

Vglut2-based glutamatergic signaling in central noradrenergic neurons is dispensable for normal breathing and chemosensory reflexes

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Abstract

Central noradrenergic (NA) neurons are key constituents of the respiratory homeostatic network. NA dysfunction is implicated in several developmental respiratory disorders including Central Congenital Hyperventilation Syndrome (CCHS), Sudden Infant Death Syndrome (SIDS) and Rett Syndrome. The current unchallenged paradigm in the field, supported by multiple studies, is that glutamate co-transmission in subsets of central NA neurons plays a role in breathing control. If true, NA-glutamate co-transmission may also be mechanistically important in respiratory disorders. However, the requirement of NA derived glutamate in breathing has not been directly tested and the extent of glutamate co-transmission in the central NA system remains uncharacterized. Therefore, we fully characterized the cumulative fate maps and acute adult expression patterns of all three Vesicular Glutamate Transporters (*Slc17a7* (Vglut1), *Slc17a6* (Vglut2), and *Slc17a8* (Vglut3)) in NA neurons, identifying a novel dynamic expression pattern for Vglut2 and an undescribed co-expression domain for Vglut3 in the NA system. Our functional studies showed that loss of Vglut2 throughout the NA system failed to alter breathing or metabolism under room air, hypercapnia, or hypoxia in unrestrained and conscious mice, which demonstrates that Vglut2-based glutamatergic signaling within the central NA system is not required for normal baseline breathing and hypercapnic, hypoxic chemosensory reflexes. These outcomes challenge the current understanding of central NA neurons in the control of breathing and suggests that glutamate may be not a critical target to understand NA neuron dysfunction in respiratory diseases.

eLife assessment

Chang et al. provide glutamate co-expression profiles in the central noradrenergic system and test the requirement of Vglut2-based glutamatergic release in respiratory and metabolic activity under physiologically relevant gas challenges. Their experiments provide **compelling** evidence that conditional deletion of Vglut2 in noradrenergic neurons does not impact steady-state breathing or metabolic activity in room air, hypercapnia, or hypoxia. This study provides an **important** contribution to our understanding of how noradrenergic neurons regulate respiratory homeostasis in conscious adult mice.

Introduction

Breathing is a vital and life-sustaining function supporting homeostatic processes, most critically maintaining blood pH/CO₂ and O₂ levels within a narrow physiological range. Respiratory homeostasis is mediated by neuron-modulated lung ventilation adjustments in response to physiological deviations resulting in high pCO₂ or low pO₂ blood and tissue levels, known as the hypercapnic and hypoxic reflexes respectively (Del Negro et al., 2018 [DOI](#); Dick et al., 2018 [DOI](#)). These reflexes are part of a complex brainstem neural network that integrates a multitude of information streams across the central and peripheral nervous systems to regulate respiratory output. Within this brainstem network, central noradrenergic (NA) neurons are known to be an important component that plays a variety of roles in modulating breathing. Furthermore, various perturbations across the central NA system have been implicated in several developmental disorders with respiratory and chemosensory features such as Central Congenital Hyperventilation Syndrome (CCHS), Sudden Infant Death Syndrome (SIDS) and Rett Syndrome (Beltrán-Castillo et al., 2017 [DOI](#); Feldman et al., 2013 [DOI](#); Gauda et al., 2007 [DOI](#); Viemari, 2008 [DOI](#)). Thus, understanding how central NA neurons modulate respiratory chemoreflexes or chemosensory breathing is critically important for the development of new diagnostic and therapeutic interventions to address respiratory pathophysiology.

Central noradrenergic neurons are commonly thought to exert their effect on breathing through their primary neurotransmitter noradrenaline and adrenaline. However, in addition to noradrenaline, several studies suggest that glutamate is co-transmitted in subsets of central NA neurons. Vesicular glutamate transporter 2 (Vglut2), a gene marker of glutamatergic signaling, has been shown to be co-expressed in subsets of central NA neurons including, C1/A1, C2/A2, A5, and LC in adult rats and mice (DePuy et al., 2013 [DOI](#); Souza et al., 2022a [DOI](#); Stornetta et al., 2002a [DOI](#), 2002b [DOI](#); Yang et al., 2021 [DOI](#)), though NA specific expression of related Vglut1 and Vglut3 transporters remains unknown. Additionally, it has been well documented that central NA neurons co-expressing Vglut2 innervate key respiratory centers, such as preBötzinger complex and parafacial region (pFRG), as well as many other autonomic brainstem and forebrain centers that could indirectly drive a change in breathing if perturbed, i.e., cardio-vascular function, the intermediolateral nucleus (IML)-sympathetic system and metabolism (Supplemental table 1). Thus, it has currently become a dominant paradigm in the field of respiratory physiology that NA-based glutamate release is a critical form of neurotransmission in the control of breathing. This paradigm is supported by multiple papers. Abbott et al. (2014) [DOI](#) showed that Vglut2 is required for an increase in respiratory rate when anterior C1 neurons are unilaterally opto-genetically stimulated. Malheiros-Lima et al. (2020) [DOI](#) showed that Vglut2-expressing C1 neurons project to the pFRG region, and hypoxic breathing was blunted after blockade of ionotropic glutamatergic receptors at the pFRG site in anesthetized rats, together supporting a role for anterior C1 neurons releasing glutamate at the pFRG site in turn to regulate breathing under hypoxia. Similarly,

Malheiros-Lima et al. (2022) [\[1\]](#) and Malheiros-Lima et al. (2018) [\[2\]](#) showed that Vglut2-expressing C1 neurons project to the NA A5 region and the preBötzing complex respectively, and again the blockade of ionotropic glutamatergic receptors at the A5 region or preBötzing complex reduced the increase in phrenic nerve activities or in respiratory frequency elicited by optogenetic stimulation of C1 cells in an anesthetized preparation. In addition, Guyenet et al. (2013) [\[3\]](#) and others speculate that the apparent lack of plasmalemmal monoamine transporter in C1 fibers indicates reduced or absent noradrenergic or adrenergic signaling due a lack of re-uptake and neurotransmitter pool depletion (Comer et al., 1998 [\[4\]](#); Lorang et al., 1994 [\[5\]](#)). Cumulatively, the studies argue for glutamate as the predominant functional neurotransmitter for C1 NA neurons in the breathing neural network. To our knowledge, this dominant perspective has not been otherwise previously challenged. Although these studies are informative, the evidence supporting the role of NA-based Vglut2 signaling in respiratory control are either indirect and circumstantial or cannot be seen as physiological given experimental limitations, such as the focal nature of optogenetic stimulation. Thus, it is not yet clear what the requirement is for NA-based glutamatergic signaling in homeostatic breathing in the awake and unrestrained animal and how that might inform upon disease.

To better understand the role of NA-based glutamatergic signaling in breathing, we sought to both fully characterize the molecular profiles of the central noradrenergic system with respect to glutamate co-expression and to determine the requirement of Vglut2-based glutamatergic release in respiratory control under physiological chemosensory challenges in awake and unrestrained mice. We first fully characterized the recombinase-based cumulative fate maps for Vglut1, Vglut2 and Vglut3 expression and compared those maps to their real time expression profiles in central NA neurons by RNA *in situ* hybridization in adult mice. We found a novel dynamic expression pattern for Vglut2 and an entirely undescribed co-expression domain for Vglut3 in the central NA system. Secondly, to determine if Vglut2-based glutamatergic signaling in NA neurons is required for respiratory homeostasis, we conditionally ablated Vglut2 in all NA neurons and tested respiratory, chemosensory, and metabolic function in unrestrained and conscious mice. Using the same genetic model in prior studies (Abbott et al., 2014 [\[6\]](#)), conditional deletion of Vglut2 in NA neurons did not significantly impact breathing under room air, hypercapnic, or hypoxic conditions. These results demonstrate, for the first time, that NA Vglut2-based glutamatergic signaling is dispensable for respiratory control, which challenges the prevalent perspective on the role of C1 and other Vglut2 expressing NA neurons in respiratory homeostasis and suggests that glutamate may not be a critical target to understand NA neuron dysfunction in respiratory diseases.

Results

Cumulative fate maps of central noradrenergic neurons co-expressing Vglut1, Vglut2 and Vglut3

To fully characterize the expression profiles of all three glutamate markers Vglut1, Vglut2 and Vglut3 in central noradrenergic neurons, we first used an intersectional genetic strategy (**Figure 1A** [\[7\]](#)). We bred three Cre drivers *Slc17a7^{Cre}* (Vglut1-Cre), *Slc17a6^{Cre}* (Vglut2-Cre) and *Slc17a8^{Cre}* (Vglut3-Cre) to *Dbh^{p2a-Flpo}* (DBH-p2a-Flpo) (targeting noradrenergic neurons) mice respectively. The three compound lines of Vglut1-Cre/+; DBH-p2a-Flpo/+, Vglut2-Cre/+; DBH-p2a-Flpo/+, and Vglut3-Cre/+; DBH-p2a-Flpo/+ were then crossed with the *Rosa26^{RC::FLTG}* (RC::FLTG) intersectional reporter mice which express tdTomato in cells expressing only flippase (Flpo) while expressing eGFP in cells co-expressing both Flpo and Cre recombinases. Thus, in each of the three intersectional reporter crosses [e.g. 1) Vglut1-Cre/+; DBH-p2a-Flpo/+; RC::FLTG/+, 2) Vglut2-Cre/+; DBH-p2a-Flpo/+; RC::FLTG/+ and 3) Vglut3-Cre/+; DBH-p2a-Flpo/+; RC::FLTG/+], NA neurons without any Vglut1/2/3 expression are labelled by red fluorescent protein (tdTomato) while NA neurons co-expressing either Vglut1, Vglut2, or Vglut3 are labeled by green fluorescent protein (eGFP). By

using this method, we characterized and quantified the expression profiles of all three vesicular glutamate transporters Vglut1, Vglut2 and Vglut3 across every NA nucleus in adult mice including A7, Locus Coeruleus (LC), sub CD/CV, A5, and throughout the anterior - posterior dimension of C1/A1, C2/A2 (**Figure 1B-D**). Vglut1-Cre was not co-expressed in any central NA nuclei in adult mice. However, Vglut2-Cre and Vglut3-Cre both showed co-expression in central NA neurons. Vglut3-Cre-expressing NA neurons are restricted to posterior C2/A2 and posterior C1/A1, and the majority is in posterior C2/A2 where $26.9 \pm 3.16\%$ NA neurons are Vglut3-Cre positive. In posterior C1/A1, only $1.26 \pm 0.559\%$ NA neurons are Vglut3-Cre positive. Surprisingly, $84.6 \pm 3.75\%$ NA neurons in total have Vglut2-Cre co-expression and each NA nucleus was predominantly labelled by Vglut2-Cre expression. The percentage of Vglut2-Cre positive NA neurons in A7, LC, A5, sub CD/CV, anterior and posterior part of C1/A1, anterior and posterior part of C2/A2 is $69.8 \pm 8.29\%$, $80.5 \pm 3.78\%$, $84.8 \pm 11.3\%$, $51.5 \pm 2.61\%$, $99.0 \pm 0.681\%$, $95.7 \pm 2.40\%$, $96.1 \pm 1.98\%$, $97.8 \pm 0.771\%$, respectively (Mean $\pm$ SEM). The presence of Vglut2-Cre co-expression in anterior NA groups was unexpected as previous *in situ* data in adult rats found Vglut2 positive NA neurons only in the posterior C2/A2 and C1/A1 (DePuy et al., 2013; Stornetta et al., 2002a, 2002b). Notably, however, the Vglut2 co-expression in the LC region agrees with Yang et al. (2021) which also showed that a high percentage of LC NA neurons are Vglut2 positive by using another intersectional strategy in mice, *Th^{Flpo}; Slc17a6^{Cre}; Rosa26^{Ai65}* (TH-Flpo; Vglut2-Cre; Ai65). The differences in our and other fate maps compared to *in situ* hybridization may either reflect early gene expression that is down regulated in the adult or may reflect low levels of expression not detectable by *in situ* hybridization but that are nonetheless sufficient to effect recombination in the intersectional genetic strategy.

