*Tg(Th-Cre)Fl172Gsat (Th-Cre)* defines neurons that are required for full hypercapnic and hypoxic reflexes.

Jenny J. Sun\textsuperscript{a} and Russell S. Ray\textsuperscript{a,b}

\textsuperscript{a} Baylor College of Medicine, Department of Neuroscience

1 Baylor Plaza

T707

Houston Texas, 77030

+1 713 798 2717

\textsuperscript{b} Corresponding author, russell.ray@bcm.edu

**KEY WORDS**

Brainstem, Catecholamine, DREADD, Hypercapnic Reflex, Hypoxic Reflex, Respiration
SUMMARY STATEMENT

DREADD mediated silencing of Tg(Th-Cre)Fl172Gsat defined neurons in adult mice results in reduced O₂ and CO₂ breathing reflexes and respiratory rhythm destabilization under hypoxic challenge resembling Cheyne-Stokes respiration.

ABSTRACT

The catecholaminergic system has been implicated in many facets of breathing control and offers an important target to better comprehend the underlying etiologies of both developmental and adult respiratory pathophysiologies. Here we used a noninvasive DREADD based pharmaco-genetic approach to acutely perturb Tg(Th-Cre)Fl172Gsat (Th-Cre) defined neurons in awake and unrestrained mice in an attempt to characterize catecholaminergic function in breathing. We report that clozapine-N-oxide (CNO)-DREADD mediated inhibition of Th-Cre defined neurons results in blunted ventilatory responses under respiratory challenge. Under a hypercapnic challenge (5%CO₂/21%O₂/74%N₂), perturbation of Th-Cre neurons results in reduced fR, VE, and VE/VO₂. Under a hypoxic challenge (10%O₂/90%N₂), we saw reduced fR, VE, and VO₂ in addition to instability in both interbreath interval and tidal volume resulting in a Cheyne-Stokes like respiratory pattern. These findings demonstrate the necessity of Th-Cre defined neurons for the hypercapnic and hypoxic ventilatory responses and breathing stability during hypoxia. However, given the expanded non-catecholaminergic expression domains of the Tg(Th-Cre)Fl172Gsat mouse line found in the brainstem, full phenotypic effect cannot be assigned solely to catecholaminergic neurons. Nonetheless, this work identifies a key respiratory population that may lead to further insights into the circuitry that maintains respiratory stability in the face of homeostatic challenges.
1 Introduction

The catecholaminergic (CA) system including the dopaminergic, noradrenergic and adrenergic systems has been implicated in respiratory homeostasis (Li et al., 2008, Viemari, 2008). A better understanding of the role of the CA system in breathing is of importance in determining the potential mechanisms in developmental disorders with respiratory features such as Congenital Central Hypoventilation Syndrome (CCHS) (Ramanantsoa and Gallego, 2013), Rett Syndrome (Katz et al., 2009, Weese-Mayer et al., 2006, Julu et al., 2001), and Sudden Infant Death Syndrome (SIDS) (Weese-Mayer et al., 2007); in pathophysiologies such as obstructive sleep apnea (Hakim et al., 2012, Zhu et al., 2007) and in Acute Respiratory Distress Syndrome (ARDS) (Beitler et al., 2016) and Chronic Obstructive Pulmonary Disorder (COPD) (Williams et al., 2014, Yamauchi et al., 2012) where pulmonary dysfunction leads to hypoxia and respiratory destabilization; as well as inform upon opiate mediated respiratory arrest (Lalley, 2008).

CA neurons are defined by their expression of tyrosine hydroxylase (Th), an early synthesizing enzyme in the catecholaminergic pathway leading to production of dopamine, noradrenaline and adrenaline, encompassing several populations in the nervous system. Here, we aimed to characterize the CA system in respiratory function in adult mice through the use of acute and targeted pharmaco-genetic neuronal inhibition.

