* and *b* in *E*) and upon subsequent application of B_aCl_2 (*c* and *d*); $^{*}p < 0.05$ (Friedman test). ***G,*** Voltage-clamp recording showing the current–voltage relationship of a Di-expressing serotonergic neuron with or without CNO. ***H,*** Average current elicited by CNO and 8-OH-DPAT at –60 mV; $^{*}p = 0.002$ (Mann–Whitney *U* test). Calibrations: ***A, B,*** 1 µM (CNO); ***C***, 100 nM (8-OH-DPAT); ***E,*** 1 µM (CNO); 100 µM ($BaCl_2$).

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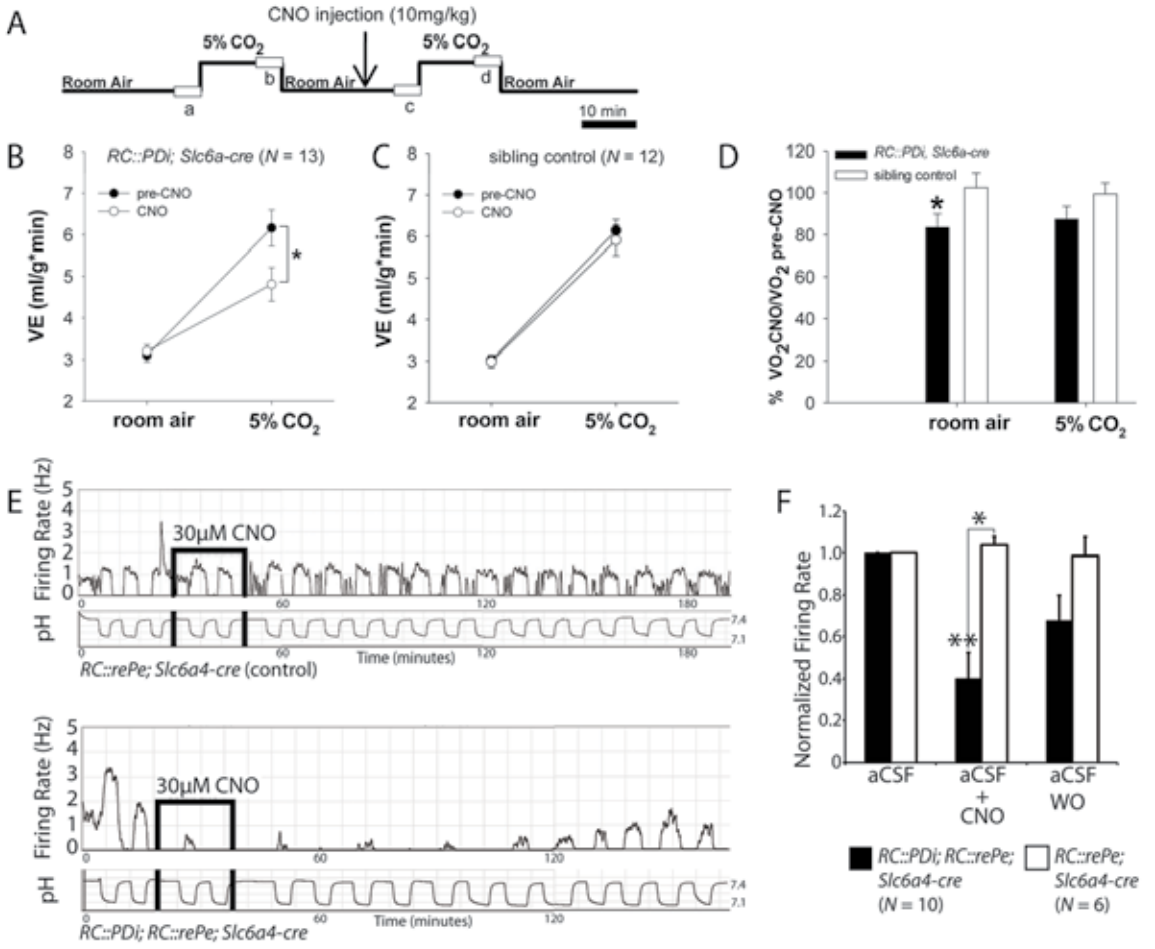