Real time mRNA expression patterns of Vglut1, Vglut2 and Vglut3 in central noradrenergic neurons in adult mice

DePuy et al. (2013), Stornetta et al. (2002a) and Stornetta et al. (2002b) showed that Vglut2 co-expression is only located in the C2/A2 and C1/A1 region in the adult rat but not in anterior NA nuclei in the brainstem by *in situ* hybridization. To verify if the real time NA-based Vglut2 expression pattern in adult mice is the same as in adult rats and to further characterize the expression patterns of the other two vesicular glutamate transporters (Vglut1 and Vglut3) in adult mice, we performed fluorescent RNA *in situ* hybridization experiments. We co-stained Vglut1, Vglut2, Vglut3 with DBH respectively in brain tissue of adult mice and characterized the colocalization of Vglut1/2/3 with DBH in brainstem NA nuclei A7, LC, A5, sub CD/CV, anterior C1/A1 and C2/A2, and posterior C1/A1 and C2/A2 (**Figure 2A-C**). No Vglut1/DBH double positive neurons were detected in any part of the central NA system, consistent with our fate map. Vglut3 mRNA colocalization with DBH is only found in the posterior part of C2/A2 where $27.1 \pm 1.86\%$ DBH positive neurons have Vglut3 co-localization, again consistent with our fate map. For Vglut2, we only found detectable levels of Vglut2 mRNA in C1/A1 and C2/A2 NA region, but not in A7, LC, A5, sub CD/CV. Quantitatively, the percentage of Vglut2/DBH double positive neurons in anterior C1/A1, anterior C2/A2, posterior C1/A1 and posterior C2/A2 is $84.7 \pm 5.82\%$, $66.3 \pm 0.335\%$, $35.7 \pm 3.01\%$ and $90.1 \pm 2.45\%$, respectively. This result is consistent with the previous *in situ* data in adult rats (DePuy et al., 2013; Stornetta et al., 2002a, 2002b) suggesting that there is no obvious expression difference of Vglut2 in the central NA system between mouse and rat. However, the Vglut2 *in situ* data did not show expression in anterior NA populations. This difference between our *in situ* data and fate map data supports our previous hypothesis that many NA neurons expressed Vglut2 at some point from early development toward adulthood but the expression of Vglut2 is turned back down during adulthood. Interestingly, Yang et al. (2021) did show adult Vglut2 co-expression in LC by a viral injection of both Cre and Flpo-dependent eYFP into the LC of a bi-transgenic mouse with both TH-Flpo and Vglut2-Cre. To verify this result, we injected an AAV virus containing Cre-dependent tdTomato (pAAV-EF1a-DIO-tdTomato-WPRE) into the LC region of adult Vglut2-Cre mice. Consistent with Yang et al. (2021), we observed sparse Vglut2-Cre and TH (immunopositive) double positive neurons in the LC (**Figure 2** supplement 1). Additionally,

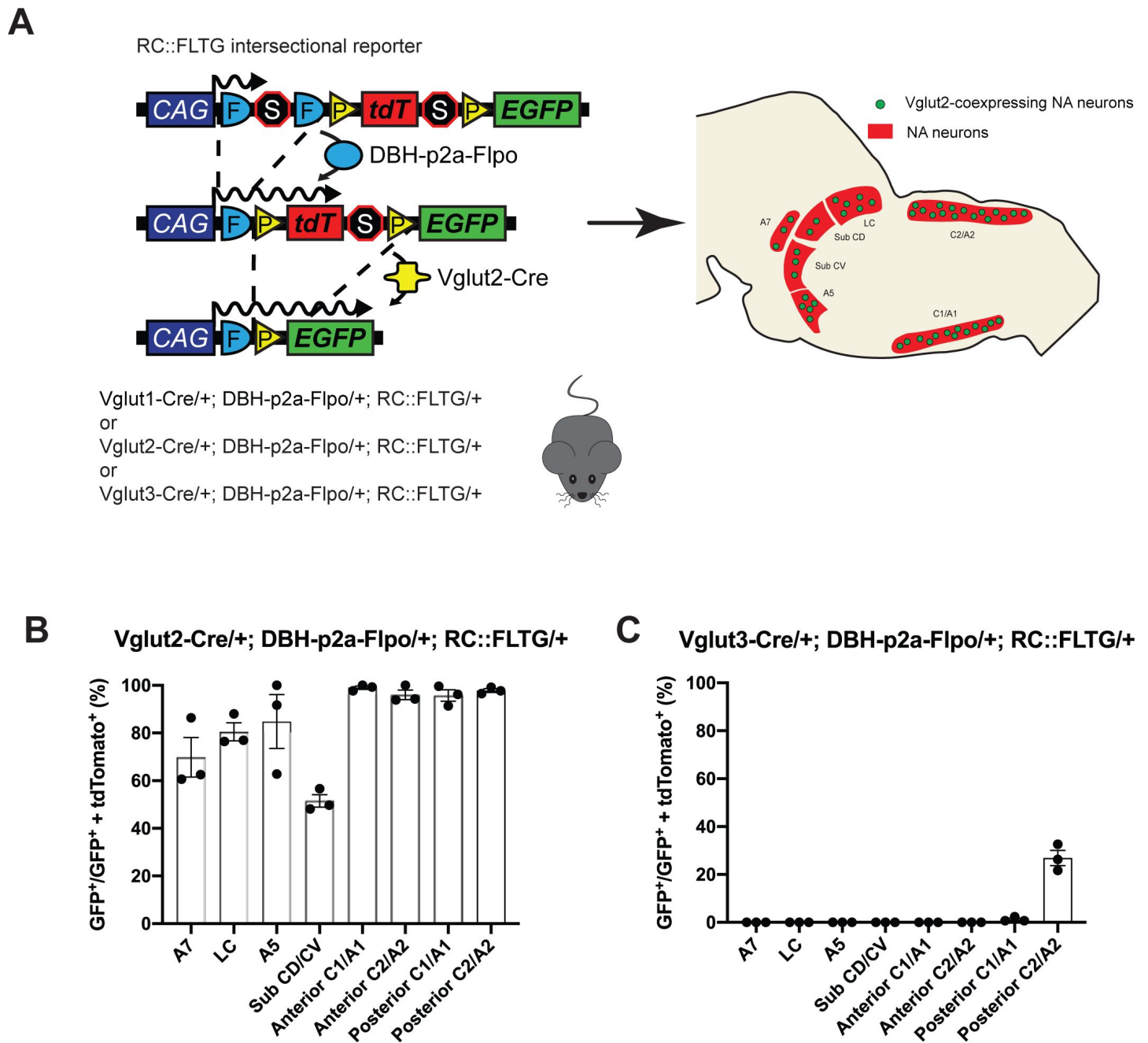


Figure 1.

Cumulative fate maps of glutamate co-expressing NA neurons characterized by intersectional genetics.

(A) Breeding schematic. Vglut1-Cre/+; Vglut2-Cre/+ and Vglut3-Cre/+ were each bred with DBH-p2a-Flpo/+ mice respectively. These Vglut1, Vglut2, or Vglut3-Cre/+; DBH-p2a-Flpo/+ compound lines were then crossed with the RC::FLTG intersectional reporter mice. In each of the three intersectional reporter crosses, NA neurons co-expressing either Vglut1, Vglut2, or Vglut3 are labeled by green fluorescent protein (eGFP) while NA neurons without any Vglut1,2, or 3 expression are labelled by red fluorescent protein (tdTomato). Shown as an example is the Vglut2-Cre/+; DBH-p2a-Flpo/+; RC::FLTG/+ intersectional reporter line. (B) Quantification of the percentage of Vglut2-coexpressing NA neurons among NA neurons in each brainstem NA nuclei including A7, LC, A5, sub CD/CV, anterior C1/A1 and C2/A2, posterior C1/A1 and C2/A2. (C) Quantification of the percentage of Vglut3-coexpressing NA neurons among NA neurons in each NA nuclei. (D) Fluorescent expression of tdTomato (red) and eGFP (green) in coronal sections of Vglut1, Vglut2, or Vglut3 intersectional reporter lines in brainstem NA nuclei. Nucleus is labeled by DAPI (blue). Scale bar 50µm.

D

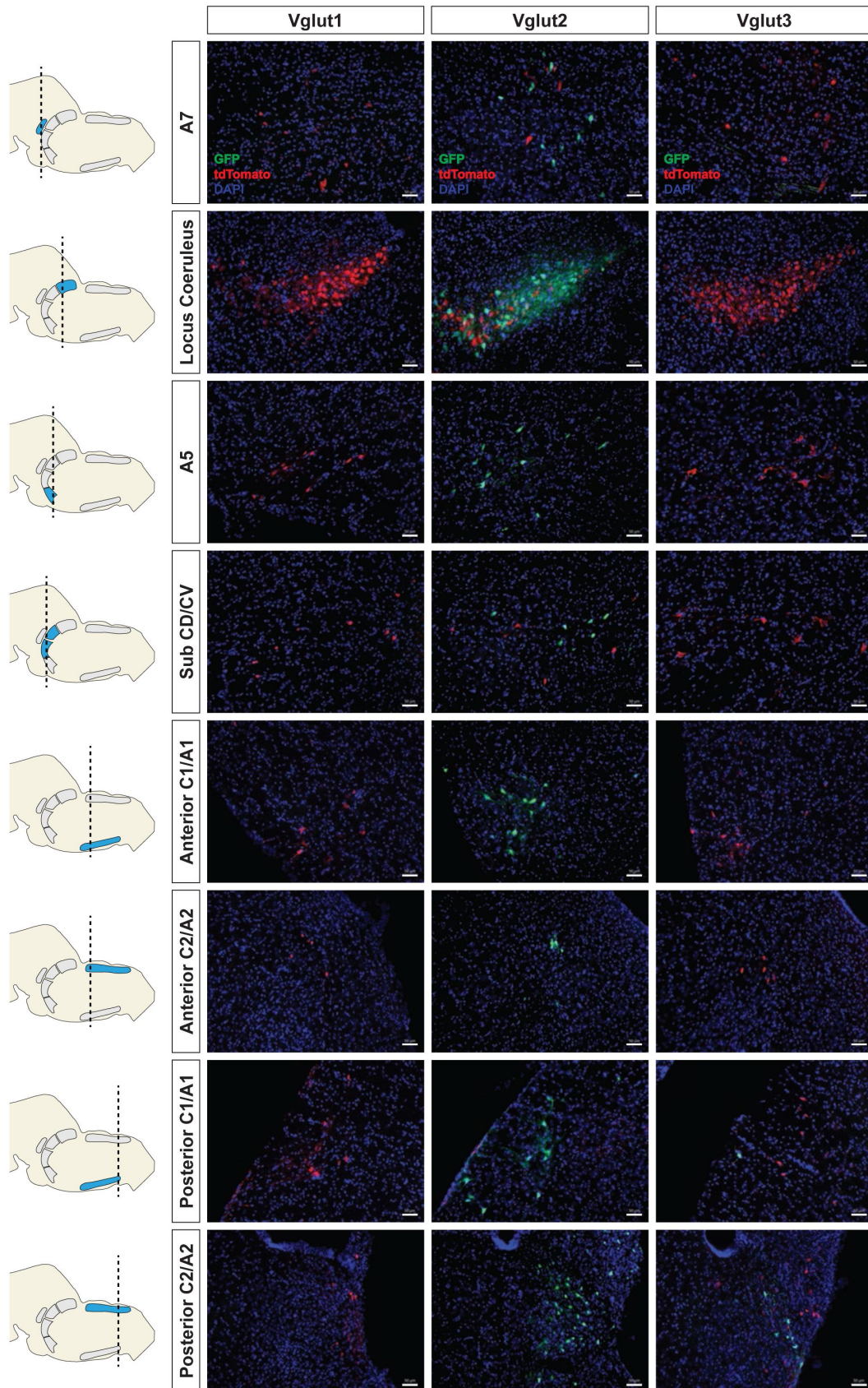


Figure 1. (continued)

Souza et al. (2022a) [\[1\]](#) showed about 28.5% A5 neurons are Vglut2 mRNA positive in adult rats by RNA scope, a method which is more sensitive than the traditional *in situ* hybridization we used here. These data suggest that some adult NA neurons in LC and A5 have Vglut2 co-expression, but the Vglut2 mRNA level cannot be detected by traditional *in situ* hybridization.

Vglut2 expression is effectively knocked down in the whole central noradrenergic system in DBH-Cre; Vglut2 cKO mice

To investigate the requirement of Vglut2-based glutamatergic signaling in the central NA system in breathing under physiological challenges, we used Dbh-Cre; *Slc17a6^{flox/flox}* (DBH-Cre; Vglut2 cKO) as the mouse model in which Vglut2 is inactivated in all noradrenergic neurons. This is the same model used by Abbott et al. (2014) [\[2\]](#) to show that Vglut2 expression in the NA system was required for a modest increase in respiratory frequency following unilateral optogenetic stimulation of anterior C1. To verify if Vglut2 expression is effectively removed from central NA neurons in DBH-Cre; Vglut2 cKO mice, we carried out fluorescent RNA *in situ* hybridization for Vglut2 and DBH in the mouse brainstems and compared the Vglut2 signal intensity in DBH positive neurons in DBH-Cre; Vglut2 cKO and its littermate controls. We found that Vglut2 mRNA expression is $96.4 \pm 0.226\%$ decreased compared to controls, indicating that the Vglut2 is effectively recombined to abrogate expression in NA neurons (**Figure 3A-B** [\[3\]](#)).