DREADD mediated neuronal manipulations combined with recombinase based targeting strategies have provided an approach for functional circuit mapping that enables acute non-invasive perturbation of targeted populations while measuring respiratory output in conscious and unrestrained adult mice (Hennessy et al., 2017, Brust et al., 2014, Ray et al., 2012, Ray et al., 2011). The inhibitory pharmacogenetic DREADD or hM4Di receptor is inactive until activated by administration of the biologically inert ligand, Clozapine-N-Oxide (CNO) to perturb neuron firing. To examine the potential aspects of respiratory physiology served by the catecholaminergic system, we utilized the Cre responsive RC::PDi mouse in combination with the B6.FVB(Cg)-Tg(Th-Cre)Fl172Gsat (hereafter Th-Cre) driver to express the hM4D receptor in catecholaminergic and other neurons defined by this frequently used Th-Cre driver. Using whole-body plethysmography, we assessed respiratory and metabolic function under baseline (21%O\textsubscript{2}/79%N\textsubscript{2}), hypercapnic (5%CO\textsubscript{2}/21%O\textsubscript{2}/74%N\textsubscript{2}) and hypoxic (10%O\textsubscript{2}/90%N\textsubscript{2}) conditions after CNO-DREADD mediated perturbation of targeted cells, examining several aspects of
respiratory and metabolic homeostasis include rate (fₚ) tidal volume (VT), minute ventilation (VE), oxygen consumption (VO₂), and waveform patterning.

Our results show that hM4Di mediated inhibition of Th-Cre defined neurons results in reduced hypercapnic and hypoxic reflexes as well as temperature deficits, identifying a population of cells that are critical for maintaining respiratory and metabolic homeostasis. However, because of additional Th-Cre expression domains in multiple populations, we cannot fully attribute the respiratory phenotypes seen here solely to the catecholaminergic system. Nonetheless, the results presented provide an important neural correlate for further study of ventilation, metabolic drive and rhythm stability in the hypoxic and hypercapnic ventilatory responses toward a better understanding of potential neural mechanisms involved in hypoxic disordered breathing.

2 Methods

2.1 Mice

Studies were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee under protocol AN-6171 and all experiments were performed in accordance with relevant guidelines and regulations.

Heterozygous B6.FVB(Cg)-Tg(Th-Cre)Fl172Gsat (Th-Cre) mice were mated to homozygous RC::PDi mice to derive sibling cohorts in which all mice carried the RC::PDi allele. RC::PDi mice was maintained in the colony by backcrossing to C57BL/6J mice before homozygosing. Sibling mice that did not inherit the Th-Cre allele were used as sibling controls to the Th-Cre; PDi offspring. All experimental animals were treated in compliance with the United States Department of Health and Human Services and the Baylor College of Medicine IUACUC guidelines.

2.2 Whole Body Plethysmography

Mouse respiration was measured in a custom built barometric whole body plethysmograph. Ventilation was calibrated to a series of 20 µl pipetman injections into an empty 140 mL water-jacketed temperature controlled chamber at a rate of 3Hz while measuring baseline gas composition for each assay. The rate of gas inflow and outflow was continuously controlled via dual rotameters. Flow rate was 198.11mL/min. Gas was humidified to 100% by passing through a water column prior to entering the chamber.
and dehumidified by passing through 20cm Nafion tubing encased in silica desiccant. Plethysmography pressure changes were measured using a Validyne DP45 differential pressure transducer and CD15 carrier demodulator in comparison to a reference chamber and recorded with LabChartPro in real time. Chamber temperature was constantly monitored using a ThermoWorks MicroThermo 2 and probe and was recorded with LabChartPro in real time. Subsequent waveforms were analyzed offline to determine respiratory rate ($f_R$), tidal volume ($V_T$), minute ventilation ($V̇_E$), oxygen consumption ($V̇_O_2$), and minute ventilation normalized to oxygen consumption ($V̇_E/V̇_O_2$).

