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***Egr2*-neurons control the adult respiratory response to hypercapnia**

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Abstract

The early growth response 2 transcription factor, *Egr2*, establishes a population of brainstem neurons essential for normal breathing at birth. *Egr2*-null mice die perinatally of respiratory insufficiency characterized by subnormal respiratory rate and severe apneas. Here we bypass this lethality using a noninvasive pharmacogenetic approach to inducibly perturb neuron activity postnatally, and ask if *Egr2*-neurons control respiration in adult mice. We found that the normal ventilatory increase in response to elevated tissue CO₂ was impaired, blunted by 63.1±8.7% after neuron perturbation due to deficits in both respiratory amplitude and frequency. By contrast, room-air breathing was unaffected, suggesting that the drive for baseline breathing may not require those *Egr2*-neurons manipulated here. Of the multiple brainstem sites proposed to affect ventilation in response to hypercapnia, only the retrotrapezoid nucleus, a portion of the serotonergic raphé, and a portion of the A5 nucleus have a history of *Egr2* expression. We recently showed that acute inhibition of serotonergic neurons en masse blunts the CO₂ chemoreflex in adults, causing a difference in hypercapnic response of ~50% after neuron perturbation through effects on respiratory amplitude only. The suppressed respiratory frequency upon perturbation of *Egr2*-neurons thus may stem from non-serotonergic neurons within the *Egr2* domain. Perturbation of *Egr2*-neurons did not affect body temperature, even on exposure to ambient 4 °C. These findings support a model in which *Egr2*-neurons are a critical component of the respiratory chemoreflex into adulthood. Methodologically, these results highlight how pharmacogenetic approaches allow neuron function to be queried in unanesthetized adult animals, reaching beyond the roadblocks of developmental lethality and compensation as well as the anatomical disturbances associated with invasive methods.

Keywords

Egr2; Pharmaco-genetics; Hypercapnic respiratory response

1. Introduction

The neural circuitry controlling breathing is protectively redundant, matures postnatally, and is thought to differentially engage distinct types of neurons depending on age and arousal state (Carroll, 2003; Feldman et al., 2003; Nattie and Li, 2009; Nattie, 2001). Physical

features further challenge its investigation, including hard-to-access locations in the brainstem, highly dispersed neuron populations difficult to capture for en masse manipulations such as electrolytic lesion or viral transgene delivery, and expression of neurotransmitters and signaling systems that offer only modest cell-type specificity for targeted probing pharmacologically. These formidable barriers are being surmounted in part through the application of pharmacogenetic approaches for inducible neuron perturbation in vivo. Here we apply one such pharmacogenetic approach involving the conditional inhibitory Designer Receptor Exclusively Activated by Designer Drug (DREADD) (Armbruster et al., 2007) allele called *RC::PDi* (Ray et al., 2011), to query if neurons defined by expression of the early growth response 2 (*Egr2*) transcription factor play a role in respiratory function in adult mice. *Egr2*-null mice typically die within 18 h of birth due to respiratory insufficiency (Jacquin et al., 1996), precluding the study of *Egr2*-defined neurons in adults. Moreover, *Egr2*-null neonates show substantial non-cell autonomous brainstem abnormalities (Schneider-Maunoury et al., 1997; Voiculescu et al., 2001), confounding attribution of respiratory functions specifically to *Egr2*-neurons even in neonates. Given the importance of *Egr2* defined cells in early postnatal respiratory function, we hypothesize that these same *Egr2* neurons play a critical role in adult respiratory homeostasis.

To test the hypothesis that *Egr2*-defined neurons are required for adult respiratory function, we employed *RC::PDi* (Ray et al., 2011) which is an allele engineered for Cre-dependent expression of Di, a synthetic Gi/o protein-coupled receptor that appears physiologically neutral until triggered by binding the synthetic ligand clozapine-N-oxide (CNO) (Armbruster et al., 2007). Di activation by CNO has been shown to hyperpolarize and inhibit the action potential firing of neurons cell-autonomously, likely via Gi/o gating of endogenously expressed Kir3 channels (Armbruster et al., 2007; Ferguson et al., 2011; Ray et al., 2011). Here, we use *Egr2^{tm2(cre)Pch}* (Voiculescu et al., 2000), referred to as *Egr2^{cre}*, to drive *RC::PDi* recombination and thus constitutive Di expression selectively in neurons with a history of *Egr2* expression, offering the capability to inducibly inhibit these neurons following administration of CNO (Fig. 1). Relative neutrality of the Di receptor in the absence of CNO permits normal development into adulthood (Ray et al., 2011). Avoided are compensatory circuitry changes or perturbations to other brainstem regions, allowing for the direct mapping of CNO/Di-triggered physiological deficits specifically to *Egr2*-neuron function. Further, because CNO can be effectively administered via intraperitoneal injection, analyses can be performed without anesthetics, which are known to perturb autonomic function and respiratory output. Anatomical disturbances associated with cannulas and stereotaxic injections are also avoided. Thus, the *RC::PDi* allele paired with the *Egr2^{cre}* driver permit testing the hypothesis that *Egr2*-defined neurons are required for respiratory function in the adult mouse.