Vglut2-based glutamatergic signaling in central NA neurons is not required for baseline breathing nor for the hypercapnic ventilatory reflex under distinct CO₂ challenges

Multiple prior studies provide indirect evidence that NA glutamate transmission may play roles in respiratory function, particularly that anterior C1 neurons mediate the hypoxic ventilatory response through the pFRG/RTN and/or the NA A5 group (Malheiros-Lima et al., 2022 [\[4\]](#), 2020 [\[5\]](#)). Additionally, the A5 group has been implicated in the hypercapnic reflex (Haxhiu et al., 1996 [\[6\]](#)). Thus, we sought to determine if Vglut2-based glutamatergic signaling in central NA neurons is necessary to regulate baseline breathing and the hypercapnic chemoreflex in awake and unrestrained animals. We used whole-body barometric plethysmography to measure the breathing and metabolism of conscious and free-moving mice. After a five-day habituation protocol, the ventilation response of DBH-Cre; Vglut2 cKO mice and their littermate controls was measured under room air and hypercapnia (5% CO₂) (**Figure 4A** [\[7\]](#)). Surprisingly, DBH-Cre; Vglut2 cKO mice didn't show significant differences in overall respiratory output (V_E/V_{O_2}), respiratory rate (V_f), tidal volume (V_T), minute ventilation (V_E) and metabolism demand (V_{O_2}) compared to their littermate controls under both room air and 5% CO₂ challenge (**Figure 4B** [\[8\]](#) and **Table 1** [\[9\]](#)–**2** [\[10\]](#)). These results suggested that Vglut2-based glutamatergic signaling is not required for regulating baseline breathing and hypercapnic ventilatory reflex under 5% CO₂.

To further investigate if Vglut2-based glutamatergic signaling in central NA neurons is required for ventilation response of higher CO₂ challenges, we also measured the breathing of DBH-Cre; Vglut2 cKO mice under 7% CO₂ and 10% CO₂. We found that under the 7% CO₂ condition, DBH-Cre; Vglut2 cKO mice showed only a significantly decreased tidal volume (V_T), but the overall respiratory output (V_E/V_{O_2}), respiratory rate (V_f), minute ventilation (V_E) and metabolism demand (V_{O_2}) didn't show a significant difference between the mutant mice and their sibling controls (**Figure 5A-B** [\[11\]](#) and **Table 3** [\[12\]](#)). This result suggests that Vglut2-based glutamatergic signaling in central NA neurons is not required or at least is not as important as previously expected for regulating the hypercapnic ventilatory reflex under 7% CO₂.

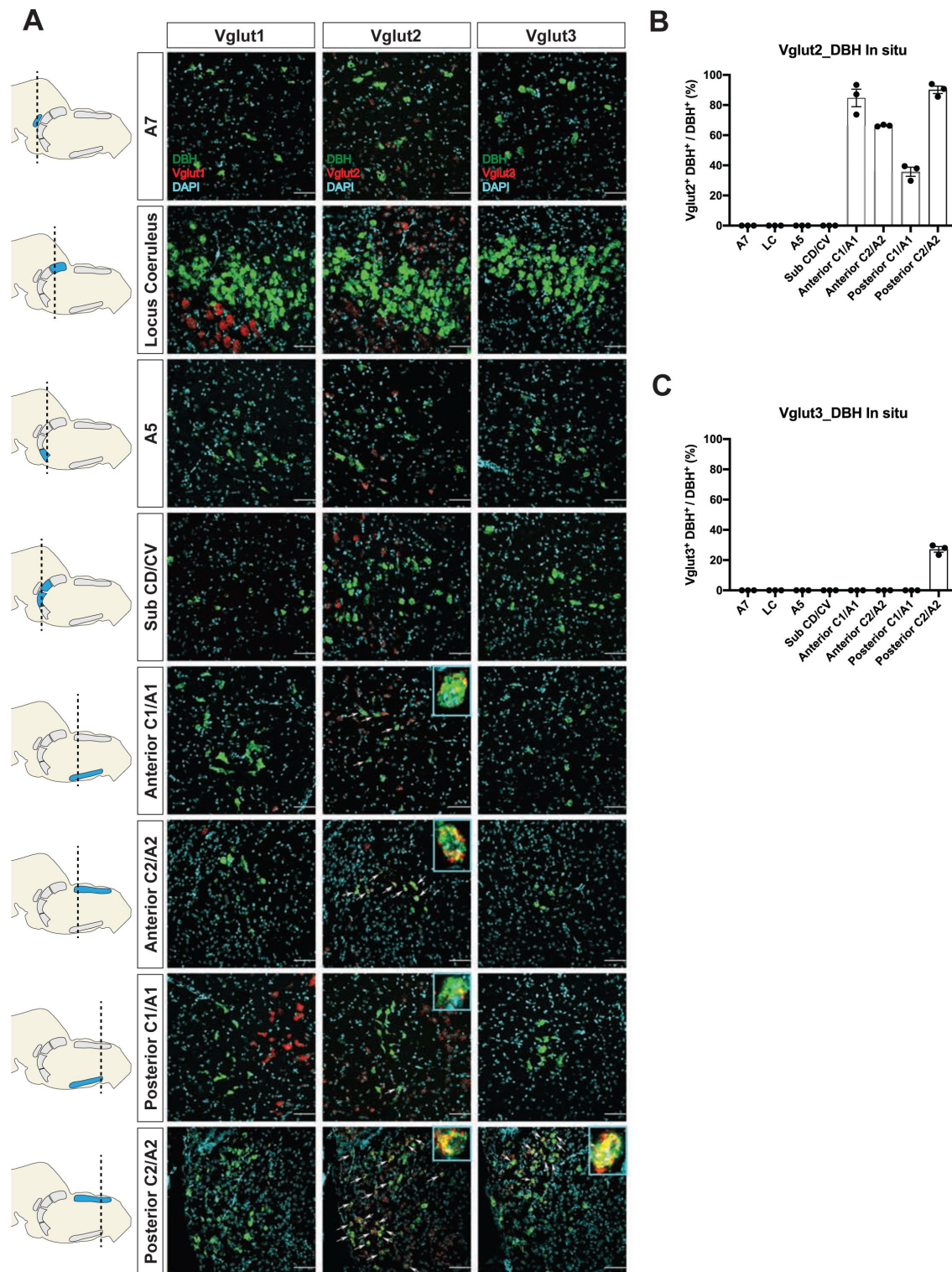


Figure 2.

Characterization of real time co-expression of Vglut1, Vglut2 and Vglut3 in brainstem NA neurons in adult mice by fluorescent *in situ* hybridization.

(A) Representative images of Vglut1, Vglut2, or Vglut3 and DBH double ISH in coronal sections of WT mice in all of brainstem NA nuclei in adult mice. DBH (green), Vglut1/2/3 (red), DAPI (cyan). Scale bar 50µm. (B) Quantification of the percentage of Vglut2 co-expression in NA neurons in each NA nuclei. (C) Quantification of the percentage of Vglut3 co-expression in NA neurons in each NA nuclei.

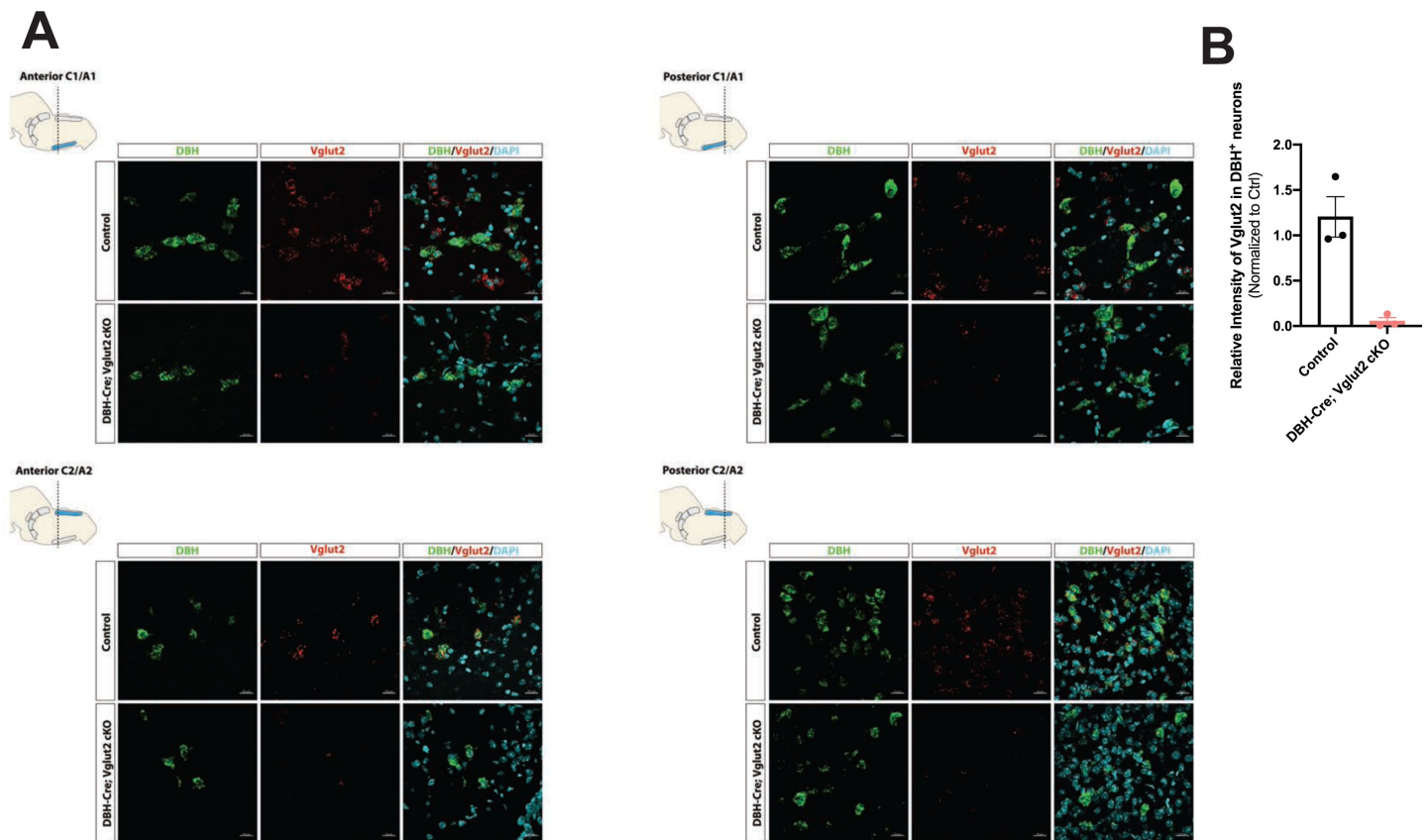


Figure 3.

DBH with Vglut2 co-staining by fluorescent *in situ* hybridization confirms that Vglut2 expression is disrupted in the whole central NA system in DBH-Cre; Vglut2 cKO mice.

(A) Representative images of Vglut2 and DBH double ISH in anterior C1/A1, posterior C1/A1, anterior C2/A2 and posterior C2/A2 in control and DBH-Cre; Vglut2 cKO mice. DBH (green), Vglut2 (red), DAPI (cyan). Scale bar 20µm. (B) Quantification of intensity of Vglut2 signal in control and DBH-Cre; Vglut2 cKO mice.