2.3 Experimental Design

2.3.1 Hypercapnic Assay. 12 $Th$-$Cre$; $PDi$ and 12 sibling control mice were assayed for their respiratory response in room air (21%$\text{O}_2$/79%$\text{N}_2$) and in 5% CO$_2$ balanced with room air before and after CNO administration (Fig. 1A). 10 $Th$-$Cre$; $PDi$ males and 2 females and 7 sibling control males and 5 females from nine litters were assayed. The animals ranged in age from 3 months to 6 months and body weight ranged from 19.7g to 45.2g. For each animal, the weight and rectal temperature was taken before the animal was placed in the plethysmography chamber (held at ~31-32°C). After acclimation (typically, 20-40 min, indicated by a steady respiratory trace free from movement artifact), a 20-minute baseline room air trace was taken, followed by a 20-minute exposure to 5% CO$_2$, followed by another 20 minutes of room air to return respiratory parameters to baseline. The animal was then taken out of the chamber for another rectal temperature measurement and intraperitoneal injection of CNO dissolved in saline at 1mg/mL for an effective concentration of 10 mg/kg. Prior studies suggest that behavioral changes from CNO in transgenic mice expressing DREADDs can take effect within 5-15 minutes of injection (Alexander et al., 2009, Ray et al., 2011) and clears from plasma within 2 hours (Guettier et al., 2009), although phenotypes can be observed for up to 10-15 hours. The animal was returned to the chamber for another 20 minutes of post-CNO room air, 20 minutes of 5% CO$_2$, and a final 20 minutes of room air before an end temperature was taken. Temperature was taken again 30 minutes after the end of the assay after the mouse was singly housed at ambient temperature (~23°C).

2.3.2 Hypoxic Assay. 11 $Th$-$Cre$; $PDi$ and 10 sibling control mice were assayed for their respiratory response in room air both before and after CNO administration, and in post-CNO 10% $\text{O}_2$. 9 $Th$-$Cre$; $PDi$ males and 2 females and 6 sibling control males and 4
females from eight litters were assayed. The animals ranged in age from 4.5 months to 7 months and body weight ranged from 24.7g to 50.1g. The protocol was similar to the hypercapnic assay, but instead of a 5% CO₂ exposure, the animal was exposed to 10% O₂ balanced with N₂ 20 min after CNO administration (Fig. 2A). Animals were not exposed to a pre-CNO 10% O₂ condition to avoid potential confounds of hypoxic plasticity.

2.4 Data Analysis

Respiratory waveforms were collected when the mouse was at rest and readings were free from movement artifacts. A minimum of 1 minute cumulative data compiled from traces at least 5 seconds long from the last 5 minutes of a given experimental condition were analyzed. No filtering or smoothing was applied to the pressure waveform. Tidal volume (V₉) was determined as described (Ray et al., 2011). Oxygen consumption was determined by comparing the gas composition between calibration in an empty chamber and live breathing using an AEI oxygen sensor and analyzer.

Poincaré plots and apnea and sigh measurements were determined using at least one minute of movement-free traces from each breathing condition. Apneas were defined as an interbreath interval (IBI) that was more than twice as long as the average IBI. Sighs were defined as a breath that had more than twice the amplitude of the average breath. The coefficients of variation (CV) of the IBI and amplitude were also calculated from the same trace compilation of each breathing condition (standard error IBI or amplitude / mean IBI or amplitude) and defined as the periodic and volume instabilities, respectively.

2.5 Statistics

Room air and hypercapnic results were compared using a linear mixed-effects regression model with animal type (experimental or control) and CNO treatment (pre or post) as fixed effects and animal ID as a random effect. Hypoxic and temperature results were compared between animal types using a linear mixed-effects regression model with animal type as a fixed effect. Individual data points and mean±s.e.m. are shown on each graph is shown in all figures.
2.6 Central Nervous System Cre Activity Pattern

Cell populations targeted by the Tg(Th-Cre)FI172Gsat driver were identified in images of coronal sections provided by the Gene Expression Nervous System Atlas (GENSAT). Structures and cell types were mapped by comparison to The Mouse Brain 3rd edition by Keith Franklin and George Paxinos (Franklin, 2008).