Here we report that CNO/Di-mediated acute perturbation of *Egr2*-neurons in adult mice blunted the ventilatory response by $63.1 \pm 8.7\%$ when breathing hypercapnic air (5% carbon dioxide (CO_2)), reflecting deficits in both respiratory frequency (f_R) and tidal volume (V_T). Room-air (0% CO_2) breathing was unaffected, as was the ability to maintain body temperature on cold exposure. This contrasts with the effects observed following CNO/Di-mediated inhibition of brainstem serotonergic neurons, some of which reside within the *Egr2*-domain, in which only the V_T response to hypercapnia was affected. The present results suggest that *Egr2*-neurons are key to respiratory function in the adult and likely include a neuron population outside the serotonergic system that modulates f_R in response to elevated levels of inhaled CO_2 . By employing this pharmacogenetic approach, we have been able to query a previously inaccessible population of neurons in the adult, bypass perinatal lethality in a manner free from both cell-autonomous and non-cell-autonomous

developmental deficits, and assess respiratory function in unanesthetized, freely behaving animals.

2. Results

2.1. Acute perturbation of *Egr2*-neurons in adult mice diminishes the hypercapnic respiratory response in both respiratory amplitude and frequency

Hypercapnic acidosis, a decrease in tissue pH caused by PCO_2 elevation from cellular metabolism or ventilatory dysfunction, is a powerful respiratory stimulus, the response to which is thought to involve the activity of multiple brainstem neuron types, including cell populations defined by expression of *Egr2* (Goridis and Brunet, 2010; Guyenet et al., 2010; Nattie and Li, 2009; Nattie, 2011). To assess the requirement for *Egr2*-neurons in the breathing response to elevations in tissue CO_2 levels in adult mice, we employed the Cre-dependent Di-expressing allele, *RC::PDi* (Fig. 1) (Ray et al., 2011). By partnering *RC::PDi* with the *cre* knock-in allele *Egr2^{cre}* (Voiculescu et al., 2000), we targeted Di expression to *Egr2*-neurons, allowing for their acute perturbation upon administration of CNO.

To assess the efficiency of recombination mediated by *Egr2^{cre}* and thus the brainstem cell populations ‘captured’ for Di expression, we analyzed cell types marked by fluorescent protein expression upon partnering *Egr2^{cre}* with either the Cre-dependent tdTomato reporter allele, *Gt(ROSA)26Sor^{tm14}(CAG-tdTomato)Hze* (Madisen et al., 2009), or the Cre-dependent eGFP reporter allele, *RC::rePe* (Ray et al., 2011). Both reporter alleles are *R26* knock-in alleles configured similarly to *RC::PDi* and show similar capacity to be recombined by Cre (Ray et al., 2011). Extensive capture of *Egr2*-derived cells was observed (Fig. 2), as reflected by swaths of marked cells in the domains of rhombomeres 3 and 5 as expected given the well established profile for *Egr2* expression and the already well documented activity of this specific *Egr2^{cre}* driver (Thoby-Brisson et al., 2009). Scores of different neuron types and glia comprise the *Egr2* territories, including three cell types that have been implicated in contributing to the CO_2 respiratory chemoreflex: a subset of serotonergic neurons (Fig. 2D) consistent in number with our previous intersectional fate mapping of the *Egr2*-derived subset of serotonergic neurons (Jensen et al., 2008), a majority, if not all, of the Phox2b RTN neurons (Fig. 2E), and a small subset of tyrosine hydroxylase-positive noradrenergic neurons in the ventral portion of the A5 nucleus (Fig. 2F). In *Egr2^{cre}; RC::PDi* mice, we expect Di expression to recapitulate the pattern seen with fluorescent reporters for the *Egr2^{cre}* driver and that a large portion of these cells will also express levels of the necessary endogenous Gi/o proteins and Kir3 channels to mediate hyperpolarization and action potential inhibition (neuronal ‘silencing’) following CNO administration.