Figure 3. CNO/Di perturbation of serotonergic neurons disrupts the central respiratory CO_2 chemoreflex. ***A,*** Protocol for plethysmographic assessment at 34°C of respiratory responses to inspired CO_2 in the awake animal before and after CNO administration. Boxes *a, b, c,* and *d* represent points analyzed from continuous recordings. ***B,*** *RC::PDi; Slc6a4-cre* mice upon CNO administration showed reduced-minute ventilation responses to inspired CO_2 compared with pre-CNO baselines; $^*p = 0.002$ (repeated-measures [RM] ANOVA followed by Tukey's test). ***C,*** Pre-CNO and CNO responses to CO_2 were indistinguishable for control mice and from the pre-CNO response of *RC::PDi; Slc6a4-cre* mice. ***D,*** *RC::PDi; Slc6a4-cre* mice exhibited reduced VO_2 upon CNO administration compared with controls; $^*p < 0.05$ (paired *t* test). ***E,*** Firing rate and simultaneous bath pH recordings from cultured sarotonergic neurons from controls (top) compared with *RC::PDi; RC::rePe; Slc6a4-cre* mice (bottom). Changing CO_2 from 5% to 9% shifted pH from ~7.4 to ~7.16 and induced an increase in firing rate. In Di neurons (bottom), this response was inhibited by CNO and reversed on return to aCSF. ***F,*** Peak firing rates (normalized to baseline) in 9% CO_2-saturated aCSF were suppressed with CNO application in *RC::PDi; RC::rePe; Slc6a4-cre* neurons; $^*p < 0.05$ (Mann–Whitney *U* test).

Room air ventilation was unaffected following CNO/Di manipulation of *Slc6a4-cre*-expressing neurons (Figs. 3*A–D*). This phenomenon is reminiscent of phenotypes reported for adult mice in which embryonic deletion of the gene *lmx1b* in Pet1-expressing cells results in developmental loss of most if not all serotonergic neurons (Hodges et al., 2008). Notably, following CNO injection, oxygen consumption in room air dropped from within- to below-normal range (0.053 ± 0.003[SEM] ml/g/min to 0.043 ± 0.003[SEM] ml/g/min) in *RC::PDi; Slc6a4-cre* mice but not in controls (Fig. 3*D*). Thus, Di-mediated perturbation of *Slc6a4-cre*-expressing neurons led to a decrease in metabolic rate without proportionately matching effects on ventilation. An additional role for serotonergic neurons may thus be supported: that of influencing metabolic rate and the ability of ventilation to properly track with metabolic state.

Triggering the chemoreflex requires sensing and transducing milieu changes in pH/PCO_2 into

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cellular activity changes, e.g., in action potential firing rates capable of affecting respiratory circuit output and breathing. We found that cultured serotonergic neurons from the lower brainstem reproducibly increased their firing rate 2- to 3-fold in response to acidosis (0.2–0.3 pH units, a magnitude of physiological relevance) caused by increasing the CO_2 bubbled in the superfusate from 5% to 9% (Fig. 3*E*). This finding is consistent with previous ones in serotonergic neurons from rats (Richerson, 1995; Wang and Richerson, 1999). Approximately 70% of medullary serotonergic neurons exhibit chemosensitivity, supporting the view that not all medullary serotonergic neurons project to brainstem respiratory centers; rather, this property likely distinguishes a specific functional subset of serotonergic neurons.

Because we performed recordings under conditions of relative synaptic isolation (blockade of $GABA_A$, NMDA, and AMPA/kainate receptors by picrotoxin, AP5, and CNQX, respectively), the observed chemosensitivity may be an intrinsic property of these serotonergic neurons. CNO administration inhibited this chemoresponsiveness (1 μM CNO [data not shown] or 30 μM CNO [Figs. 3*E*, *F*]) at concentrations less than or comparable with that delivered *in vivo*, which could be restored upon return to control superfusate. Control, non–Di-expressing serotonergic neurons showed no response to CNO (Fig. 3*E*, upper panel). CNO was continuously applied over 2–3 pH cycles for an average of 20 min (versus the ~5 min exposure during firing rate assays) (Figs. 2*A–D*). Recovery times were longer (76.3 ± 60.7 [SEM] min) following this extended CNO exposure.