3 Results

3.1 The B6.FVB(Cg)-Tg(Th-Cre)FI172Gsat driver defines cells required for the hypercapnic ventilatory response.

To assess the requirement of Th-Cre defined neurons for the increased ventilatory response to hypercapnic (high CO₂) conditions, we employed the established RC::PDi inhibitory DREADD system and partnered it with the transgenic Th-Cre allele that captures catecholaminergic neurons in addition to several other brainstem populations, listed in Table 1. Using whole-body plethysmography, we measured the ventilatory response of animals under room air and 5% CO₂ before and after CNO administration (Fig. 1A). Th-Cre; PDi animals had baseline respiratory parameters similar to sibling controls during room air and 5% CO₂ conditions prior to CNO administration. In contrast, after CNO administration, Th-Cre; PDi mice showed significant respiratory and metabolic changes, especially under hypercapnic conditions (Fig. 3A,B). Under room air conditions, compared to pre-CNO values, we saw a statistically insignificant trend of increased V̇E (Fig. 1D, +35.9%, p=0.83) mediated by small increases in both fR (Fig. 1B, +6.28%, p=0.68) and VT (Fig. 1C, +26.2%, p=0.83). However, overall V̇E/VO₂ was unchanged as VO₂ increased as well (Fig. 1E, +27.9%, p=0.01). We also observed a reduction in periodic instability (Fig. 3F, -40.5% reduction in interbreath interval [IBI] coefficient of variation [CV], p=0.017). Upon a post-CNO hypercapnic challenge, V̇E was significantly reduced compared to pre-CNO values (Fig. 1D, -43.6%, p=0.01), mediated by a large reduction in fR (Fig. 1B, -26.3%, p<0.001) and a small but statistically insignificant reduction in VT (Fig. 1C, -22.7%, p=0.12). Overall V̇E/VO₂ (Fig. 1F, -69.9%, p<0.001) was also significantly reduced as post-CNO VO₂ levels were similar to pre-CNO levels. Other than the decrease in periodic instability seen under room air conditions, Th-
*Cre; PDi* animals did not show significant variability between pre- and post CNO respiratory patterns under either room air conditions or the 5% CO$_2$ hypercapnic challenge (Fig. 3).

Sibling control *PDi* mice showed no significant difference before or after CNO administration in $\dot{V}_E/\dot{V}O_2$, or constituent $f_R$, $V_T$, $\dot{V}_E$, or $\dot{V}O_2$ parameters or waveform pattern under room air or hypercapnic challenge.

### 3.2 The *B6.FVB(Cg)-Tg(Th-Cre)Fl172Gsat* driver defines cells required for the hypoxic ventilatory response.

To assess the requirement of *Th-Cre* defined neurons for the increased ventilatory response to hypoxic (low oxygen) conditions, we also measured respiration of *Th-Cre; PDi* animals under room air and 10% O$_2$ conditions (Figure 2A). Mirroring the data from the hypercapnic challenge, *Th-Cre; PDi* mice showed a statistically insignificant trend of increased $\dot{V}_E$ (Fig. 2D, +21.6%, p=0.22) mediated by small increases in $f_R$ (Fig. 2B, +5.1%, p=0.57) and $V_T$ (Fig. 2C, +14.0%, p=0.15) under room air after CNO administration as compared to pre-CNO values (Figure 2B). Overall $\dot{V}_E/\dot{V}O_2$ was unchanged once again as there was a parallel increase in $\dot{V}O_2$ (Fig. 2E, +26.6%, p=0.22). Compared to post-CNO sibling controls, *Th-Cre; PDi* mice showed a robustly reduced ventilatory hypoxic response (Fig. 2) with reductions in $f_R$ (Fig. 2B, -49.8%, p<0.001), $\dot{V}_E$ (Fig. 2D, -46.3%, p=0.0013) and $\dot{V}O_2$ (Fig. 2E, -39.8%, p<0.001). The change in $\dot{V}_E$ and $\dot{V}O_2$ was matched at 15 minutes as the $\dot{V}_E/\dot{V}O_2$ ratio was not significantly different (Fig. 2F). Poincaré analysis at 15 minutes of 10% O$_2$ reveals significant respiratory instability (Fig. 4A) in the *Th-Cre; PDi* animals as compared to sibling controls in both interbreath interval (Fig. 4E, p<0.001 for CV comparison) and tidal volume (Fig. 4F, p<0.001), culminating in a significant increase in apneas, (Fig. 4B, p=0.01), apnea length (Fig. 4C, p<0.001), and sighs (Fig. 4D, p=0.01).

Sibling control *PDi* mice showed no significant differences before or after CNO administration in $\dot{V}_E/\dot{V}O_2$, or constituent $f_R$, $V_T$, $\dot{V}_E$, or $\dot{V}O_2$ parameters or waveform pattern under room air.
3.2 The *B6.FVB(Cg)-Tg(Th-Cre)Fl172Gsat* driver defines cells required for temperature maintenance.