Given the efficacy of this *Egr2^{cre}* driver, we partnered it with *RC::PDi* and measured, using whole body plethysmography, the ventilatory response to an increase in inspired CO_2 from 0% (room air) to 5% (a modest rise) before and after CNO administration (Fig. 3). The typical increase in respiratory tidal volume (V_T ; ml/g), respiratory frequency (f_R , breaths per minute), and minute ventilation ($\dot{V}_E = V_T f_R$; ml/g/min) that would tend to restore normal arterial pH/p CO_2 levels was observed in double transgenic *RC::PDi; Egr2^{cre}* adults ($n=14$) prior to CNO administration (Fig. 3). By contrast, after CNO administration (10 mg/kg intraperitoneal injection, i.p.) this response to inspired CO_2 was substantially blunted: increased $27.8 \pm 3.1\%$ before CNO, but only $9.5 \pm 3.6\%$ following CNO injection and f_R increased $62.8 \pm 4.6\%$ before CNO, but only $36.4 \pm 8.0\%$ after CNO. Overall, the percent change in $\dot{V}_E$ during a 5% CO_2 challenge was reduced by $63.1 \pm 8.7\%$ after CNO injection (* $P < 0.001$ two-way repeated-measures analysis of variance (RM ANOVA, with % CO_2 and CNO treatment both as factors within a genotype) followed by Tukey post-hoc). Room-air ventilation was notably unaffected after CNO/Di manipulation of *Egr2*-neurons (Fig. 3).

The ventilatory responses to inspired CO₂ elicited in sibling single-transgenic control mice that did not express Di were indistinguishable whether or not CNO had been administered (Fig. 3, $n=10$). Thus, consistent with previous work, the ligand CNO appears neutral in this assay. Further, the pre-CNO control baseline responses in V_T and f_R were indistinguishable from the pre-CNO responses of the double transgenic $RC::PDi; Egr2^{cre}$ mice ($P=0.088$ Student's t -test for V_T , and $P=0.208$ Mann–Whitney rank sum for the non-parametric f_R values); likewise, the pre-CNO control responses (V_T , f_R , and $\dot{V}_E$) to hypercapnia were indistinguishable from those of the double transgenics (Student's t -test, $P=0.059$, $P=0.663$, and $P=0.281$ respectively). These findings indicate that expression of Di in the absence of CNO also had no appreciable effect. Thus, only when both the Di receptor and CNO were combined in *Egr2*-defined neurons was a respiratory phenotype observed.

Because newborn *Egr2*^{-/-} pups have severe apneas and disruptions in respiratory rhythm (Jacquin et al., 1996), we examined respiratory traces from CNO-treated double transgenic $RC::PDi; Egr2^{cre}$ adults as well as single transgenic sibling controls for variation in respiratory periodicity that might indicate perturbations in inspiratory or expiratory regulation. However, in all cases, regardless of genotype, waveform periodicity was continuous, showing no indication of irregularity or episodic periods of apnea (Fig. 4A). We also generated Poincaré plots where the inter-breath interval (IBI) is plotted as a function of the previous inter-breath interval to determine if individual double transgenic $RC::PDi; Egr2^{cre}$ mice exhibited any breathing irregularities or periods of apnea (Fig. 4B). The tight clusters of data points reflect a consistently similar IBI. Apneas would be represented by data points outside this cluster, which are absent in the individuals shown. As a quantitative measure of variation in periodicity, we calculated the coefficient of variation of the IBI. In the adult double transgenic $RC::PDi; Egr2^{cre}$ mice, this value was 0.181 ± 0.015 before CNO and 0.153 ± 0.015 after CNO ($P=0.082$, paired t -test), indicating no significant change in periodicity of the respiratory rhythm as a result of neuron perturbation.

We also examined metabolic rate as reflected in oxygen (O₂) consumption ($\dot{V}_{O_2}$; ml/g/min). Double transgenic $RC::PDi; Egr2^{cre}$ mice following CNO administration showed a modest but significant decrease in $\dot{V}_{O_2}$ when challenged with 5% CO₂ (post-CNO, 5% CO₂ $\dot{V}_{O_2} = 0.050 \pm 0.003$ ml/g/min) as compared to room air (post-CNO, RA $\dot{V}_{O_2} = 0.057 \pm 0.003$ ml/g/min) or to pre-CNO measurements (pre-CNO, RA $\dot{V}_{O_2} = 0.056 \pm 0.004$ ml/g/min; pre-CNO, 5% CO₂ $\dot{V}_{O_2} = 0.057 \pm 0.004$ ml/g/min) ($P=0.002$, 2-way RM ANOVA followed by Tukey posthoc). No differences in $\dot{V}_{O_2}$ were observed in single transgenic sibling controls under any of the conditions (pre-CNO, RA 0.048 ± 0.003 ; pre-CNO, 5% CO₂ 0.046 ± 0.004 ; post-CNO, RA 0.049 ± 0.003 ; post-CNO, 5% CO₂ 0.049 ± 0.004).

