Th-Cre neurons results in reduced fR, hypoxic challenge (10% O2/90% N2), we saw reduced fR, maintains respiratory stability in the face of homeostatic challenges. Nonetheless, this work identifies a key respiratory population that may lead to further insights into the circuitry that CA neurons. The catecholaminergic (CA) 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 pathophysiology. Here, we used a noninvasive DREADD-based pharmacogenetic approach to acutely perturb Tg (Th-Cre)F1172Gsat (Th-Cre)-defined neurons in awake and unrestrained mice in an attempt to characterize CA 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 VC. 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-CA expression domains of the Tg(Th-Cre)F1172Gsat mouse line found in the brainstem, full phenotypic effect cannot be assigned solely to CA 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.

KEY WORDS: Brainstem, Catecholamine, DREADD, Hypercapnic reflex, Hypoxic reflex, Respiration

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 for 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); pathophysiology such as obstructive sleep apnea (Hakim et al., 2012; Zhu et al., 2007), Acute Respiratory Distress Syndrome (ARDS) (Detter 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 to 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 CA 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 pharmacogenetic neuronal inhibition.

DREADD-mediated neuronal manipulations combined with recombinase based targeting strategies have provided an approach for functional circuit mapping that enables acute noninvasive 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, 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 CA system, we utilized the Cre-responsive RC::PDi mouse in combination with the B6.FVB(Cg)-Tg(Th-Cre)F1172Gsat (hereafter Th-Cre) driver to express the hM4D receptor in CA and other neurons defined by this frequently used Th-Cre driver. Using whole-body plethysmography, we assessed respiratory and metabolic function under baseline (21% O2/79% N2), hypercapnic (5% CO2/21% O2/74% N2) and hypoxic (10% O2/90% N2) conditions after CNO-DREADD-mediated perturbation of targeted cells, examining several aspects of respiratory and metabolic homeostasis, including rate (fR), 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 CA 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.

RESULTS
The B6.FVB(Cg)-Tg(Th-Cre)F1172Gsat 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 CA 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. 2A,B). Under room air conditions, compared to pre-CNO values, we saw a statistically insignificant trend of increased $V_e$ (Fig. 1D, +35.9%, $P<0.83$) mediated by small increases in both $f_R$ (Fig. 1B, +6.28%, $P=0.68$) and $V_T$ (Fig. 1C, +26.2%, $P=0.83$). However, overall $V_e/V_O_2$ was unchanged as $V_O_2$ increased (Fig. 1E, +27.9%, $P=0.01$). We also observed a reduction in periodic instability [Fig. 2F, −40.5% reduction in interbreath interval (IBI) coefficient of variation (CV), $P=0.017$]. Upon a post-CNO hypercapnic challenge, $V_e$ was significantly reduced compared to pre-CNO values (Fig. 1D, −43.6%, $P=0.01$), mediated by a large reduction in $f_R$ (Fig. 1B, −26.3%, $P<0.001$) and a small but statistically insignificant reduction in $V_T$ (Fig. 1C, −22.7%, $P=0.12$). Overall $V_e/V_O_2$ (Fig. 1F, −69.9%, $P<0.001$) was also significantly reduced as post-CNO $V_O_2$ 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% CO2 hypercapnic challenge (Fig. 2).

Sibling control PDi mice showed no significant difference before or after CNO administration in $V_e/V_O_2$, or constituent $f_R$, $V_T$, $V_e$, or $V_O_2$ parameters or waveform pattern under room air or hypercapnic challenge.