Temperature was taken as an element of the respiratory protocol at the beginning of the assay (pre-CNO at ambient temperature, ~23°C), immediately before CNO injection (pre-CNO at 30°C), immediately after the end of the assay (post-CNO at 30°C), and 30 minutes after the end of the assay (post-CNO at ambient temperature, ~23°C). In both the hypercapnic and hypoxic assays, *Th-Cre; PDi* animals showed a significant reduction in body temperature 30 minutes after the end of the assay as compared to sibling controls (Fig. 5, p=0.025 and p<0.001). Additionally, they also showed a reduced temperature immediately after hypoxic exposure (p<0.001).

4 Discussion

The goal of this project was to build a model system that captures catecholaminergic populations for functional perturbation to identify and study circuits involved in respiratory homeostasis. To access the catecholaminergic system, we utilized the *RC::PDi* mouse line to express the inhibitory DREADD receptor in *B6.FVB(Cg)-Tg(Th-Cre)Fl172Gsat* defined neurons and cells. Upon intraperitoneal injection of CNO, DREADD activation is expected to activate downstream endogenous Kir channels through G protein signaling and hyperpolarize neurons, disrupting their function and enabling acute whole body barometric plethysmography studies on conscious and unrestrained animals.

The *Th-Cre* driver mouse line captures populations that are critical to both the hypercapnic and hypoxic ventilatory responses when perturbed by exogenous Gi signaling via CNO activated DREADD (hM4D or Di). During hypercapnic challenges, CNO-DREADD mediated perturbation results in reduced $f_R$, $\dot{V}_E$, and $\dot{V}_E/\dot{V}_O_2$, and during hypoxic challenges, perturbation results in reduced $f_R$, $\dot{V}_E$, and $\dot{V}_O_2$. Additionally, while statistically insignificant, in room air conditions of both studies perturbation of *Th-Cre* cells resulted in a trend of increased $\dot{V}_E$ with a matched increase in $\dot{V}_O_2$ (that was significant in the hypercapnic assay). These results suggest that *Th-Cre* captures a
population of cells that may directly or indirectly influence metabolic rate, but does not disrupt, under baseline conditions, $\dot{V}E/\dot{V}O_2$ regulation.

Interestingly, perturbation of Th-Cre neurons under hypoxic conditions also results in Cheyne-Stokes like respiration with instability in both interbreath interval and tidal volume and an increased number of apneas and sighs. This suggests that the Th-Cre driver captures neuron populations involved in central breathing pattern generation, whose functions are most critical under hypoxic stress as the phenotype is not seen in normoxic nor hypercapnic conditions. The shape of the waveform suggests that post-inspiratory and or expiratory populations are affected.

To avoid the potential confound of a hypoxic plasticity response affecting post-CNO room air and hypoxic ventilation, we did not include a pre-CNO hypoxic exposure in our protocol. Thus, we cannot technically rule out the possibility that the hypoxic phenotype we observe is not due to CNO-mediated perturbation of the targeted cells, but a background interaction between the genetic background of the Th-Cre mouse and hypoxia, or the genetic background and injection or CNO. We find this highly unlikely, however, considering the type and magnitude of the phenotype, which we have never observed in a multitude of our own DREADD-based assays, including other drivers on the Gensat genetic background, and several previous reports showing CNO to be otherwise inert in these assays.

The Th-Cre driver captures several different populations of neurons in the brainstem and elsewhere in the CNS beyond catecholaminergic neurons, including subsets of the serotonergic raphé nuclei, the vestibular nucleus, and the reticulo- and pedunculo-tegmental nuclei of the pons (Table 1). Our data using the same hM4D-CNO system in combination with a dopaminergic specific Dopamine-active-transporter (DAT)-Cre that is a gene knock in with high fidelity and a noradrenergic/adrenergic specific Dopamine-beta-hydroxylase (DBH)-Cre also show deficits in the hypercapnic response but do not phenocopy the hypoxic deficit and instability (data not shown), suggesting that distinct aspects of the phenotype seen in Th-Cre; PDi animals may arise from a non-catecholaminergic population of cells.