2.2. Perturbation of *Egr2* defined neurons in adults does not alter thermoregulation during cold challenge

Adult thermal homeostasis in the face of ambient cold exposure is known to involve brainstem regulation of sympathetic output, allowing for thermogenesis via shivering and brown fat metabolism (Cano et al., 2003; Morrison et al., 2008). To explore whether *Egr2*-neurons influence thermo-regulatory circuits, we measured core body temperature before and after CNO administration to double transgenic $RC::PDi; Egr2^{cre}$ mice ($n=10$) and sibling single transgenic controls ($n=10$) initially at ~23 °C (room temperature well below thermoneutrality) and then following exposure to 4 °C. Prior to and 30 min following CNO administration at 23 °C, core body temperature was not significantly different between double transgenic $RC::PDi; Egr2^{cre}$ mice and sibling single-transgenic controls (Fig. 5). Following 4 °C exposure for two additional hours, we observed no differences in core body temperature between individually housed $RC::PDi; Egr2^{cre}$ mice and sibling controls

(unpaired *t*-test). Thus, acute CNO/Di perturbation of *Egr2*-neurons does not appear to appreciably affect thermal homeostasis, contrasting the observed respiratory effects.

3. Discussion

Within the adult brainstem, the lineage of *Egr2*-expressing neurons comprises two large domains, one in the pons and one in the medulla, reflective of their hindbrain segments of origin embryologically—rhombomeres 3 and 5 (Wilkinson et al., 1989). Many different cell types are encompassed by this population (Dubreuil et al., 2009; Jacquin et al., 1996; Jensen et al., 2008; Nattie, 2011; Schneider-Maunoury et al., 1993, 1997), including neurons implicated in respiratory control such as glutamatergic retrotrapezoid neurons, a subset of raphe serotonergic neurons, a small ventral portion of the noradrenergic A5 nucleus, and perhaps others yet to be assigned respiratory function. Ventrolateral glia important to respiratory control (Mulkey and Wenker, 2011) may also have a history of *Egr2* expression, although this remains to be fate mapped. Delineating the adult role of *Egr2*-neurons has been a significant challenge because most genetic manipulations involving the *Egr2*-cell lineage result in mortality at birth: *Egr2*^{-/-} mice, which exhibit developmental loss of rhombomeres 3 and 5 as well as secondary non-cell-autonomous brainstem derangements (Jacquin et al., 1996; Schneider-Maunoury et al., 1993) die perinatally of respiratory dysfunction, as do mice harboring the more restricted genetic lesion of deleting the transcription factor-encoding gene *Phox2B* solely in *Egr2*-neurons (*Egr2*^{cre}; *Phox2B*^{fl/fl} mice) (Dubreuil et al., 2009). To reach beyond neonatal stages and test our hypothesis that *Egr2*-neurons are required for full respiratory function in the adult, we examined respiratory output in awake and unanesthetized mice while exploiting the Di system (Armbruster et al., 2007) by way of the Cre-dependent allele *RC::PDi* (Ray et al., 2011) coupled with *Egr2*^{cre} for selective inducible neuron inhibition.

In adult double transgenic *RC::PDi*; *Egr2*^{cre} mice, we found that CNO/Di-induced perturbation of *Egr2*-cells decreased significantly the respiratory response to breathing a hypercapnic (5% CO₂) mixture, showing deficits in both *V_T* and *f_R* responses, but did not affect baseline breathing during eupnea. These results confirm our hypothesis that *Egr2*-defined neurons are required for at least one aspect of respiratory homeostasis, the hypercapnic ventilatory response. While we acknowledge that our CNO/Di strategy may fall short in inhibiting certain *Egr2*-neurons, depending on the achieved levels of Di driven from *RC::PDi* following Cre recombination and on the levels of endogenous Gi/o proteins and Kir3 channels needed for Di-induced hyperpolarization, the present findings, nonetheless, indicate that the global response to hypercapnia is regulated in adult mice to a great extent by the *Egr2*-cell lineage.