These data support the parsimonious model whereby a subset of serotonergic neurons in the lower brainstem acts as central respiratory chemoreceptors capable of regulating the downstream respiratory network (and thus, lung ventilation) in an attempt to restore normal arterial pH/PCO_2 (Dias et al., 2008; Hodges et al., 2008; Corcoran et al., 2009; Hodges and Richerson, 2010; Nattie, 2011). PCO_2 stabilization is also served by Phox2B-expressing glutamatergic neurons of the brainstem retrotrapezoid nucleus (Dubreuil et al., 2009; Marina et al., 2010). It will be important to determine how these and likely other (Mitchell et al., 1963; Elam et al., 1981; Williams et al., 2007; Marina et al., 2010; Nattie, 2011) neural systems integrate to control breathing, and under what developmental stages, arousal states, and conditions they may be differentially employed. Astrocytes along the brainstem surface have also been implicated as respiratory chemosensors capable of influencing retrotrapezoid neuron activity (Gourine et al., 2010; Wenker et al., 2010). So it will be important to assess whether astrocytes also influence serotonergic neurons and whether their effects on chemoreception persist in conscious animals.

Assessing serotonergic neuron activity in body temperature change

During the whole-body plethysmography (Fig. 3), chamber temperature was held at ~34°C to reduce effects of body temperature fluctuations on respiratory parameters and oxygen consumption. Under these conditions, core body temperature remained within ± 0.65°C (SEM) of baseline for controls and Di mice. Absent this thermoneutral environment, double-transgenic *RC::PDi; Slc6a4-cre* mice's body temperature dropped within minutes of CNO administration. While housed individually at room temperature (~23°C), *RC::PDi; Slc6a4-cre* mice's body temperature dropped from 36.9 ± 0.2°C (SEM) to 30.33 ± 0.2°C (SEM) within 30 min of CNO injection, dipping to 27.1 ± 0.9°C (SEM) by ~2.5 h (Fig. 4*A*). After this time, recovery ensued, with restoration to within normal range by 11.8 ± 1.3 h (SEM). Sibling controls receiving CNO showed normal thermoregulation (37.2 ± 0.03°C [SEM]).

These findings establish that *Slc6a4-cre*–expressing neuron activity is required for adult thermal homeostasis and that most thermoregulatory capacity is lost, given the near equilibration of body temperature to ambient room temperature. The kinetics of body temperature change (Fig. 4*A*) reflect, to a large degree, body thermal inertia, contrasting with the more rapid response observed for individual neurons (Fig. 2). Similar body temperature dysregulation was observed on replacing *Slc6a4-cre; RC::PDi* with *Pet1::Flpe; RC::FDi* (data not shown), thereby independently confirming attribution of function to serotonergic neurons. (Note, however, that in the latter case, the extent of temperature dysregulation is less severe, likely because the *Pet1::Flpe* driver shows mosaicism in recombinase expression in medullary serotonergic neurons.) Delineating the effectors of body temperature homeostasis acting downstream of serotonergic circuitry will be an important next step.

In contrast to the response evoked upon acute serotonergic neuron inhibition, adult mice devoid of serotonin-producing neurons from midgestation onward are able to maintain a near wild-type body temperature (~36°–38°C) while housed at room temperature (~24°C) (Hodges et al., 2008; Hodges