Given the large number of anatomically defined populations captured in the central nervous system as well as possible peripheral populations, it becomes difficult to
speculate on which populations may contribute to which aspects of the observed hypercapnic and hypoxic phenotypes. Further confounds comes from differences in approach. While we use a cell autonomous acute pharmacogenetic perturbation, many approaches utilize genetic, pharmacological, and electrolytic lesions that may produce cell non-autonomous effects, induce developmental compensatory mechanisms, and may capture fibers of passage and other intercalated cell types. Lastly, our respiratory measurements are normalized to oxygen consumption, which can partially account for indirect or secondary respiratory changes that may result from increased or decreased metabolic drive. Nonetheless, it is likely that we are perturbing several neuron populations that are important to different aspects of breathing in developmental disorders, pathophysiologies, and neurodegenerative disorders.

Several developmental disorders such as Rett Syndrome (Katz et al., 2009, Weese-Mayer et al., 2006, Viemari et al., 2005), Pit-Hopkins Syndrome (Peippo and Ignatius, 2012) and CCHS (Ramanantsoa and Gallego, 2013) show respiratory instability and reduced chemosensory function. In these disorders central serotonergic and noradrenergetic chemosensory function has been shown to be affected and appear to be captured by this driver (Toward et al., 2013, Viemari et al., 2005). Although this is an adult model, the findings may offer some insight into the potential role of CA in Sudden Infant Death Syndrome, both autonomously and non-autonomously where CA levels have been shown to be lowered in PACAP -/- mice that have a higher level of sudden death in the first two weeks of life and show reduced catecholamine levels and chemosensory deficits (Arata et al., 2013, Cummings et al., 2004). Additional pathophysiologies such as obstructive sleep apnea, ARDS, and COPD present with respiratory instability while engaging the hypoxic reflex circuitry to maintain vital functions. A series of studies on Parkinson’s disease using 6-OHDA in the striatum showed a loss of CA dopamine neurons and RTN neurons resulting in chemosensory dysfunction that was mitigated by potential compensation by the Locus Coeruleus (Tuppy et al., 2015, Oliveira et al., 2017). Given that this model only shows respiratory instability under hypoxic challenges, the B6.FVB(Cg)-Tg(Th-Cre)Fl172Gsat driver defines new populations that either as a whole or separately may play a role in the circuitry that is developmentally perturbed in these pathophysiologies, or engaged when responding to hypoxic stress from obstruction, compromised pulmonary function, or neurodegeneration.
Finally, perturbation of *Th-Cre; PDi* defined neurons resulted in significant temperature deficits 30 minutes after removal from the chamber (~70 minutes after CNO injection) and a deficit immediately after hypoxic exposure (~40 minutes after CNO injection). As thermogenesis is a crucial component of physiological homeostasis, this suggests that the *Th-Cre* driver captures cells critical for temperature maintenance and may play a role in metabolic homeostasis.

5 Conclusions

The results presented here define a set of neurons that encompass the catecholaminergic system and other distinct populations in the brainstem that play a role in room air respiration and metabolic regulation, and are also required for the hypercapnic and hypoxic ventilatory reflexes and respiratory stability while facing a homeostatic challenge. This work sets the stage for future studies to more closely model respiratory pathophysiologies to determine the critical subsets of neurons required for respiratory stability under hypoxic conditions, yielding key insights as to the neural substrates that underlie congenital and adult respiratory pathophysiologies.

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7 Competing interests

No competing interests declared.