These findings are in agreement with related studies performed in adult rats in which cells of the ventrolateral medulla (VLM) were acutely perturbed either via injection of a cell-ablating toxin (Akilesh et al., 1997) or allatostatin-mediated inhibition of virally transduced *Phox2b*-expressing neurons in the area of the RTN and C1 nuclei (Marina et al., 2010). A common target in each of these brainstem perturbations, including the *RC::PDi*; *Egr2*^{cre} approach presented here, is the RTN which lies within the *Egr2*-cell population (Dubreuil et al., 2009; Thoby-Brisson et al., 2009). C1 neurons, while implicated in respiratory control (Li et al., 2008), do not have a history of *Egr2*^{cre} expression and thus are not directly perturbed in our assays, lending support to the idea that respiratory responses elicited by hypercapnia may be independent of C1 neurons, as suggested from phrenic nerve responses to VLM stimulation using channelrhodopsin2, which are indistinguishable whether C1 has been lesioned or left intact (Abbott et al., 2009).

A subset of serotonergic neurons are included within the *Egr2* lineage (Jensen et al., 2008) and they may contribute to the overall respiratory response to CO₂ given in our previous findings in which CNO/Di-mediated inhibition of all serotonergic neurons en masse, inclusive of the *Egr2* serotonergic neuron subset, results in a 50% blunting of the ventilatory response to inspired 5% CO₂ (Ray et al., 2011). Notably, this serotonergic-mediated response derives from deficits mainly in V_T [Fig. 6, double transgenic *RC::PDi*; *Slc6a4-cre* mice following CNO injection (Ray et al., 2011)]. By contrast, CNO/Di-mediated perturbation of *Egr2*-cells disrupts both V_T and f_R responses to inspired 5% CO₂ (Fig. 3). These findings suggest involvement of nonserotonergic *Egr2*-cells in f_R regulation during hypercapnia, perhaps a task performed in part by RTN neurons.

Additional studies will be needed to explore whether the *Egr2*-cell lineage also includes respiratory-relevant peripheral cells, such as cells in the carotid bodies, which might also be manipulated here and contribute to the chemoreflex deficit. It is also important to note that rodents are able to detect low levels of CO₂ by olfactory chemoreceptors (Coates, 2001) although this has an inhibitory as opposed to an excitatory effect on breathing. On visual inspection, we observed no gross changes in sniffing behavior during plethysmography as a function of genotype, CNO administration, or CO₂ exposure.

Resolving which of the various subpopulations of *Egr2* cells are critical for breathing responses during hypercapnia is the next step and one that can be addressed using the parent allele, *RC::FPDi* (Ray et al., 2011), an intersectional dual recombinase (Cre and Flp) responsive allele from which the Cre-dependent allele *RC::PDi* used here was derived. CNO/Di-mediated inhibition can be targeted to specific *Egr2*-cell subsets by partnering the combination of *Egr2^{cre}* and *RC::FPDi* alleles with various Flp transgenics as outlined in Ray et al. (2011). Further, it should be possible to explore whether the roles played by different *Egr2*-neuron subpopulations change as a function of age, gender, and arousal state—variables relevant to respiratory instability and function (Carroll, 2003; Feldman et al., 2003; Nattie, 2001).

Lack of a normal ventilatory response to inhaled CO₂ and to elevated PCO₂ along with life-threatening apneas characterizes a human disease called congenital central hypoventilation syndrome (CCHS). The most frequent CCHS-causing mutation lies in the *PHOX2B* gene, is referred to as the *PHOX2B^{27Ala}* mutation, and results in a 20-residue polyalanine expansion in *PHOX2B* (Amiel et al., 2009). A conditional *Phox2b^{27Alaki}* allele has been generated and partnered with *Egr2^{cre}* to restrict the *Phox2b^{27Ala}* mutation solely to *Egr2*-cells. In these *Egr2^{cre}*, *Phox2b^{27Alaki}* mice, RTN neurons fail to develop, consistent with their arising from *Egr2*-expressing progenitors in rhombomere 5 and with requiring wild-type *Phox2B* cell-autonomously for their proper differentiation (Ramanantsoa et al., 2011). These mice, however, do not die perinatally like *Egr2^{cre}*; *Phox2b^{fl/fl}* mice or germline *Phox2b^{27Ala/+}* mice, but instead survive into adulthood, exhibiting normal rhythmic baseline breathing and a diminished response to inspired 8% CO₂, similar to the deficiency observed in *RC::PDi*; *Egr2^{cre}* mice following CNO induced neuronal silencing. Because *Egr2^{cre}*, *Phox2b^{27Alaki}* mice survive into adulthood while *Egr2^{cre}*; *Phox2b^{fl/fl}* mice do not (Dubreuil et al., 2009), yet both exhibit impaired RTN development, it has been proposed that the defect underlying perinatal lethality must lie outside the RTN but within the *Egr2*-cell lineage (Ramanantsoa et al., 2011). Intersectional *RC::FPDi* strategies, for which the present work is foundational, offer the possibility for identifying such cell subtype(s) critical for perinatal viability.