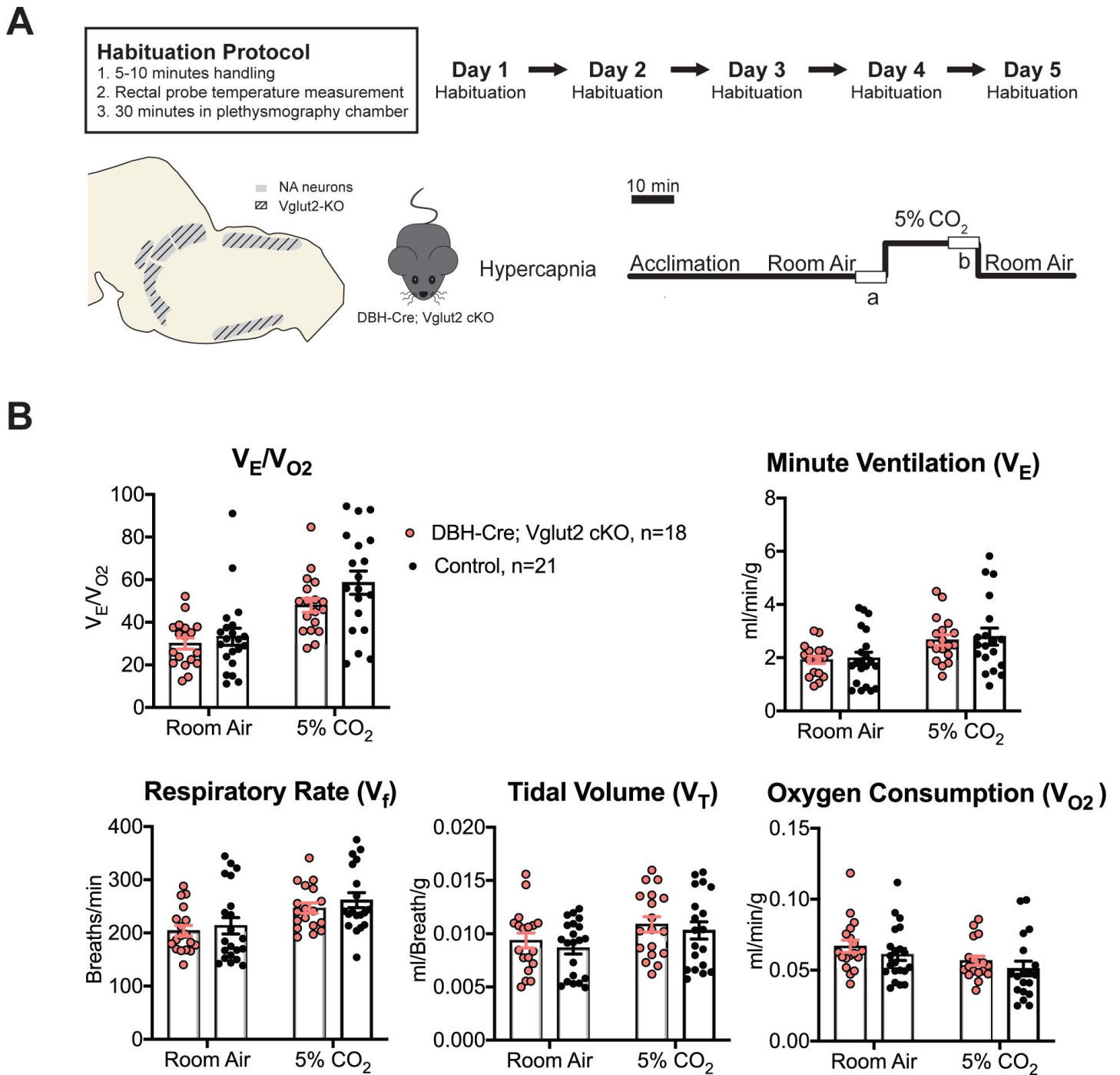


Figure 4.

Vglut2 conditional knockout in central noradrenergic neurons fails to alter baseline breathing and the hypercapnic ventilatory reflex (5% CO₂).

(A) Mouse model schematic and experimental protocol including habituation and hypercapnia protocol. (B) Under both room air and hypercapnia (5% CO₂), DBH-Cre; Vglut2 cKO mice didn't show significant changes in respiratory output (V_E/V_{O_2}), minute ventilation (V_E), respiratory rate (V_f), tidal volume (V_T), and metabolism demand (V_{O_2}). Linear mixed-effects regression model, * $p < 0.05$.

	DBH-Cre; Vglut2 cKO Mean $\pm$ SEM	Control Mean $\pm$ SEM	DBH-Cre; Vglut2 cKO vs Control linear mixed-effects regression model
V_E/V_{O_2}	30.01 $\pm$ 2.58	33.22 $\pm$ 3.97	P = 0.98
V_f	204.08 $\pm$ 10.04	213.58 $\pm$ 15.36	P = 0.98
V_T	0.009 $\pm$ 0.0007	0.009 $\pm$ 0.0006	P = 0.88
V_E	1.93 $\pm$ 0.14	1.98 $\pm$ 0.22	P = 0.995
V_{O_2}	0.067 $\pm$ 0.004	0.061 $\pm$ 0.004	P = 0.78

Table 1.

Respiratory and metabolic values under room air conditions and the statistical tests.

	DBH-Cre; Vglut2 cKO Mean $\pm$ SEM	Control Mean $\pm$ SEM	DBH-Cre; Vglut2 cKO vs Control linear mixed-effects regression model
V_E/V_{O_2}	47.99 $\pm$ 3.28	58.60 $\pm$ 5.44	P = 0.33
V_f	246.72 $\pm$ 9.78	261.66 $\pm$ 14.00	P = 0.24
V_T	0.011 $\pm$ 0.0007	0.010 $\pm$ 0.0008	P = 0.81
V_E	2.67 $\pm$ 0.20	2.80 $\pm$ 0.32	P = 0.98
V_{O_2}	0.057 $\pm$ 0.003	0.051 $\pm$ 0.005	P = 0.42

Table 2.

Respiratory and metabolic values under 5% CO₂ conditions and the statistical tests.

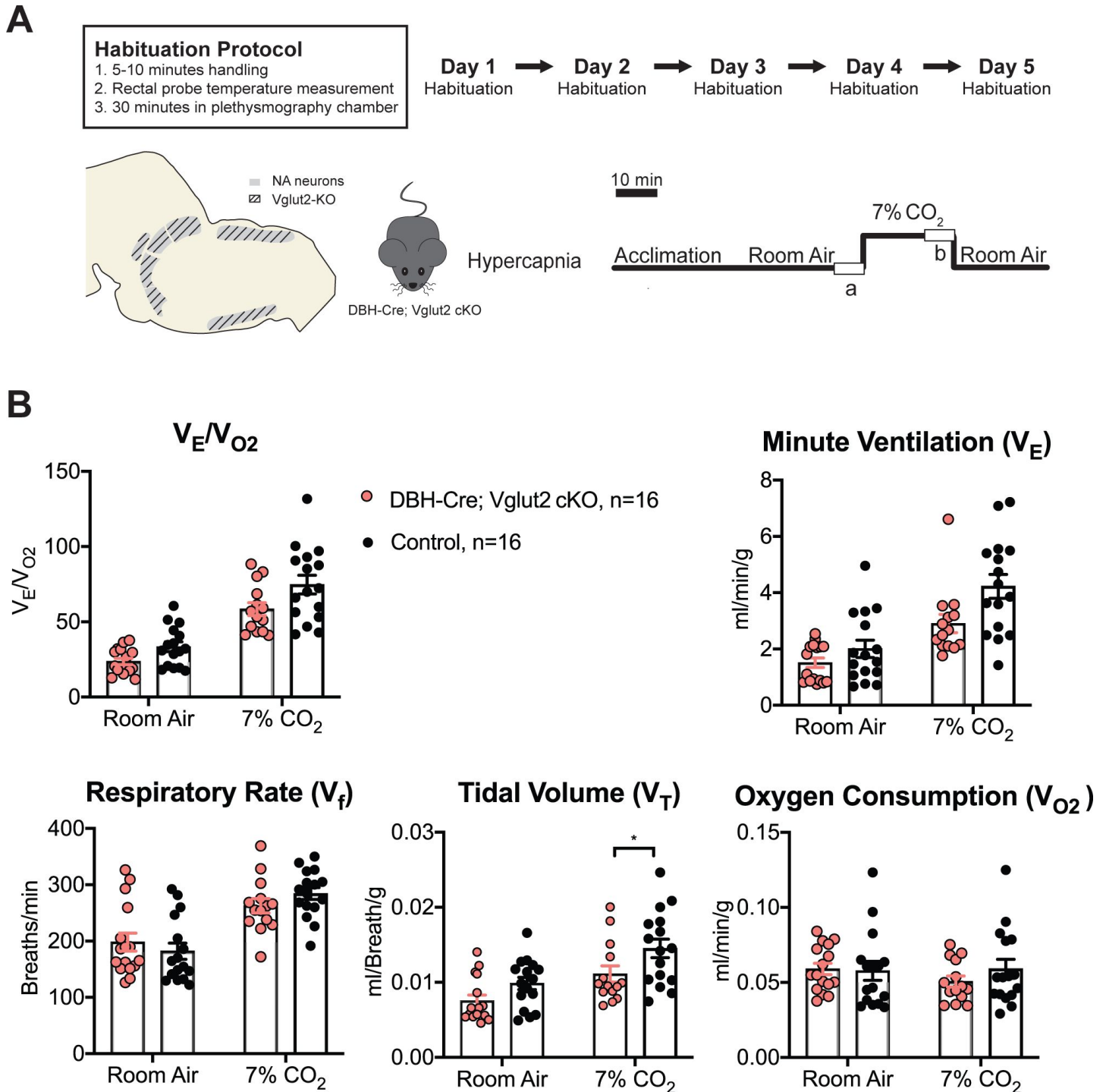


Figure 5.

Vglut2 conditional knockout in central noradrenergic neurons fails to alter the majority of hypercapnic ventilation parameters and metabolism under 7% CO₂.

(A) Mouse model schematic and experimental protocol including habituation and hypercapnia protocol. (B) Knocking out Vglut2 in the whole NA system failed to alter ventilation (V_E/V_{O_2} , V_f , V_E) and metabolism (V_{O_2}) but only showed a significantly reduce in V_T under 7% CO₂ condition. Linear mixed-effects regression model, * $p < 0.05$.

	DBH-Cre; Vglut2 cKO Mean $\pm$ SEM	Control Mean $\pm$ SEM	DBH-Cre; Vglut2 cKO vs Control linear mixed-effects regression model
V_E/V_{O_2}	58.48 $\pm$ 4.29	74.70 $\pm$ 6.22	P = 0.23
V_f	262.69 $\pm$ 12.82	284.44 $\pm$ 10.59	P = 0.98
V_T	0.011 $\pm$ 0.0011	0.015 $\pm$ 0.0012	P = 0.023*
V_E	2.90 $\pm$ 0.32	4.23 $\pm$ 0.43	P = 0.16
V_{O_2}	0.051 $\pm$ 0.004	0.059 $\pm$ 0.006	P = 0.64

Table 3.

Respiratory and metabolic values under 7% CO₂ conditions and the statistical tests.

Under 10% CO₂ challenge, none of the overall respiratory outputs (V_E/V_{O_2}), respiratory rate (V_f), tidal volume (V_T), minute ventilation (V_E) and metabolism demand (V_{O_2}) were significantly different between DBH-Cre; Vglut2 cKO mice and their littermate controls, suggesting that Vglut2-based glutamatergic signaling in central NA neurons is not required for the hypercapnic ventilatory reflex under 10% CO₂ (**Figure 6A-B** [↗](#) and **Table 4** [↗](#)).

Vglut2-based glutamatergic signaling in central NA neurons is not required for the hypoxic ventilatory reflex under 10% O₂

To determine if Vglut2-based glutamatergic signaling in central NA neurons is necessary to regulate the hypoxic chemoreflex, we measured the ventilation response of DBH-Cre; Vglut2 cKO mice under 10% O₂. We analyzed the five respiratory parameters in three 5-min time periods under 10% O₂ challenge. No significant difference was found in the overall respiratory output (V_E/V_{O_2}), respiratory rate (V_f), tidal volume (V_T), minute ventilation (V_E) and metabolism demand (V_{O_2}) between DBH-Cre; Vglut2 cKO mice and controls in either of the three 5-min 10% O₂ periods (**Figure 7A-B** [↗](#) and **Table 5** [↗](#)). These data suggest that Vglut2-based glutamatergic signaling in central NA neurons is not required for the hypoxic ventilatory reflex under 10% O₂.

Discussion

Neuron co-transmission of two or more neurotransmitters across excitatory, inhibitory, and neuro-modulatory facets is becoming an increasingly appreciated phenomenon in the central nervous system. Functional interrogation of co-transmission is essential not only to understand the distinct and cooperative roles that multiple signaling molecules may play at the synapse but also to determine the most critical targets for potential therapeutics. To our knowledge, the unopposed paradigm in the field until now has been that vesicular glutamate transporter 2 (Vglut2)-based glutamate transmission from central noradrenergic neurons is important in respiratory homeostasis. Anatomically, it has long been appreciated that central NA neurons co-express Vglut2, the major glutamate marker among three glutamate transporters (DePuy et al., 2013 [↗](#); Souza et al., 2022a [↗](#); Stornetta et al., 2002a [↗](#), 2002b [↗](#); Yang et al., 2021 [↗](#)). Also, it has been well documented that NA Vglut2 positive fibers innervate known central respiratory centers or other autonomic centers, that, when perturbed, could disrupt respiratory homeostasis (Supplemental Table 1). Functionally, multiple reports have argued that Vglut2-based glutamate transmission plays a role in respiratory homeostasis. It has also been suggested that, at least for C1 neurons, the apparent lack of a plasmalemmal monoamine transporter may attenuate or eliminate noradrenergic or adrenergic release from these fibers. However as discussed below, much of the functional evidence supporting a role for Vglut2-based glutamate transmission from central noradrenergic neurons is circumstantial or of a non-physiological nature, and absent or attenuated release of adrenaline and noradrenaline has not been demonstrated. Nonetheless, the dominant perspective that NA-Vglut2 glutamate transmission plays a role in breathing has remained unchallenged. Our studies here uncover a novel dynamic expression pattern for Vglut2 and an entirely undescribed co-expression domain for Vglut3 in the central NA neurons, and in contrast to prior work, our results show that loss of Vglut2 in NA neurons does not appreciably change baseline or chemosensory breathing in conscious and free-moving mice.