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REFERENCES


Figures

A

B

Th-Cre; PDI, n=12

Sibling controls, n=12

C

D

E

F
Figure 1. *Th-Cre; PDi* mice show a blunted response to hypercapnic conditions after DREADD-mediated acute perturbation. A) Hypercapnic protocol. B-F) CNO-DREADD mediated inhibition of *Th-Cre; PDi* defined neurons results in changes in room air ventilation and decreased hypercapnic ventilation. Upon CNO administration, *Th-Cre; PDi* animals (n=12) show an increase in $\dot{V}O_2$ (p=0.01) but no overall change to $\dot{V}E/\dot{V}O_2$. Under 5% CO$_2$ conditions, CNO administration in *Th-Cre; PDi* animals results in dramatically reduced $f_R$ (p=0<0.001), $\dot{V}E$ (p=0=0.01), and $\dot{V}E/\dot{V}O_2$ (p=0<0.001). Sibling controls (n=12) show no significant difference between pre- and post-CNO conditions. Individual data points and mean±s.e.m. are shown on each graph. Statistical significance was determined using a linear mixed-effects regression model with animal type (experimental or control) and CNO treatment (pre or post) as fixed effects and animal ID as a random effect. *p<0.05, **p<0.01, ***p<0.001, ns=not significant.
Figure 2. *Th-Cre; PDi* mice show a blunted response to hypoxic conditions after DREADD-mediated acute perturbation. A) Hypoxic protocol. B-F) CNO-DREADD mediated inhibition of *Th-Cre; PDi* defined neurons results in decreased hypoxic ventilation. Upon CNO administration, no change is seen under room air conditions in *Th-Cre; PDi* mice (n=11). Under hypoxic conditions, *RC::PDi; Th-Cre* show dramatically reduced $f_R$ ($p=0<0.001$), $\dot{V}E$ ($p=0=0.0013$), and $\dot{V}O_2$ ($p=0<0.001$) as compared to sibling controls (n=10). Sibling controls show no significant difference before or after CNO administration under room air conditions. Individual data points and mean±s.e.m. are shown on each graph. Statistical significance was determined using a linear mixed-effects regression model with animal type as a fixed effect. *p<0.05, **p<0.01, ***p<0.001, ns=not significant.
Figure 3. *Th-Cre; PDi* mice have minimal changes in waveform pattern after DREADD-mediated acute perturbation under room air and hypercapnic conditions. A) Representative respiratory traces pre- and post-CNO during room air breathing conditions in *Th-Cre; PDi* animals. B) Representative respiratory traces pre- and post-CNO during hypercapnic breathing conditions in *Th-Cre; PDi* animals. C-G) *Th-Cre; PDi* animals (n=12) showed a reduction in periodic instability (F) after CNO administration under baseline conditions (p=0.017) and no change in waveform parameters (apnea frequency, average apnea length, sigh frequency, periodic and volume instability) under hypercapnic conditions. Sibling controls (n=12) showed no significant difference before or after CNO administration. Individual data points and mean±s.e.m. are shown on each graph. Statistical significance was determined using a linear mixed-effects regression model with animal type (experimental or control) and CNO treatment (pre or post) as
fixed effects and animal ID as a random effect. *p<0.05, **p<0.01, ***p<0.001, ns=not significant.
Figure 4. *Th-Cre; PDi* mice show dramatic changes in waveform pattern under hypoxic conditions after DREADD-mediated acute perturbation. A) Representative respiratory traces post-CNO during hypoxic breathing conditions in *Th-Cre; PDi* animals and sibling controls. In addition to reductions in amplitude and frequency, significant
instability in both periodicity and amplitude can be seen in Th-Cre; PDi animals. B-F) Th-Cre; PDi animals (n=11) showed a dramatic change in waveform pattern after CNO administration under hypoxic conditions, with increases in apnea number (p=0.01), average apnea length (p<0.001), sigh number (p=0.01), and periodic (p<0.001) and volume instability (p<0.001) as compared to sibling controls (n=10). Individual data points and mean±s.e.m. are shown on each graph. Statistical significance was determined using a linear mixed-effects regression model with animal type as a fixed effect. *p<0.05, **p<0.01, ***p<0.001, ns=not significant. G) Poincaré plots (the inter-breath interval (IBI) plotted as a function of the previous inter-breath interval) for two Th-Cre; PDi mice (left) and two sibling controls (right) after CNO administration while breathing 10% O2. H) Poincaré plots (the amplitude plotted as a function of the previous breath’s amplitude) for two Th-Cre; PDi mice (left) and two sibling controls (right) after CNO administration while breathing 10% O2.
A

**Hypercapnic Assay**

- Graph showing temperature changes over time (T_start, T_mid, T_end, T_post 30)
- Data points for Th-Cre; PD1 (n=12) and Sibling controls (n=12)