While the CNO/Di-induced perturbation of the *Egr2*-cell lineage profoundly disrupted the respiratory response to hypercapnia, it did not derail body temperature homeostasis upon ambient cold exposure. Core body temperatures of CNO-treated double transgenic *RC::PDi*; *Egr2^{cre}* mice versus single transgenic sibling controls were indistinguishable from each

other whether exposed to an ambient 23 °C or 4 °C (Fig. 4). This contrasts with the extreme drop in core body temperature to near 27 °C following CNO/Di-mediated inhibition of serotonergic neurons in double transgenic *Slc6a4-cre; RC::PDi* mice housed individually at 23 °C (Ray et al., 2011). These findings suggest that thermoregulatory modulators reside largely outside the *Egr2*-cell lineage, although we cannot yet rule out that key thermoregulatory neurons may reside within the *Egr2*-lineage but are poorly inhibited by this CNO/Di approach. Further functional mapping is required.

In summary, these findings indicate, as hypothesized, that within the *Egr2* neuron population lays one or more cell subsets critical to respiratory function in adults, in particular, the CO₂ chemoreflex. Coupled with findings from previous studies of neonates, a model is emerging in which a subset of *Egr2*-neurons are required for a normal respiratory CO₂ chemoreflex throughout life, perhaps even different subsets depending on age. Present data also supports the provocative possibility that *Egr2* neurons and a normal CO₂ chemoreflex are not required to regulate adult breathing under conditions of eupnea; further studies though are needed as it is also possible that not all *Egr2* neurons are effectively inhibited in this CNO/Di paradigm, such as those that might be involved in modulating eupnic breathing. Methodologically, these results highlight how pharmacogenetic approaches, such as the *RC::PDi* inhibitory DREADD system, allow neuron function to be queried in unanesthetized adult animals, overcoming the obstacles of developmental lethality and non-cell-autonomous compensation as well as the anatomical disturbances required for optogenetic and other invasive approaches. Building upon the work presented here, use of the parental allele *RC::FPDi* will allow more selective neuron subtypes within the *Egr2*-cell population to be resolved and functionally manipulated in vivo to assess their specific contribution to respiratory regulation.

4. Experimental procedures

4.1. Transgenic mouse lines

All procedures were carried out in accordance with Harvard Medical School and the Geisel School of Medicine at Dartmouth IACUC policies and were housed under standard conditions (12 h light/dark cycle, 23 °C room temperature, food and water ad libitum). For histology experiments, heterozygous *Egr2^{cre}* mice were mated with homozygous *RC::rePe* (Ray et al., 2011) or *Gt(ROSA)26Sor^{tm14}(CAG-tdTomato)^{Hze}* (Madisen et al., 2009) mice. For physiological experiments, *RC::PDi* homozygous mice were mated to heterozygous *Egr2^{cre}* mice to derive sibling cohorts in which all mice carried the *RC::PDi* allele. Sibling mice that did not inherit the *Egr2^{cre}* allele, were used as controls to the *RC::PDi; Egr2^{cre}* offspring. Similarly, *Slc6a4-cre* mice were mated with *RC::PDi* homozygous mice to produce experimental and sibling control cohorts for plethysmographic assessment.

4.2. Plethysmography

Plethysmography on awake, freely moving mice was carried out as in Ray et al. (2011). Briefly, mice were placed in a room-air plethysmograph chamber, acclimatized for 20 min of which recordings from the last 5 min 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 were used for data analyses. Chamber gas was then switched back to room air for 15 min, mice were then removed briefly for an intra-peritoneal injection of CNO dissolved in saline (1 mg/ml) to an effective concentration of 10 mg/kg CNO and then returned immediately to the room-air chamber for an additional 10 min of recordings, of which the last 5 min 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 were used for data analyses. Rectal body temperature was recorded prior to the initial placement in the chamber, during the period where the animal

was removed for injection, and upon removal from the chamber at the termination of the experiment.