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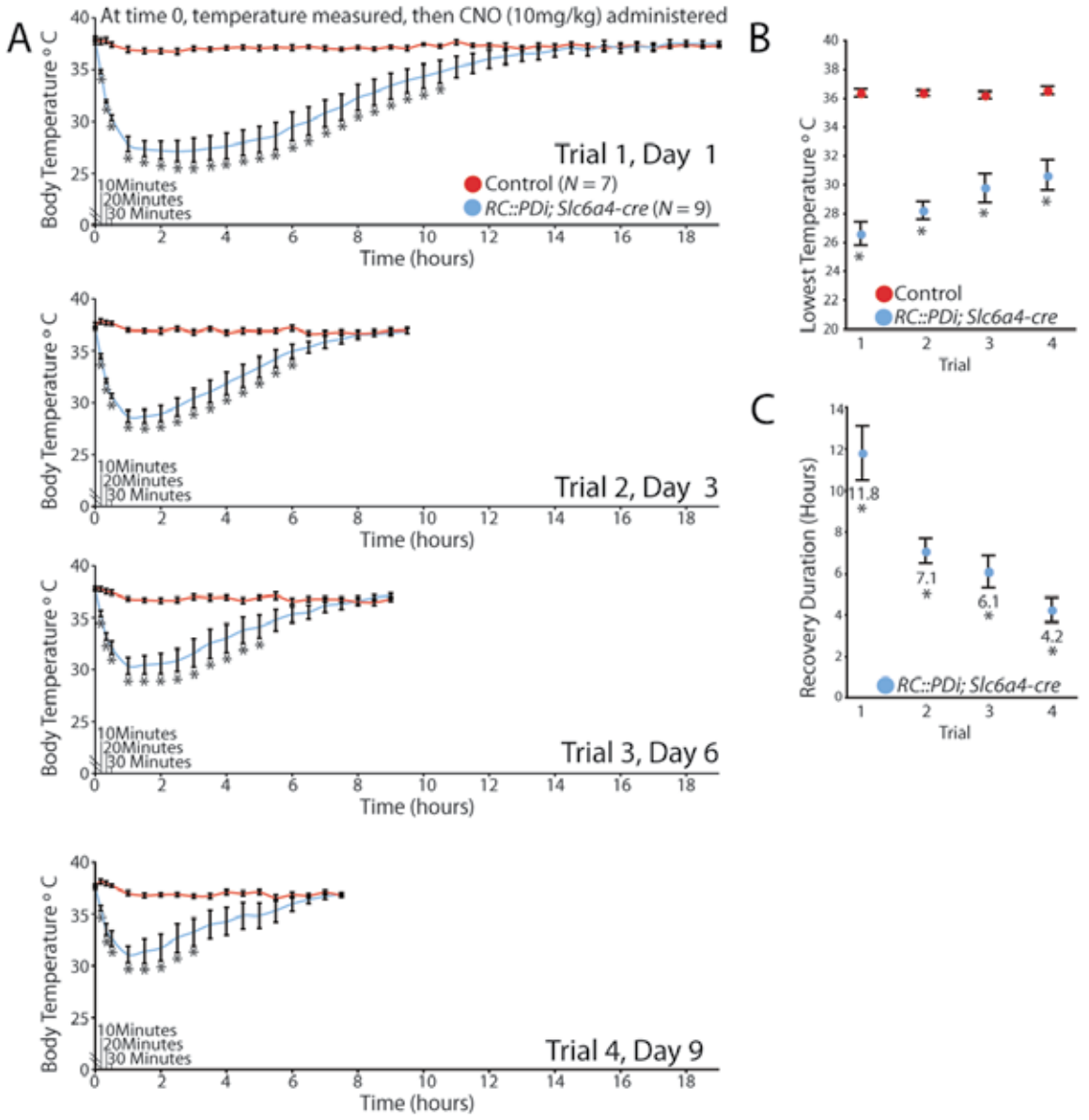

Figure 4. CNO/Di inhibition of serotonergic neurons induced severe yet reversible and repeatable hypothermia. Trials consisted of body temperature assessments taken at room temperature just before a single CNO administration and then every 10 min for the first one-half hour, followed by every 30 min until recovery. Animals underwent 4 sequential trials. ***A,*** Body temperature averages of *RC::PDi; Slc6a4-cre* mice versus controls before and after CNO injection; *$p < 0.05$ (unpaired *t* test). ***B,*** Average lowest temperatures achieved per trial; *$p < 0.05$ (a one-way RM ANOVA). ***C,*** Average duration to return to 36°C following CNO injection; *$p < 0.01$ (a one-way RM ANOVA).

and Richerson, 2010). This phenotypic difference indicates that compensatory circuitry arises in the face of developmental loss of serotonergic neurons. It also highlights how acute neuron perturbation avoids such confounds.