Vglut2 has been shown to be co-expressed in subsets of central NA neurons, however, to our knowledge, the potential for the central NA neurons to express the other glutamate transporters Vglut1 or Vglut3 has not been reported at the anatomical level. To determine if central NA neurons express either Vglut1 or Vglut3 and to confirm the Vglut2 expression, we carried out cumulative

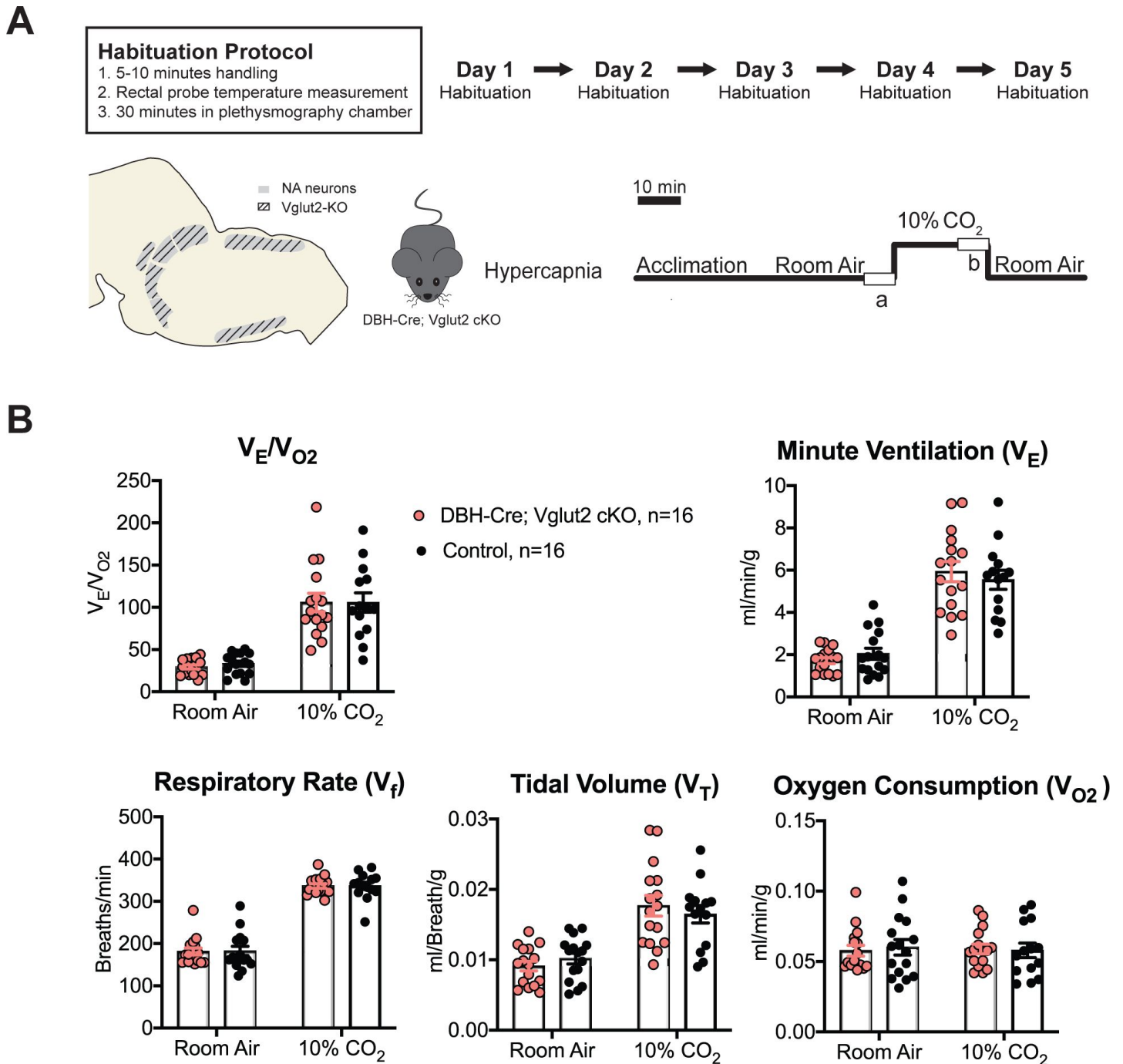


Figure 6.

Vglut2 conditional knockout in central noradrenergic neurons fails to alter the hypercapnic ventilatory reflex (10% CO₂).

(A) Mouse model schematic and experimental protocol including habituation and hypercapnia protocol. (B) Knocking out Vglut2 in the whole NA system didn't significantly alter ventilation (V_E/V_{O_2} , V_f , V_T , V_E) and metabolism (V_{O_2}) under 10% CO₂ condition. Linear mixed-effects regression model, * $p < 0.05$.

	DBH-Cre; Vglut2 cKO Mean $\pm$ SEM	Control Mean $\pm$ SEM	DBH-Cre; Vglut2 cKO vs Control linear mixed-effects regression model
V_E/V_{O_2}	105.77 $\pm$ 10.80	105.69 $\pm$ 11.51	P = 0.9996
V_f	336.31 $\pm$ 5.26	336.38 $\pm$ 8.52	P = 0.9997
V_T	0.018 $\pm$ 0.0015	0.016 $\pm$ 0.0012	P = 0.98
V_E	5.94 $\pm$ 0.48	5.54 $\pm$ 0.45	P = 0.9996
V_{O_2}	0.059 $\pm$ 0.003	0.058 $\pm$ 0.005	P = 0.9999999

Table 4.

Respiratory and metabolic values under 10% CO₂ conditions and the statistical tests.

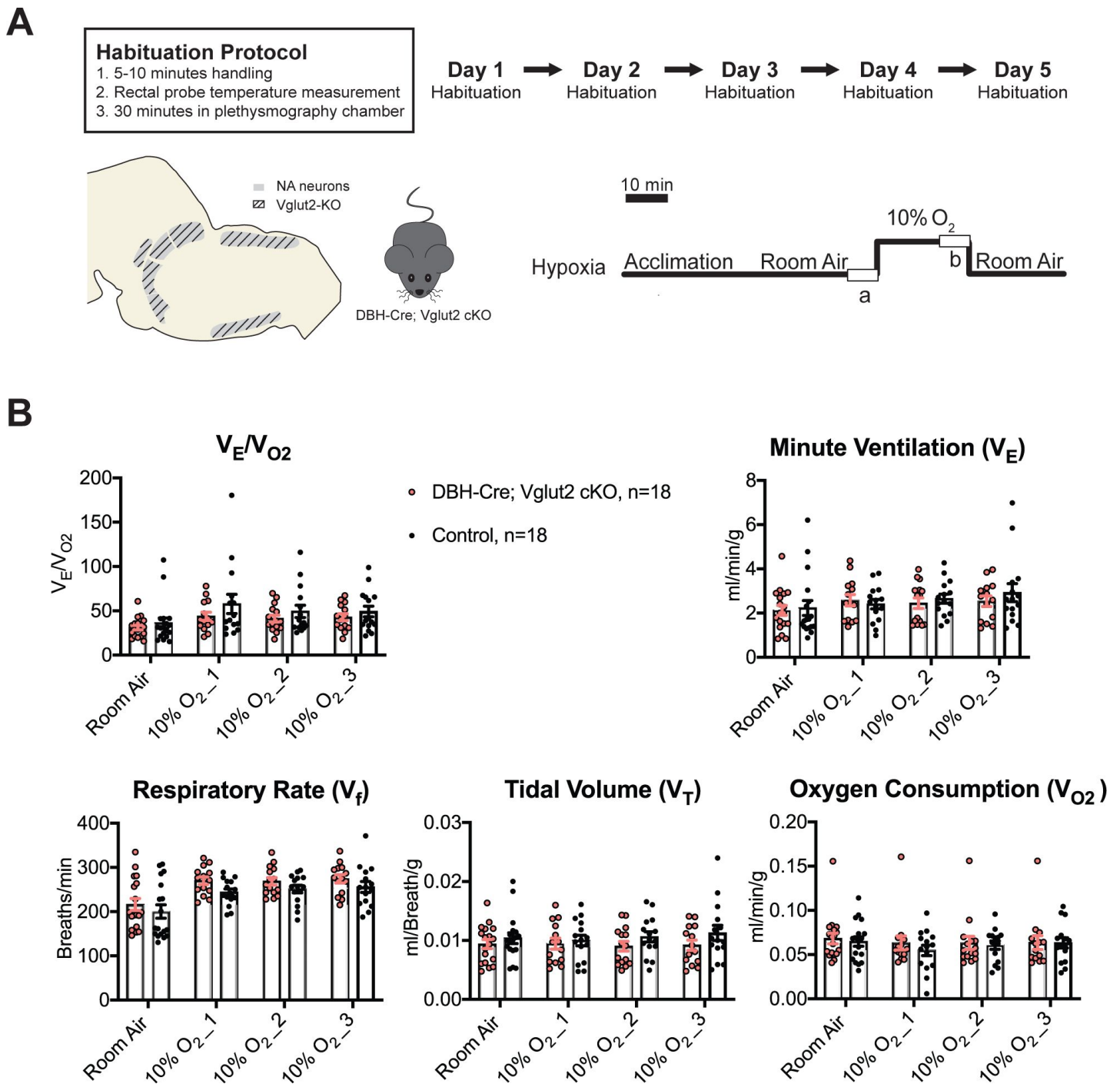


Figure 7.

Vglut2 conditional knockout in central noradrenergic neurons fails to alter the hypoxic ventilatory reflex (10% O_2).

(A) Mouse model schematic and experimental protocol including habituation and hypoxia protocol. (B) Knocking out Vglut2 in the whole NA system didn't show significant breathing changes including V_E/V_{O_2} , V_E , V_f , V_T , and V_{O_2} in either of the three five-minute time periods under hypoxia (10% O_2). Linear mixed-effects regression model, * $p < 0.05$.

	DBH-Cre; Vglut2 cKO Mean ± SEM	Control Mean ± SEM	Linear mixed- effects regression model	DBH-Cre; Vglut2 cKO Mean ± SEM	Control Mean ± SEM	Linear mixed- effects regression model	DBH-Cre; Vglut2 cKO Mean ± SEM	Control Mean ± SEM	Linear mixed- effects regression model
	10% O ₂ _1			10% O ₂ _2			10% O ₂ _3		
V _E /V _{O₂}	43.79 ± 4.57	57.75 ± 10.88	P = 0.99	41.30 ± 3.85	49.36 ± 6.93	P = 0.99	42.82 ± 3.97	49.19 ± 6.02	P = 0.99
V _I	269.71 ± 8.49	243.89 ± 7.64	P = 0.67	268.38 ± 8.40	251.15 ± 8.67	P = 0.83	273.96 ± 9.31	255.84 ± 12.19	P = 0.80
V _T	0.009 ± 0.0009	0.010 ± 0.0009	P = 0.89	0.009 ± 0.0008	0.011 ± 0.0009	P = 0.85	0.009 ± 0.0009	0.011 ± 0.0013	P = 0.64
V _E	2.58 ± 0.25	2.40 ± 0.21	P = 0.999998	2.45 ± 0.24	2.65 ± 0.21	P = 0.9998	2.53 ± 0.24	2.92 ± 0.41	P = 0.998
V _{O₂}	0.063 ± 0.008	0.055 ± 0.006	P = 0.995	0.063 ± 0.007	0.061 ± 0.005	P = 0.9998	0.064 ± 0.008	0.063 ± 0.006	P = 0.99999999

Table 5.

Respiratory and metabolic values under 10% O₂ conditions and the statistical tests.

expression of Vglut1, Vglut2 and Vglut3 using fluorescent RNA *in situ* hybridization. Our *in situ* results for Vglut2 agreed with earlier published outcomes (DePuy et al., 2013 [DOI](#); Stornetta et al., 2002a [DOI](#), 2002b [DOI](#)) showing as much as 80% expression in posterior NA neurons. However, there was no appreciable signal in anterior groups (A7, LC, A5, sub CD/CV). This lack of expression was in stark contrast to the cumulative fate map for Vglut2, showing as much as 75% of the anterior central NA neurons were recombined by the Vglut2-Cre over the lifetime of the animal. To further interrogate the acute activity of the Vglut2-Cre in the Locus Coeruleus in the adult mouse, we injected a Cre responsive virus in LC of Vglut2-Cre mice. The outcomes from the cumulative fate map and acute injections are in agreement with published results from Yang et al. (2021) [DOI](#). Notably our use of the DBH-p2a-Flpo avoided expression in the PBN that was seen in the TH-Flpo mouse. In addition, 28.5% A5 neurons were shown to be Vglut2 positive in adult rats by RNA scope (Souza et al., 2022b [DOI](#)). It is notable that both our lab and work published by (DePuy et al., 2013 [DOI](#); Stornetta et al., 2002a [DOI](#), 2002b [DOI](#)) failed to detect Vglut2 expression in anterior groups, but that apparently very low levels can be detected by more sensitive recombinase and RNA scope methods. Additional electrophysiological evidence suggests that this expression is functional (Yang et al., 2021 [DOI](#)). Our data suggests that Vglut2 co-expression in anterior NA groups is time dependent, though the underlying temporal dynamics of Vglut2 remain unknown. It is notable that we see nearly 80% of the LC and 85% of A5 recombined in the intersectional strategy representing lifetime expression, but our and other studies find only 20% of the LC and 28.5% of A5 may be expressing Vglut2 at any given moment in adults. This suggests either a temporal restriction in expression as the animal matures or may reflect dynamic change driven by behavioral or physiological experiences.