B

**Hypoxic Assay**

- Graph showing temperature changes over time (T_start, T_mid, T_end, T_post 30)
- Data points for Th-Cre; PD1 (n=10) and Sibling controls (n=11)
- Significant differences indicated by ***
Figure 5. *Th-Cre; PDi* mice show a reduced temperature following CNO administration. **A)** 30 minutes after the end of the hypercapnic assay ($T_{\text{post 30}}$), *Th-Cre; PDi* animals showed a small reduction in temperature as compared to sibling controls ($p=0.025$). **B)** After hypoxic exposure, *Th-Cre; PDi* animals showed a reduction in temperature at the end of the assay ($T_{\text{end}}$, $p<0.001$) and a dramatic drop 30 minutes after the end of the assay ($T_{\text{post 30}}$, $p<0.001$). Individual data points and mean±s.e.m. are shown on each graph. Statistical significance was determined using a linear mixed-effects regression model with animal type as a fixed effect. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns=not significant.*
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<td>Reticular thalamic nucleus</td>
<td>GABAergic neurons</td>
</tr>
<tr>
<td>P39</td>
<td>-0.94 mm</td>
<td>Lateral hypothalamus</td>
<td>Glutamatergic, orexin neurons</td>
</tr>
<tr>
<td>P41</td>
<td>-1.22 mm</td>
<td>Para- and paro- ventricular hypothalamus</td>
<td>Magnocellular, parvocellular neurosecretary neurons</td>
</tr>
<tr>
<td>P52</td>
<td>-2.54 mm</td>
<td>Posterior hypothalamus</td>
<td>Sympatho-excitatory neurons</td>
</tr>
<tr>
<td>P53</td>
<td>-2.70 mm</td>
<td>Substantia nigra</td>
<td>Dopaminergic neurons</td>
</tr>
<tr>
<td>P56</td>
<td>-3.08 mm</td>
<td>Ventral tegmental area</td>
<td>Dopaminergic neurons</td>
</tr>
<tr>
<td>P56</td>
<td>-3.08 mm</td>
<td>Periaqueductal gray (sparse)</td>
<td>Enkaphalin neurons</td>
</tr>
<tr>
<td>P65</td>
<td>-4.16 mm</td>
<td>Dorsal raphé</td>
<td>Dopaminergic, serotonergic, substance P neurons</td>
</tr>
<tr>
<td>P65</td>
<td>-4.16 mm</td>
<td>Retrorubal fields / A8 dopamine cells</td>
<td>Dopaminergic neurons</td>
</tr>
<tr>
<td>P65</td>
<td>-4.16 mm</td>
<td>B9 serotonergic cells</td>
<td>Serotonergic neurons</td>
</tr>
<tr>
<td>P65</td>
<td>-4.16 mm</td>
<td>Reticulo- and pedunculo tegmental nucleus of the pons</td>
<td>Cholinergic, glutamatergic neurons</td>
</tr>
<tr>
<td>P71</td>
<td>-4.84 mm</td>
<td>Raphé magnus nucleus</td>
<td>Serotonergic neurons</td>
</tr>
<tr>
<td>P75</td>
<td>-5.34 mm</td>
<td>Locus coeruleus</td>
<td>Noradrenergic neurons</td>
</tr>
<tr>
<td>P79</td>
<td>-5.80 mm</td>
<td>Vestibular nucleus</td>
<td>Vestibular projection cells</td>
</tr>
<tr>
<td>P82</td>
<td>-6.12 mm</td>
<td>Cerebellar deep nuclei (sparse)</td>
<td>Cerebellar projection cells</td>
</tr>
</tbody>
</table>

**Table 1. B6.FVB(Cg)-Tg(Th-Cre)Fl172Gsat Cre activity marks catecholaminergic and other neuron populations in the central nervous system.** In addition to catecholaminergic neurons, the *Th-Cre* driver also marks areas in the hypothalamus, amygdala, and serotonergic nuclei. Cell populations targeted by the *Th-Cre* driver were identified in images of coronal sections provided by the Gene Expression Nervous System Atlas (GENSAT). Structures and cell types were mapped by comparison to The Mouse Brain 3rd edition by Keith Franklin and George Paxinos (Franklin, 2008). GABA = gamma-Aminobutyric acid, GnRH = gonadotropin-releasing hormone-expressing, GHRH = growth hormone-releasing hormone, AgRP = agouti-related peptide, NPY = neuropeptide Y.