### Table 1. B6.FVB(Cg)-Tg(Th-Cre)F1172Gsat Cre activity marks CA and other neuron populations in the CNS

| Slice | Bregma | Area | Putative Neurons |
|-------|--------|------|------------------|
| P16   | 1.78 mm | Olfactory areas (tubule, nucleus, piriform cortex) | Olfactory projection neurons |
| P25   | 0.74 mm | Septal nucleus and nucleus accumbens (sparsely) | GABAergic medium spiny neurons, cholinergic interneurons |
| P25   | 0.74 mm | Caudate putamen | GABAergic, dopaminergic neurons |
| P27   | 0.50 mm | Lateral and medial preoptic area of the hypothalamus | GnRH neurons, GABAergic neurons |
| P31   | 0.02 mm | Periventricular hypothalamic nucleus | Magnocellular, parvocellular neurosecretory neurons |
| P32   | −0.01 mm | Bed nucleus of the stria terminalis (sparsely) | Glutamatergic neurons |
| P37   | −0.70 mm | Amygdala (various nuclei) | GABAergic neurons |
| P37   | −0.70 mm | Reticular thalamic nucleus | GABAergic neurons |
| P39   | −0.94 mm | Lateral hypothalamus | Glutamatergic, orexin neurons |
| P41   | −1.22 mm | Para- and paraventricular hypothalamus | Magnocellular, parvocellular neurosecretory neurons |
| P41   | −1.22 mm | Arcuate nucleus | Dopaminergic neurons, GHRH neurons, AgRP, NPY, GABAergic neurons |
| P52   | −2.54 mm | Posterior hypothalamus | Sympatho- excitatory neurons |
| P53   | −2.70 mm | Substantia nigra | Dopaminergic neurons |
| P56   | −3.08 mm | Ventral tegmental area | Dopaminergic neurons |
| P56   | −3.08 mm | Periaqueductal gray (sparsely) | Enkephalin neurons |
| P65   | −4.16 mm | Dorsal raphé | Dopaminergic, serotonergic, substance P neurons |
| P65   | −4.16 mm | Retronubral fields / A8 dopamine cells | Dopaminergic neurons |
| P65   | −4.16 mm | B9 serotonergic cells | Serotonergic neurons |
| P65   | −4.16 mm | Reticulo- and pedunculo segmental nucleus of the pons | Cholinergic, glutamatergic neurons |
| P71   | −4.84 mm | Raphé magnus nucleus | Serotonergic neurons |
| P75   | −5.34 mm | Locus coeruleus | Noradrenergic neurons |
| P79   | −5.80 mm | Vestibular nucleus | Vestibular projection cells |
| P82   | −6.12 mm | Cerebellar deep nuclei (sparsely) | Cerebellar projection cells |

In addition to CA 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 GENSAT. Structures and cell types were mapped by comparison to ‘The Mouse Brain in Stereotaxic Coordinates’ (3rd edition) (Franklin and Paxinos, 2008). GABA, gamma-aminobutyric acid; GnRH, gonadotropin-releasing hormone-expressing; GHRH, growth hormone-releasing hormone; AgRP, agouti-related peptide; NPY, neuropeptide Y.

### The B6.FVB(Cg)-Tg(Th-Cre)F1172Gsat 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% O2 conditions (Fig. 3A). Mirroring the data from the hypercapnic challenge, Th-Cre; PDi mice showed a statistically insignificant trend of increased $V_e$ (Fig. 3D, +21.6%, $P=0.22$), mediated by small increases in $f_R$ (Fig. 3B, +5.1%, $P=0.57$) and $V_T$ (Fig. 3C, +14.0%, $P=0.15$), under room air after CNO administration as compared to pre-CNO values. Overall $V_e/V_O_2$ was unchanged once again as there was a parallel increase in $V_O_2$ (Fig. 3E, +26.6%, $P=0.22$). Compared to post-CNO sibling controls, Th-Cre; PDi mice showed a robustly reduced ventilatory hypoxic response (Fig. 3) with reductions in $f_R$ (Fig. 3B, −49.8%, $P<0.001$), $V_e$ (Fig. 3D, −46.3%, $P=0.0013$) and $V_O_2$ (Fig. 3E, −39.8%, $P=0.001$). The change in $V_e$ and $V_O_2$ was matched at 15 min as the $V_e/V_O_2$ ratio was not significantly different (Fig. 3F).

Poincaré analysis at 15 min of 10% O2 showed significant respiratory instability (Fig. 4A,G,H) 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 $V_e/V_O_2$, or constituent $f_R$, $V_T$, $V_e$, or $V_O_2$ parameters or waveform pattern under room air.

### The B6.FVB(Cg)-Tg(Th-Cre)F1172Gsat 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 min 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 min 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).

Fig. 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 $V_{O_2}$ (P=0.01) but no overall change to $V_{E}/V_{O_2}$. Under 5% CO$_2$ conditions, CNO administration in Th-Cre; PDi animals results in dramatically reduced $f_R$ (P<0.001), $V_{E}$ (P=0.01), and $V_{E}/V_{O_2}$ (P=0.001). Sibling controls (n=12) show no significant difference between pre- and post-CNO conditions. Individual data points and means±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.