For Vglut1, neither fate map nor *in situ* hybridization revealed any co-expression within the central NA system. However, Vglut3 expression was found in the posterior part of NA neurons including C2/A2 and C1/A1, which is first characterized by this study. The majority of this Vglut3 co-expression is restricted to the posterior part of the C2/A2 NA population. This NA region has not been heavily implicated in respiratory control nonetheless it readily lends itself to interrogation by intersectional genetic methods and *in situ* hybridization. Given its distal expression from anterior C1, it is unlikely that Vglut3 would compensate for loss of Vglut2 in anterior C1, though this has not been ruled out. Further, while Vglut3 is a marker of glutamatergic neurons, its role in neurotransmission is not wholly defined as Vglut3 is typically found in soma and dendrites (Fremeau et al., 2004 [DOI](#)).

Based on the Vglut2 co-expression in NA neurons and their projections to multiple brain regions important in respiration, several studies suggest that glutamate in NA populations is important in breathing control. Those studies provide a mix of circumstantial, indirect, or limited evidence for the potential role of Vglut2-based glutamatergic signaling in central NA neurons in breathing regulation. However, it has not yet been shown whether or not NA-based Vglut2 signaling is required for respiratory control due to experimental caveats or limitations.

Abbott et al. (2014) [DOI](#) showed that conditionally knocking out Vglut2 in the whole central NA system attenuated a modest increase in respiratory rate resulting from unilateral C1 optogenetic stimulation. Absolute values and other respiratory parameters, including tidal volume (V_T) and minute ventilation (V_E) were not reported, making direct comparison difficult. While the optogenetic stimulation did drive a Vglut2 dependent increase in respiratory rate, it is not clear if the resulting change in breathing reflects a native feature of the network that might be engaged in chemosensory responses, exercise, or volitional control. Thus, it remains possible that strong optogenetic stimulation of a small, isolated population drives a Vglut2 dependent but ectopic function that is not typically engaged in the normal operation of the breathing network. Such ectopic outcomes could come from several potential, non-exclusive mechanisms. First, unilateral stimulation could drive a network hysteresis or dysregulation akin to a focal injury that is not a typical biological feature (Ducros et al., 2003 [DOI](#)). This point is well made in Abbott et al. (2013) [DOI](#) in their discussion of C1 optogenetic stimulation where they state, “resulting cardiorespiratory

response pattern should not be considered strictly “physiological,” because a physiological response is never initiated by selective activation of a single cluster of CNS neurons and the various subsets of C1 neurons are presumably never recruited en bloc under any physiological condition”. Second, strong stimulation could result in overwhelming synaptic mechanisms (i.e., glial uptake) resulting in glutamate spillover and unintended cross talk to affect extra-synaptic neurons proximal to targeted NA fibers. Activity dependent spillover transmission has been documented elsewhere in the nervous system (Henneberger et al., 2020 [↗](#); Hülsmann et al., 2000 [↗](#)). Third, the outcomes could be secondary to a different autonomic or behavioral function requiring NA-derived glutamatergic signaling involving metabolism or circulation (DePuy et al., 2013 [↗](#); Yang et al., 2021 [↗](#)).

More circumstantially, Malheiros-Lima et al. (2020) [↗](#) suggested C1 neurons potentially release glutamate at the pFRG site to regulate breathing under hypoxia by providing three indirect lines of evidence: 1) Vglut2-expressing C1 neurons project to the pFRG region; 2) Increased breathing was blunted after blockade of ionotropic glutamatergic receptors at the pFRG site under anesthesia using chemical hypoxia; 3) Depletion of C1 neurons eliminated the increased breathing elicited by hypoxia. However, 1) the glutamatergic signaling targeting the pFRG region is not necessarily from C1 NA neurons since glutamatergic neurons from other regions also project to the pFRG site (Yang and Feldman, 2018 [↗](#)). Thus, it is possible that the glutamatergic signaling that is required for hypoxic response is derived from other glutamatergic neurons such as the preBötzinger complex and the RTN itself; 2) C1 neuron depletion knocks out all the signaling modalities in the C1 population including both noradrenaline and glutamate. It is not clear which signaling drives the hypoxic response.

Similarly, Malheiros-Lima et al. (2022) [↗](#) and Malheiros-Lima et al. (2018) [↗](#) showed that Vglut2-expressing C1 neurons project to the NA A5 region and the preBötzinger complex respectively. The blockade of ionotropic glutamatergic receptors at the A5 region or preBötzinger complex reduced the increase in phrenic nerve activities or the breathing frequency caused by optogenetic stimulation of C1 cells in anesthetized preparation. Together these results suggest that Vglut2-expressing C1 neurons communicate with A5 or preBötzinger complex neurons by releasing glutamate in turn to regulate phrenic nerve activities or breathing frequency. Again, these two studies showed that C1 neurons are important to regulate phrenic nerve activities or breathing frequency, however, it is not necessarily through a direct C1-A5 or C1-preBötzinger complex glutamatergic pathway, whereas an indirect multi-synaptic pathway originating with C1 noradrenaline and ending with glutamate transmission from an intermediate to A5 or preBötzinger complex cannot be ruled out. Importantly, in all four studies noted here, respiratory measurements were limited or proxies of breathing under anesthesia were used. The role of NA-Vglut2 signaling was not reported in the context of an awake, unrestrained animal under normoxic, hypoxic, or hypercapnic breathing.

Thus, the limitations and indirect lines of the evidence in these studies raised the question as to whether or not glutamatergic signaling in central NA neurons is required or necessary to modulate respiratory homeostasis under more physiologically relevant circumstances and if NA-derived glutamate is an important mechanism to understand the pathophysiology of NA neurons in respiratory diseases. The goal of our study was to test the requirement of NA-based glutamatergic signaling in breathing homeostasis in the awake and unrestrained animal under conditions that would engage the whole respiratory control network rather than a singular component that may drive hysteresis or artifactual outcomes. Our *in vivo* breathing data under room air, hypoxic, and multiple hypercapnic conditions failed to show the requirement of NA-derived Vglut2 in normal breathing and respiratory chemoreflexes directly. To ensure a rigorous and robust conclusion, first we use exactly the same mouse model, DBH-Cre; Vglut2 cKO, as Abbott et al. (2014) [↗](#) used, and confirmed, by a second method (fluorescent RNA *in situ* hybridization) that Vglut2 expression was indeed abrogated in the whole central NA system. Our results are in strong agreement with DePuy et al. (2013) [↗](#) that recombination is efficient. Notably, we assayed

for expression at the cell body, rather than the fibers and see no singular cell body that is scored positive. Second, we measured the breathing of conscious, unrestrained, and habituated mice under physiological challenges including normoxia, hypercapnia and hypoxia. Third, multiple respiratory and metabolic parameters were measured and reported in absolute values including respiratory rate, tidal volume, minute ventilation, oxygen consumption, overall respiratory output (minute ventilation normalized to oxygen consumption) in order to visualize breathing function comprehensively. Fourth, our experimental design was overpowered (i.e., $n=16-21$ vs $5-13$ needed for power based on power analysis and $n=7-8$ reported in other manuscripts). Each respiratory/metabolic parameter for each physiological condition including room air, hypercapnia, and hypoxia was derived by quantifying the entire respiratory trace and analyzed between mutant and sibling controls by using a powerful linear mixed-effects regression model with animal type (experimental vs. control) as fixed effects, animal ID as a random effect and including sex as a variable (there was no sex specific effect) (Lusk et al., 2022 [DOI](#)).

Despite our experimental design and the extensive nature of our measurements, there remain several considerations in the interpretation of our data. First, we uncovered a novel NA co-expressed glutamate transporter, Vglut3. It remains a formal possibility that Vglut3 somehow compensates for the loss of Vglut2, however we argue that this is unlikely for the following reasons. First, our model is the same used in the other study where an effect was seen with optogenetic stimulation. Second, focus has been on anterior C1 whereas Vglut3 is expressed in the posterior CA domains. Another consideration is that pre- or post-natal developmental compensation corrects for the loss of NA derived glutamate. Again, we argue that is not likely, as we are using the same adult model that provided the most direct evidence and first functionally demonstrated a potential role for C1-derived glutamate in breathing. Second, Abbott et al. (2014) [DOI](#) highlight that Vglut2 negative NA neurons showed no obvious abnormalities in number, morphology, and projection patterns. Third, we have examined the requirement of NA-expressed Vglut2 in P7 neonate mice in the auto-resuscitation reflex and saw no differences (data not shown). However, this could be better tested by using the Dbh-CreERT2 (DBH-CreERT2) (Stubbusch et al., 2011 [DOI](#)) to more acutely remove Vglut2 expression (though this leaves a window of 2-3 weeks for compensation to occur). Yet another consideration in interpreting our results is that we only address the loss of NA-Vglut2 based signaling across the entire NA system. It remains a formal possibility that we have removed Vglut2 dependent signaling from two counter balancing regions of the NA system and are therefore left with null results whereas removal from anterior C1 alone would show a requirement. To test this possibility, it would require an intersection conditional approach, something that has not yet been shown to be efficient and effective and would be beyond the scope of these studies. Additionally, in our studies, we account for potential metabolic changes that may underlie or be coordinate with changes in breathing. However, in this study we did not test for potential changes in blood pressure or other autonomic functions as well as affective state that may, in some obscure way, compensate for changes in breathing. Lastly, our results are loss of function in nature. We do not test sufficiency or gain of function and cannot fully rule out a role for NA-Vglut2 based glutamatergic signaling in the control of breathing.

From a translational perspective, our data questions whether or not glutamatergic signaling in NA neurons is likely to be a key mechanism and therefore a therapeutic target for breathing disorders, such as Rett syndrome and SIDS. *Mecp2*-deficient mice (a mouse model of Rett Syndrome that phenocopies many human symptoms) showed both a deficiency of NA populations (reduced number of NA neurons in C2/A2 and C1/A1 group) and highly variable respiratory rhythm at around 4-5 weeks of age (Roux et al., 2007 [DOI](#); Viemari et al., 2005 [DOI](#)). 80% Rett Syndrome patients experience breathing issues, such as unstable breathing, episodes of hyperventilation and breathing holds, during the whole lifespan including both childhood and adulthood (Ramirez et al., 2020 [DOI](#)). SIDS decedents show NA abnormalities and are hypothesized to ultimately succumb from a failure in cardiorespiratory autoresuscitation (Chigr et al., 1989 [DOI](#); Garcia et al., 2013 [DOI](#); Kopp et al., 1993 [DOI](#); Mansouri et al., 2001 [DOI](#); Ozawa et al., 2003 [DOI](#); Takashima and Becker, 1991 [DOI](#)). However, DBH-Cre; Vglut2 cKO mice, have normal baseline and chemosensory respiratory

parameters as adults and neonate mice show normal autoresuscitation indicating that perturbed NA based glutamatergic signaling may not be a key driver in these or other related respiratory pathophysiology.

In conclusion, our studies demonstrated the extent of glutamate as a functional vesicular glutamate transporter co-expression in central noradrenergic system. We were able to characterize a novel Vglut3-expressing NA population in the posterior C2/A2 nuclei and found a different Vglut2 co-expression pattern in anterior NA groups from a cumulative intersectional fate map compared to a viral fate map and *in situ* hybridization in the adult, suggesting that anterior NA populations may have a time-dependent or experience-dependent manner for Vglut2 expression. Furthermore, despite prior studies providing indirect and circumstantial evidence that noradrenergic-based glutamatergic signaling may play a role in control of breathing, our studies that remove Vglut2 expression from noradrenergic neurons failed to perturb room air breathing, the hypercapnic ventilatory reflex, and the hypoxic ventilatory reflex, which challenges the current dominant perspective and provides further insight into the potential role of NA-glutamate transmission in the control of breathing. Based on our data, we suggest that noradrenergic-based glutamatergic signaling is not required for regulating baseline breathing or the hypercapnic and hypoxic respiratory chemoreflexes, and glutamate may not be a critical mechanistic component of NA neuron dysfunction in respiratory disorders.