Respiratory airflow was recorded in a 140 ml water-jacketed temperature controlled glass chamber attached to a differential pressure transducer and reference chamber. Chamber temperature was maintained at 34 °C by adjusting the circulating water temperature (Thermo Scientific NESLAB RTE 7). Gas flow rate was ~325 ml/min. Volume calibrations were performed by repeated injections of a known volume from a 1 ml syringe. Pressure transducer data were digitized at 1000 Hz and recorded using a PowerLab data acquisition system (AD Instruments) and analyzed off-line. 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 (AEI Technologies). 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.

Results were compared within each genotype using two-way RM ANOVA with room air to CO₂ and CNO treatment as factors (each individual animal underwent all treatments) followed by Tukey post-hoc analysis for *RC::PDi*; *Egr2^{cre}* ventilation experiments, and paired *t*-test was used for comparison of oxygen consumption. Standard error of the mean is shown on all plethysmographic data. We also calculated the coefficient of variation (CV) of the inter-breath interval (standard error IBI/mean IBI) in 30 s sections of continuous data. IBI was calculated as the time between the peak inspiration of one breath and the peak inspiration of the following breath. A paired *t*-test was used to compare the CV IBI during room air before CNO injection and during room air after CNO injection. Statistical comparisons of CNO effects on the CO₂ response were made within each genotype.

4.3. Temperature assessment

Temperature was taken rectally with a lubricated thermocouple probe. Mice were weighed and temperature was taken before 10 mg/kg CNO administration. After CNO administration, mice were left at room temperature for 30 min, after which their temperature was taken and they were then moved to pre-chilled cages in a 4 °C cold room. At this point, temperature was taken every 10 min for the first hour and every half hour for the second hour. The experiment was concluded after 2 h of 4 °C exposure.

The results between *RC::PDi*; *Egr2^{cre}* and control siblings were compared using an unpaired *t*-test for each temperature assessment at each time point.

4.4. Histology

For embryonic tissue collection, embryos were soak fixed in ice cold 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) and incubated at 4 °C for 2 h. For postnatal tissue collection, anesthetized mice were perfused with PBS followed by 4% PFA. Brains were then removed and soak fixed in 4% PFA at 4 °C overnight. After fixation, all tissue was rinsed with PBS, then cryoprotected in 30% sucrose/PBS. Embryonic and P0.5 tissue was cryosectioned (20 μM) and collected directly onto slides. Adult tissue was cryosectioned (40 μM) and processed as floating sections. For immunostaining, slides or floating sections were washed with PBS and PBS with 0.1% TritonX-100 (PBS-T) prior to blocking with 5% normal donkey serum in PBS-T for 1 h at room temperature. Tissue was then incubated with the following primary anti-bodies for 24–72 h at 4 °C: Chicken-anti-GFP (Abcam ab13970-100, 1:5000), Rabbit-anti-DsRed (Clontech 632496, 1:3000), Goat-anti-Phox2b (Santa Cruz sc-13224, 1:500), Rabbit-anti-Tyrosine Hydroxylase (Millipore ab152, 1:5000), Rabbit-anti-Tph2 (Novus Biologicals NB100-74555, 1:2000). Primary anti-bodies were then detected using the following secondary antibodies for 2 h at room temperature (all secondary

anti-bodies from JacksonImmuno): 488 Donkey-anti-Chicken, Cy3 Donkey-anti-Rabbit, 488 Donkey-anti-Rabbit, Cy2 Donkey-anti-Goat. The endogenous tdTomato fluorescence in fixed, processed tissue was extremely robust and was not improved by additional staining with DsRed antibody. Cell nuclei were visualized with DAPI.

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Fig. 1.

The *RC::PDi* allele (Ray et al., 2011) is combined with the *Egr2^{cre}* (Voiculescu et al., 2000) (top schema) to achieve cell specific *Di* expression in rhombomeres (r)3 and (r)5 in the brainstem and elsewhere. Cre mediated recombination of *loxP* sites removes the mCherry-stop cassette and results in *Di* expression under the control of the *CAG* and *R26* regulatory elements (gray areas represent *Egr2* defined brainstem neurons; middle schema). Intraperitoneal (I.P.) injection of Clozapine-N-Oxide (CNO) activates the Di receptor to inhibit neuron function (black area, lower schema).

Fig. 2.