**DISCUSSION**

The goal of this project was to build a model system that captures CA populations for functional perturbation to identify and study circuits involved in respiratory homeostasis. To access the CA system, we utilized the RC::PDi mouse line to express the inhibitory DREADD receptor in B6.FVB(Cg)-Tg(Th-Cre)FI172Gsat-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 D). During hypercapnic challenges, CNO-DREADD-mediated perturbation results in reduced $f_R$, $V_{E}$ and $V_{E}/V_{O_2}$, and during hypoxic challenges, perturbation results in reduced $f_R$, $V_{E}$, and $V_{O_2}$. Additionally, while statistically insignificant, in room air conditions of both studies, perturbation of Th-Cre cells resulted in a trend of increased $V_{E}$ with a matched increase in $V_{O_2}$ (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, $V_{E}/V_{O_2}$ 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, the functions of which are most critical under hypoxic stress as the phenotype is not seen in normoxic or hypercapnic conditions. The shape of the waveform suggests that postinspiratory 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 central nervous system.
system (CNS) beyond CA 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, 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-CA population of cells.

Given the large number of anatomically defined populations captured in the CNS 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 arise 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 nonautonomous effects, induce developmental compensatory mechanisms, and 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, pathophysiology and neurodegenerative disorders.

Several developmental disorders such as Rett Syndrome (Katz et al., 2009; Weese-Mayer et al., 2006; Viemari et al., 2005), Pit...
Fig. 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 (IBI plotted as a function of the previous IBI) 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.
Fig. 5. Th-Cre; PDi mice show a reduced temperature following CNO administration. (A) 30 min after the end of the hypercapnic assay (Tpost 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 (Tend, P<0.001) and a dramatic drop 30 min after the end of the assay (Tpost 30, P<0.001). Individual data points and means±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.
with room air before and after CNO administration (Fig. 1A). Ten male and two female Th-Cre; Pdi mice, and seven male and five female sibling controls, from nine litters were assayed. The animals ranged in age from 3 months to 6 months, and body weight ranged from 19.7 g to 45.2 g. 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-min baseline room air trace was taken, followed by a 20-min exposure to 5% CO2, followed by another 20 min 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 1 mg/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 min of injection (Alexander et al., 2009; Ray et al., 2011), and clears from plasma within 2 h (Guettier et al., 2009), although phenotypes can be observed for up to 10-15 h. The animal was returned to the chamber for another 20 min of post-CNO room air, 20 min of 5% CO2, and a final 20 min of room air before an end temperature was taken. Temperature was taken again 30 min after the end of the assay after the mouse was singly housed at ambient temperature (∼23°C).

Hypoxic assay

 Eleven Th-Cre; Pdi mice 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% O2. Nine male and two female Th-Cre; Pdi mice, and six male and four female sibling controls, from eight litters were assayed. The animals ranged in age from 4.5 months to 7 months, and body weight ranged from 24.7 g to 50.1 g. 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. 3A). Animals were not exposed to a pre-CNO 10% O2 condition to avoid potential confounds of hypoxic plasticity.

Data analysis

Respiratory waveforms were collected when the mouse was at rest, and readings were free from movement artifacts. A minimum of 1 min cumulative data collected from traces at least 5 s long from the last 5 min of a given experimental condition were analyzed. No filtering or smoothing was applied to the pressure waveform. Tidal volume (Vt) was determined as described (Ray et al., 2011). Oxygen consumption was determined by comparing the gas composition between calibration in an empty chamber described (Ray et al., 2011). Oxygen consumption was determined by mouse was singly housed at ambient temperature (Temperature was taken again 30 min after the end of the assay after the animal was returned to room air to return respiratory parameters to baseline. The animal was then placed in the plethysmography chamber (held at ∼23°C). After acclimation (typically 20-40 min, indicated by a steady respiratory trace free from movement artifact), a 20-min baseline room air trace was taken, followed by a 20-min exposure to 5% CO2, followed by another 20 min 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 1 mg/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 min of injection (Alexander et al., 2009; Ray et al., 2011), and clears from plasma within 2 h (Guettier et al., 2009), although phenotypes can be observed for up to 10-15 h. The animal was returned to the chamber for another 20 min of post-CNO room air, 20 min of 5% CO2, and a final 20 min of room air before an end temperature was taken. Temperature was taken again 30 min after the end of the assay after the mouse was singly housed at ambient temperature (∼23°C).

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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 in all figures.

CNS Cre activity pattern

Cell populations targeted by the Tg(Th-Cre)FlI72Gsat 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 Franklin and Paxinos (2008).
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