Materials and Methods

Ethical Approval

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

Breeding, Genetic Background, and Maintenance of Mice

We maintained all our heterozygous mouse strains by backcrossing to wildtype C57BL/6J mice and homozygous mouse strains by sibling crosses. For immunofluorescence experiments, heterozygous B6;129S-*Slc17a7*^{tm1.1(cre)Hze}/J (*Slc17a7*^{Cre}, Vglut1-Cre) (Jax Stock No: 023527), *Slc17a6*^{tm2(cre)Lowl}/J (*Slc17a6*^{Cre}, Vglut2-Cre) (Jax Stock No: 016963) and B6;129S-*Slc17a8*^{tm1.1(cre)Hze}/J (*Slc17a8*^{Cre}, Vglut3-Cre) (Jax Stock No: 018147) were mated with heterozygous *Dbh*^{em2.1(flo)Rray} (*Dbh*^{p2a-Flpo}, DBH-p2a-Flpo) (MMRRC ID: 41575) (Sun and Ray, 2016 [\[4\]](#)) mice respectively to derive compound lines with both Cre and Flpo alleles (Vglut1-Cre/+; DBH-p2a-Flpo/+, Vglut2-Cre/+; DBH-p2a-Flpo/+, and Vglut3-Cre/+; DBH-p2a-Flpo/+). Then these three compound lines were each mated with homozygous B6.Cg-*Gt(Rosa)26Sor*^{tm1.3(CAG-tdTomato,-EGFP)Pjen}/J (*Rosa26*^{RC::FLTG}, RC::FLTG) (Jax Stock No: 026932) to derive three different intersectional mouse lines Vglut1-Cre/+; DBH-p2a-Flpo/+; RC::FLTG/+, Vglut2-Cre/+; DBH-p2a-Flpo/+; RC::FLTG/+ and Vglut3-Cre/+; DBH-p2a-Flpo/+; RC::FLTG/+. For *in situ* hybridization experiments, wildtype C57BL/6J mice were ordered from the Center of Comparative Medicine (CCM), Baylor College of Medicine. For NA-Vglut2 conditional loss of function *in situ* hybridization and plethysmography experiments, hemizygous transgene Tg(*Dbh*-cre)KH212Gsat (*Dbh*-Cre, DBH-Cre) (MMRRC ID: 036778-UCD GENSAT) mice were mated with homozygous *Slc17a6*^{tm1Lowl}/J (*Slc17a6*^{flox/flox}) (Jax Stock No: 012898) to derive *Dbh*-Cre; *Slc17a6*^{flox/+}. *Dbh*-Cre; *Slc17a6*^{flox/+} mice were mated with *Slc17a6*^{flox/flox} to derive *Dbh*-Cre; *Slc17a6*^{flox/flox} (DBH-Cre; Vglut2 cKO). Sibling mice that lacked the Cre allele or carried the Cre allele but lacked the floxed Vglut2 alleles were used as controls. Rosa26 specific primers for the RC::FLTG mice were 5'-GCACCTGCTCTCCCAAAGTC, 5'-GGGCGTACTTGGCATATGAT, and 5'-CTTTAAGCCTGCCAGAAGA (Ray et al., 2011 [\[4\]](#)) and yield a 495 bp band (targeted) and 330 bp band (wt). Cre-specific primers for all Cre drivers were 5'-ATCGCCATCTTCCAGCAGGCGCACCATTGCCC and 5'-GCATTTCTGGGATTGCTTA and yielded a 550 bp band if positive. Flpo-specific primers for DBH-p2a-Flpo are 5'-CACGCCAGGTACTTGTCT and 5'-

CCACAGCAAGAAGATGCTGA (Sun and Ray, 2016 [DOI](#)) and yielded a 226 bp band if positive. *Slc17a6^{fllox}* specific primers for Vglut2-floxed mice are available at The Jackson Laboratory website (<https://www.jax.org/strain/012898> [DOI](#)).

Immunofluorescence staining

Vglut1, Vglut2, or Vglut3-Cre/+; DBH-p2a-Flpo/+; RC::FLTG/+ adult mice were sacrificed and transcardially perfused with 0.1 M phosphate-buffered saline (PBS) then with 4% paraformaldehyde (PFA) in PBS. Mouse brains were dissected out and fixed for 2h in 4% PFA before a PBS rinse and dehydration in 30% sucrose in PBS. Brains were embedded in OCT blocks, sectioned at the thickness of 30µm, mounted on slides, and stored at -80°C until they were ready for staining. The slides were hydrated in 0.1% Triton-X in PBS (PBST) for 15 mins, blocked with 5% donkey serum in 0.1% PBST for 1h at room temperature and then incubated with primary antibodies for 72 hours at 4°C in 0.1% PBST with 5% donkey serum. Tissues were washed in 0.1% PBST 3 times for 10 min each and then incubated with secondary antibodies for 2hs at room temperature in 0.1% PBST with 5% donkey serum. Slides were washed with 0.1% PBST for 10 min and washed in PBS twice for 10 min each, stained for DAPI, washed 3 times for 10 min each with PBS and mounted in ProLong Glass (Invitrogen). The following primary and secondary antibodies were used: chicken anti-GFP (1:1,000, Abcam ab13970), rabbit anti-dsRed (1:1,000, Clontech 632496), donkey anti-chicken Cy2 (1:500, Jackson 703-225-155), donkey anti-rabbit Cy3 (1:500, Jackson 711-165-152).

In situ hybridization

Mouse brains were dissected out from adult mice with 6-8 week of age, sectioned into 25µm brain sections and mounted on slides. We generated a digoxigenin (DIG)-labeled mRNA antisense probe against Vglut1, Vglut2, and Vglut3 and fluorescein (FITC)-labeled mRNA against DBH using reverse-transcribed mouse cDNA as a template and an RNA DIG or FITC-labeling kits from Roche (Sigma). Primer and probe sequences for the Vglut1, Vglut2, Vglut3 and DBH probes are available in the Allen Brain Atlas (<http://www.brain-map.org> [DOI](#)). For the Vglut2 and DBH double ISH in DBH-Cre; Vglut2 cKO and their littermate controls, we generated a new Vglut2 probe targeting exon 2 of Vglut2 specifically (Tong et al., 2007 [DOI](#)) and the probe sequence is 892-1144bp as *Slc17a6* transcript variant 1 and the size is 253bp. ISH was performed by the RNA In Situ Hybridization Core at Baylor College of Medicine using an automated robotic platform as previously described (Yaylaoglu et al., 2005 [DOI](#)) with modifications of the protocol for double ISH. Modifications in brief [see Yaylaoglu et al. (2005) [DOI](#) for buffer descriptions]: both probes were hybridized to the tissue simultaneously. After the described washes and blocking steps the DIG-labeled probes were visualized using tyramide-Cy3 Plus (1/75 dilution, 15-minute incubation, Akoya Biosciences). After washes in TNT, the remaining HRP-activity was quenched by a 10-minute incubation in 0.2M HCl. The sections were then washed in TNT, blocked in TNB for 15 minutes, and incubated at room temperature for 30 minutes with HRP-labeled sheep anti-FITC antibody (1/500 in TNB, Roche/Sigma). Following washes in TNT, the FITC-labeled probe was visualized using tyramide-FITC Plus (1/50 dilution, 15-minute incubation, Akoya Biosciences). Following washes in TNT, the slides were stained with DAPI (Invitrogen), washed again, removed from the machine, and mounted in ProLong Diamond (Invitrogen).

Viral Injection

To verify Vglut2 co-expression in LC in adult mice (Yang et al., 2021 [DOI](#)), 6-week-old Vglut2-Cre mice were injected with pAAV-EF1a-DIO-tdTomato-WPRE virus (RRID:Addgene_133786, obtained from Joshua Ortiz at the Optogenetics and Viral Vectors Core at the Jan and Dan Duncan Neurological Research Institute, 1µl at 7.90E+10 Gc/mL) into the LC (coordinates from bregma anteroposterior -5.4 mm, lateral + 0.8 mm and dorsoventral - 4.0 mm) and allowed to incubate for 4 weeks.

Plethysmography

Plethysmography on conscious and free-moving mice was carried out as described in (Ray et al., 2011) on 6-8-week-old adult animals with at least 16 animals in both the experimental and control group for each experiment. Animals were subjected to a five-day habituation protocol with each day including several minutes of handling, temperature taken by rectal probe and at least 30 min exposure in the plethysmography chamber (Martinez et al., 2019). Plethysmography was then performed for each mouse within 1 week of the last day of habituation. On the day of testing, mice were taken out from their home cage, weighed, and rectal temperature was taken. Animals were then placed into a flow-through, temperature-controlled (about 32°C with real-time temperature recording) plethysmography chamber and allowed to acclimate for at least 45 min in room air (21% O₂/79% N₂) conditions. After acclimation and measurement under room air, the chamber gas was switched to a hypercapnic or hypoxic mixture of 5% CO₂/21% O₂/74% N₂, 7% CO₂/21% O₂/72% N₂, 10% CO₂/21% O₂/69% N₂, or 10% O₂/90% N₂, depending on the protocol, for 20 min. Chamber gas was then switched back to room air for another 20 min. The animals were removed from the chamber and rectal temperature was measured immediately after the mice were taken out from the chamber.

Plethysmography data analysis and statistics

Details were previously described in (Martinez et al., 2019; Ray et al., 2011). Plethysmography pressure changes were measured using a Validyne DP45 differential pressure transducer, CD15 carrier demodulator and a reference chamber, and were recorded with LabChart Pro in real time. Respiratory waveforms were analyzed by the BASSPRO module of the Breathe Easy software to determine respiratory frequency (V_f), tidal volume (V_T), minute ventilation (V_E), oxygen consumption (V_{O_2}) and ventilatory equivalents for oxygen (V_E/V_{O_2}) (Lusk et al., 2022). A power analysis was performed using the reported effect size in Abbott et al. (2014) which used the same mouse model as we used here and 5-13 mice were necessary to observe a statistically significant result (Abbott et al. (2014) was able to see a significant difference between two groups with $n=7$ mice). In our experiments, the sample size for each group (DBH-Cre; Vglut2 cKO and control) exceeded 13. Results (V_f , V_E , V_T , V_{O_2} , V_E/V_{O_2}) for room air and hypercapnic or hypoxic data were compared between DBH-Cre; Vglut2 cKO cohorts and sibling controls using a linear mixed-effects regression model with animal type (experimental vs. control) as fixed effects and animal ID as a random effect (Lusk et al., 2022). A $p < 0.05$ was used to indicate statistical significance, and individual data points, mean, and standard error of the mean are shown on all charts. The graphs were plotted by Prism 8.

Image quantification

Images were taken by using a Zeiss upright epifluorescent microscope and a Zeiss LSM 880 with Airyscan FAST confocal microscope. Images were captured using Zen software with z-stack function from top to bottom with 0.34µm intervals, exported, and then analyzed in Imaris using the spots and surface functions. For quantification of immunofluorescence staining, each GFP positive area coincident with DAPI (to denote nuclear localization) was defined as Vglut2 or Vglut3-expressing NA neurons while each tdTomato positive area coincident with DAPI was defined as NA neurons without any Vglut2 or Vglut3 co-expression. For quantification of Vglut2 or Vglut3 with DBH double *in situ* hybridization in adult WT mice, DBH positive areas coinciding with DAPI were identified as NA neurons and the DBH positive areas overlapped with Vglut2/3 positive pixels and DAPI were defined as NA neurons colocalized with Vglut2/3. The number of Vglut2/3 positive NA neurons and Vglut2/3 negative NA neurons was counted in each image for both immunofluorescence and *in situ* experiments and the percentage of Vglut2/3 positive NA neurons among all NA neurons in each NA nucleus in each mouse brainstem was calculated every other brain section unilaterally. For quantification of Vglut2 and DBH double ISH in DBH-Cre; Vglut2 cKO and their littermate controls, Vglut2 pixel intensities in DBH positive areas coincident with DAPI (NA neurons) were measured in mutant and control images separately. The Vglut2 pixel intensity in NA neurons of control brains was normalized as 1 and the relative Vglut2 pixel intensity of NA

neurons in mutant brains compared to that in control brains was calculated. At least three mouse brains were examined for each set of experiments in each group. All quantitative results were graphed using Prism 8.

Data availability

All the data supporting this study is available from the corresponding author upon request.

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Author contributions

YC designed and completed immunofluorescence staining, imaging, and plethysmography experiments, analyzed data, wrote the initial manuscript and revised the manuscript.

SL completed the viral injection experiments and revised the manuscript.

RR conceptualized and designed the study, analyzed the data, and revised the manuscript.

Competing interests

No competing interests are declared.