Repeated neuron perturbation in the same animal is possible: With each round of CNO administration, body temperature plummeted within minutes (Fig. 4A). The severity of the induced hypothermia, though, showed adaptation with each subsequent trial (Fig. 4B); also, the time required for return to baseline body temperature showed adaptation (Fig. 4C). We observed no overt behavioral deficits (short- or long-term) following these bouts of CNO/Di-triggered hypothermia, perhaps not surprising, given rodents' capacity for daily torpor.

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Implications: Serotonin Abnormalities in SIDS and Other Syndromes

Using this *RC::FPDi* strategy, we have revealed impaired respiratory and body temperature control upon acute perturbation of serotonergic neuron activity. These results provide direct evidence that serotonergic neurons play key roles in the central chemoreflex and thermoregulation. These findings offer potential mechanistic explanation for fatal or life-threatening disorders of homeostasis that are associated with serotonin abnormalities, such as SIDS and the serotonin syndrome.

In SIDS, the leading cause of death in children between 1 month and 1 year of age (Kinney et al., 2009), multiple abnormalities in the brainstem serotonergic system have been identified, including serotonin insufficiency (Kinney et al., 2009; Duncan et al., 2010). Our findings suggest that such insufficiency might compromise an infant's respiratory response to hypercapnic acidosis, which may occur upon rebreathing exhaled air as a result of sleeping prone, in the face-down position (SIDS infants are often found prone), contributing to respiratory failure and death. By contrast, serotonin syndrome is a disorder of serotonin excess and extreme hyperthermia, shivering, seizures, coma, and in some cases, death that can result acutely from serotonin drug interactions (Sternbach, 1991). This association between serotonin excess and hyperthermia is consistent with our reciprocal findings of serotonergic inhibition inducing hypothermia.

We presume that *in vivo*, CNO/Di signaling suppresses action potential firing, resulting in net inhibition of serotonergic neuron activity (Innis and Aghajanian, 1987; Pineyro and Blier, 1999). Other scenarios are possible, such as net excitation, but seem improbable owing to the whole-animal phenotypes observed upon CNO/Di signaling in serotonergic neurons, the molecular mechanism by which Di appears to act, and the paucity of evidence that net excitation could occur. For example, such net excitation has not been observed in cultured serotonergic neurons nor in extensive studies on $5HT_{1A}$ receptor agonists, either *in vivo* or *ex vivo*, which act mechanistically like CNO/Di (Innis and Aghajanian, 1987; Pineyro and Blier, 1999).

Conclusion

Assessing the precise *in vivo* electrophysiological consequences of CNO/Di signaling on serotonergic neuron activity, including chemoresponsiveness, awaits the means to record from individual medullary serotonergic neurons over the course of hours (compare Fig. 3*E*) in unanesthetized, conscious mice. By contrast, recordings under either anesthesia (Patel et al., 1999; Gobbi et al., 2007; Depuy et al., 2011) or decerebration (Hayashi and Sinclair, 1991; St-John and Paton, 2000) suffer from the confound of perturbing serotonergic neuron activity and chemoreflex properties—the very processes under examination.

The present studies, as well as our analyses of broad constitutive Di expression and activity, establish *RC::FPDi* as a neuronal perturbation tool. This tool features *in vivo* ligand inducibility within seconds to minutes, inhibition for minutes to hours, reversibility within hours, intra-animal repeatability, and high cell-subtype selectivity, and thus resolution, for functional mapping. It can be applied in the awake, freely behaving animal without adding confounding interference from anesthesia, surgical procedures (e.g., cannulation or intracranial fiber optics), or compensatory changes in circuitry that can develop in response to constitutive genetic alterations. *RC::FPDi* can be applied to map other behaviors in which dysregulation of specific populations has been implicated.

Acknowledgments

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