(A) Brainstem schematic depicting selected hindbrain cell populations particularly relevant to respiratory function and their relative relationships to r3 and r5. Note that this cartoon does not convey the mixing of *Egr2*-derived and non-*Egr2*-derived cells even in regions of overlap, nor the considerable cell migration of some *Egr2*-derived cell populations, but is designed as a general anatomical framework. DRN, dorsal raphe nucleus; MRN, median raphe nucleus; LC, locus coeruleus; RTN, retrotrapezoid nucleus; 7N, facial nucleus; NTS, nucleus of the solitary tract; PBC, preBötzinger complex. Inset embryonic schematic shows the location of r3 and r5 in inset of B. (B) Sagittal brainstem section showing *Egr2^{cre}* dependent Tdtomato expression (red) and DAPI (blue) at postnatal day 33 (P33) and embryonic day 12.5 (e12.5, inset) in double transgenic *Egr2^{cre};Gt(ROSA)26Sor^{tm14(CAG-tdTomato)Hze}*. (C) Coronal section depicting *Egr2^{cre}* dependent Tdtomato expression (red) and DAPI (blue) at P33. (D) *Egr2^{cre}* dependent Tdtomato expression in the caudal raphe (red, left panel) co-stained for tryptophan hydroxylase 2 (Tph2; green, middle panel). Tdtomato, Tph2 and DAPI (blue) overlay in right panel. (E) *Egr2^{cre}* dependent Tdtomato expression below the 7 N in the RTN region (red, left panel), costained for Phox2b (green, middle panel). Tdtomato, Phox2b and DAPI (blue) overlay in right panel. Arrowheads point to Phox2b positive nuclei that co-express Tdtomato. (F) *Egr2^{cre}* dependent eGFP expression in the ventral region of the A5 nucleus (green, left panel) in double transgenic *Egr2^{cre}; RC::rePe* mice, co-stained for tyrosine hydroxylase (TH; red, middle panel). eGFP, TH and DAPI (blue) overlay in right panel. Arrow points to a neuron positive for GFP and TH.

Fig. 3.

(A) Protocol for assessing mice in a plethysmograph. Room air (RA) baseline measurements are followed by baseline 5% CO₂ exposure and return to room air. After CNO administration, the sequence is repeated. (B) CNO/Di perturbation of neurons with a history of *Egr2* expression reduces the ventilatory response to inhaled hypercapnia. Exposure to 5% CO₂ induced an increase in tidal volume (V_T), respiratory rate, and minute ventilation ($\dot{V}_E$) in both sibling controls and *RC::PDi; Egr2^{cre}* mice (pre-CNO). Upon administration of CNO (10 mg/kg; i.p.) sibling controls displayed an increase in respiratory measures similar to pre-CNO baseline in response to inhaled CO₂, however the response in *RC::PDi; Egr2^{cre}* mice was severely blunted. * $P < 0.001$ (Two-way RM ANOVA applied within each genotype with RA to CO₂ and pre- to post-CNO as factors and repeated-measures in each subject followed by Tukey post-hoc).

Fig. 4.

(A) Representative breathing traces from a *RC::PDi; Egr2^{cre}* mouse under room air (RA) conditions and exposure to 5% CO₂ challenge. Apart from changes in amplitude and frequency, no other changes in periodicity or waveform shape were observed across genotypes and treatments. Sibling controls showed no effect after CNO administration (data not shown). (B) Poincaré plots (the inter-breath interval (IBI) plotted as a function of the previous inter-breath interval) for four *RC::PDi; Egr2^{cre}* mice before (left panels) and after (right panels) CNO administration while breathing room air. No distinguishable pattern was observed to indicate irregular, random or episodic breathing before or after CNO administration, as evidenced by the tight clusters of data points. No significant difference in the coefficient of variation between pre-CNO and post-CNO IBI in *RC::PDi; Egr2^{cre}* mice. $P=0.082$ [paired t -test].

Fig. 5.

CNO/Di inhibition of *Egr2^{cre}* defined neurons does not result in any thermoregulatory deficiency while mice are housed at 23 °C (room temperature) and 4 °C after CNO administration. Temperature was taken prior to CNO administration ($t=0$). After CNO administration, mice were left at room temperature for 30 min. Their temperature was assayed again before being moved to a 4 °C cold room, where temperature was measured every 10 min for the first hour and every 30 min for the second hour. No significant difference in core body temperature was found between *RC::PDi; Egr2^{cre}* and sibling control mice $P = 0.1$ [unpaired t -test for each time point].

Fig. 6.

Data derived from previously published experiments in Ray et al. (2011) showing *Di* mediated inhibition of serotonergic neurons in ventilatory assays. Inhibition of serotonergic neurons resulted in a respiratory phenotype only under hypercapnic, 5% CO₂ conditions, not room air (RA). There was no significant change in respiratory rate (lower left panel), however, a significant change in V_T was observed after CNO administration during CO₂ exposure (upper left panel) * $P < 0.001$ [repeated-measures analysis of variance (ANOVA) followed by Tukey post-hoc].