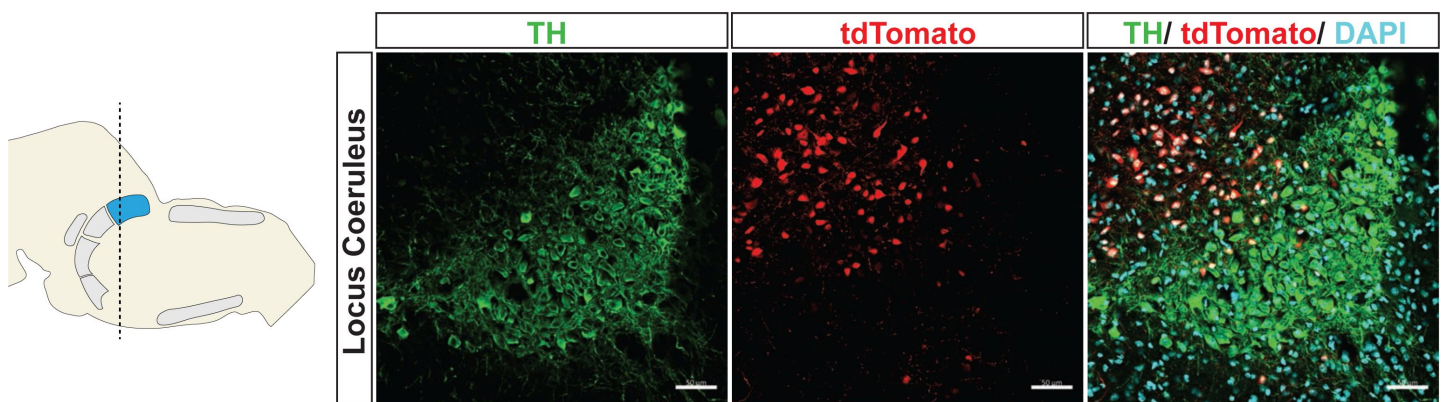


Figure 2 Supplement 1.

Virus injection of Cre-dependent tdTomato into LC of Vglut2-Cre mice.

Vglut2 ⁺ noradrenergic nuclei	Downstream target of Vglut2 ⁺ NA neurons	The role of the downstream target in breathing control	Reference
C1	preBötzinger complex	Generate inspiratory rhythm	Malheiros-Lima et al. (2018)
	parafacial region (pFRG)	Regulate active expiration during chemosensory stimulation	Malheiros-Lima et al. (2020)
	Locus Coeruleus (LC)	Chemosensitive, regulate chemosensation	Abbott et al. (2013) ; Holloway et al. (2013)
	A5	Inhibitory drive for chemosensation and respiratory rhythm	Abbott et al. (2013) ; Malheiros-Lima et al. (2022)
	A1, A2	Modulate chemosensation, stabilize respiratory rhythm	Abbott et al. (2013) ; Holloway et al. (2013)
	Lateral parabrachial nucleus (PBN)	Regulate chemosensation and arousal	Abbott et al. (2013)
	Medullary raphe	Regulate chemosensation and expiration	Abbott et al. (2013)
	Nucleus of the solitary tract (NTS)	Receive input from the carotid bodies (a key center for O ₂ chemosensation) and other cardiopulmonary afferents	Abbott et al. (2013)
	Dorsal motor nucleus of the vagus	Control respiration rate, regulate airway tone and defense	Abbott et al. (2013)
	Dorsomedial hypothalamus (DMH)	Modulate baseline respiration, chemosensation, arousal	Abbott et al. (2013)
	lateral hypothalamic area (LHA)		
	Paraventricular thalamic nucleus (PVT)	Regulate arousal	Abbott et al. (2013)
	Paraventricular nucleus (PVN)	Modulate baseline respiration, respiratory response to hypoxia	Abbott et al. (2013)
Locus Coeruleus (LC)	Lateral parabrachial nucleus	Regulate chemosensation and arousal	Yang et al. (2021)

Supplemental table 1.

Vglut2 positive innervations from central noradrenergic neurons to the brain nuclei important in breathing control.

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Reviewer #1 (Public Review):

Summary: Chang et al. provide glutamate co-expression profiles in the central noradrenergic system and test the requirement of Vglut2-based glutamatergic release in respiratory and

metabolic activity under physiologically relevant gas challenges. Their experiments show that conditional deletion of Vglut2 in NA neurons does not impact steady-state breathing or metabolic activity in room air, hypercapnia, or hypoxia. Their observations challenge the importance of glutamatergic signaling from Vglut2 expressing NA neurons in normal respiratory homeostasis in conscious adult mice.

Strengths: The comprehensive Vglut1, Vglut2, and Vglut3 co-expression profiles in the central noradrenergic system and the combined measurements of breathing and oxygen consumption are two major strengths of this study. Observations from these experiments provide previously undescribed insights into (1) expression patterns for subtypes of the vesicular glutamate transporter protein in the noradrenergic system and (2) the dispensable nature of Vglut2-dependent glutamate signaling from noradrenergic neurons to breathing responses to physiologically relevant gas challenges in adult conscious mice.

Weaknesses: Although the cellular expression profiles for the vesicular glutamate transporters are provided, the study fails to document that glutamatergic-based signaling originating from noradrenergic neurons is evident at the cellular level under normal, hypoxic, and/or hypercapnic conditions. This limits the reader's understanding of why conditional Vglut2 knockdown is dispensable for breathing under the conditions tested.

Reviewer #2 (Public Review):

The authors characterized the recombinase-based cumulative fate maps for vesicular glutamate transporters (Vglut1, Vglut2 and Vglut3) expression and compared those maps to their real-time expression profiles in central NA neurons by RNA in situ hybridization in adult mice. Authors have revealed a new and intriguing expression pattern for Vglut2, along with an entirely uncharted co-expression domain for Vglut3 within central noradrenergic neurons. Interestingly, and in contrast to previous studies, the authors demonstrated that glutamatergic signaling in central noradrenergic neurons does not exert any influence on breathing and metabolic control either under normoxic/normocapnic conditions or after chemoreflex stimulation. Also, they showed for the first-time the Vglut3-expressing NA population in C2/A2 nuclei. In addition, they were also able to demonstrate Vglut2 expression in anterior NA populations, such as LC neurons, by using more refined techniques, unlike previous studies.

A major strength of the study is the use of a set of techniques to investigate the participation of NA-based glutamatergic signaling in breathing and metabolic control. The authors provided a full characterization of the recombinase-based cumulative fate maps for Vglut transporters. They performed real-time mRNA expression of Vglut transporters in central NA neurons of adult mice. Further, they evaluated the effect of knocking down Vglut2 expression in NA neurons using a DBH-Cre; Vglut2cKO mice on breathing and control in unanesthetized mice. Finally, they injected the AAV virus containing Cre-dependent Td tomato into LC of v-Glut2 Cre mice to verify the VGlut2 expression in LC-NA neurons. A very positive aspect of the article is that the authors combined ventilation with metabolic measurements. This integration holds particular significance, especially when delving into the exploration of respiratory chemosensitivity. Furthermore, the sample size of the experiments is excellent.

Despite the clear strengths of the paper, some weaknesses exist. It is not clear in the manuscript if the experiments were performed in males and females and if the data were combined. I believe that the study would have benefited from a more comprehensive analysis exploring the sex specific differences. The reason I think this is particularly relevant is the developmental disorders mentioned by the authors, such as SIDS and Rett syndrome, which could potentially arise from disruptions in central noradrenergic (NA) function, exhibit varying degrees of sex predominance. Moreover, some of the noradrenergic cell groups are sexually dimorphic. For instance, female Wistar rats exhibit a larger LC size and more LC-NA neurons than male subjects (Pinos et al., 2001; Garcia-Falgueras et al., 2005).

More recently, a detailed transcriptional profiling investigation has unveiled the identities of over 3,000 genes in the LC. This revelation has highlighted significant sexual dimorphisms, with more than 100 genes exhibiting differential expression within LC-NA neurons at the transcript level. Furthermore, this investigation has convincingly showcased that these distinct gene expression patterns have the capacity to elicit disparate behavioral responses between sexes (Mulvey et al., 2018). Therefore, the authors should compare the fate maps, Vglut transporters in males and females, at least considering LC-NA neurons. Even in the absence of identified sex differences, this information retains significant importance.

An important point well raised by the authors is that although suggestive, these experiments do not definitively rule out that NA-Vglut2 based glutamatergic signaling has a role in breathing control. Subsequent experiments will be necessary to validate this hypothesis.

An improvement could be made in terms of measuring body temperature. Opting for implanted sensors over rectal probes would circumvent the need to open the chamber, thereby preventing alterations in gas composition during respiratory measurements. Further, what happens to body temperature phenotype in these animals under different gas exposures? These data should be included in the Tables.

Is it plausible that another neurotransmitter within NA neurons might be released in higher amounts in DBH-Cre; Vglut2 cKO mice to compensate for the deficiency in glutamate and prevent changes in ventilation?

Continuing along the same line of inquiry is there a possibility that Vglut2 cKO from NA neurons not only eliminates glutamate release but also reduces NA release? A similar mechanism was previously found in VGLUT2 cKO from DA neurons in previous studies (Alsio et al., 2011; Fortin et al., 2012; Hnasko et al., 2010). Additionally, does glutamate play a role in the vesicular loading of NA? Therefore, could the lack of effect on breathing be explained by the lack of noradrenaline and not glutamate?

Reviewer #3 (Public Review):

Summary:

The authors, Y Chang and colleagues, have performed elegant studies in transgenic mouse models that were designed to examine glutamatergic transmission in noradrenergic neurons, with a focus on respiratory regulation. They generated 3 different transgenic lines, in which a red fluorophore was expressed in dopamine-B-hydroxylase (DBH; noradrenergic and adrenergic neurons) neurons that did not express a vesicular glutamate transporter (Vglut) and a green fluorophore in DBH neurons that did express one of either Vglut1, Vglut2 or Vglut3.

Further experiments generated a transgenic mouse with knockout of Vglut2 in DBH neurons. The authors used plethysmography to measure respiratory parameters in conscious, unrestrained mice in response to various challenges.

Strengths:

The distribution of the Vglut expression is broadly in agreement with other studies, but with the addition of some novel Vglut3 expression. Validation of the transgenic results, using *in situ* hybridization histochemistry to examine mRNA expression, revealed potential modulation of Vglut2 expression during phases of development. This dataset is comprehensive, well-presented and very useful.

In the physiological studies the authors observed that neither baseline respiratory parameters, nor respiratory responses to hypercapnea (5, 7, 10% CO₂) or hypoxia (10% O₂) were different between knockout mice and littermate controls. The studies are well-designed

and comprehensive. They provide observations that are supportive of previous reports using similar methodology.

Weaknesses:

In relation to the expression of Vglut2, the authors conclude that modulation of expression occurs in adulthood and that this explains differences in expression patterns in some (nor)adrenergic cell groups. Altered sensitivity is provided as an explanation for different results between studies examining mRNA expression. These are likely explanations; however, the conclusion would really be definitive with inclusion of a conditional cre expressing mouse. Given the effort taken to generate this dataset, it seems to me that taking that extra step would be of value for the overall understanding of glutamatergic expression in these catecholaminergic neurons.

The respiratory physiology is very convincing and provides clear support for the view that Vglut2 is not required for modulation of the respiratory parameters measured and the reflex responses tested. It is stated that this is surprising. However, comparison with the data from Abbott et al., *Eur J Neurosci* (2014) in which the same transgenic approach was used, shows that they also observed no change in baseline breathing frequency. Differences were observed with strong, coordinated optogenetic stimulation, but, as discussed in this manuscript, it is not clear what physiological function this is relevant to. It just shows that some C1 neurons can use glutamate as a signaling molecule. Further, Holloway et al., *Eur J Neurosci* (2015), using the same transgenic mouse approach, showed that the respiratory response to optogenetic activation of Phox2 expressing neurons is not altered in DBH-Vglut2 KO mice. The conclusion seems to be that some C1 neuron effects are reliant upon glutamatergic transmission (C1-DMV for example), and some not.

Further contrast is made in this manuscript to the work of Malheiros-Lima and colleagues (eLife 2020) who showed that the activation of abdominal expiratory nerve activity in response to peripheral chemoreceptor activation with cyanide was dependent upon C1 neurons and could be attenuated by blockade of glutamate receptors in the pFRG - i.e. the supposition that glutamate release from C1 neurons was responsible for the function. However, it is interesting to observe that diaphragm EMG responses to hypercapnia (10% CO₂) or cyanide, and the expiratory activation to hypercapnia, were not affected by the glutamate receptor blockade. Thus, a very specific response is affected and one that was not measured in the current study.

In my view these observations are not at odds and the physiology described in the current study stands as informative. A more nuanced discussion of the data and acknowledgement of the differences, which are not actually at odds, would improve the paper and place the information within a more comprehensive model.