Neuroscience

A brainstem circuit controls cough-like airway defensive behaviors in mice

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eLife Assessment

This **valuable** study by Xu and colleagues investigates brainstem circuits mediating evoked respiratory reflexes that they define as cough-like in a freely behaving mouse model. They have applied multiple circuit mapping and manipulation approaches to suggest that the caudal spinal trigeminal nucleus (SP5C) nucleus can play a novel role in generating a reflex cough-like behavior in mice. The authors give **incomplete** evidence that the reflex behavior produced in their mouse model is definitively cough, limiting functional interpretation of the putative circuit identified and requiring more thorough experimental interrogation of the behavior studied.

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Abstract

The respiratory tract is subject to complex neural control for eupneic breathing and distinct airway defensive reflexes. Growing evidence has highlighted significant heterogeneity of airway-innervating vagal sensory neurons in mediating various respiratory functions, however, the central neuronal pathways and neural circuits involved in the airway regulation remain less understood. Combining whole-body plethysmography (WBP), audio, and video tracking to access breathing and airway defensive behaviors in conscious animals, we developed a quantitative paradigm implementing the mouse as a model to study cough-like defensive behaviors. Using TRAP2 transgenic mice and in vivo fiber photometry, we found that the neural activity in the caudal spinal trigeminal nucleus (SP5C) is strongly correlated with tussigen-evoked cough-like responses. Impairing synaptic outputs or chemogenetic inhibition of the SP5C effectively abolished these cough-like reflexes. Optogenetic stimulation of SP5C excitatory neurons or their projections to the ventral respiratory group (VRG) triggered robust cough-like behaviors without tussive stimuli. Notably, tonic elevation of SP5C excitability caused spontaneous cough-like activities chronically in mice. Together, our data provide strong evidence for a previously unrecognized brainstem circuit that controls cough-like defensive behaviors in mice.

Introduction

Breathing is essential for maintaining homeostasis of O_2 and CO_2 in mammals and is subject to precise neural control. Various innate defensive reflexes, such as apnea, sneezing, coughing, and expiratory reflexes, are crucial for protecting the airways from harmful pathogens and irritants. Dysregulation of the airway-to-brain axis can lead to prevalent and severe clinical problems, including dysphagia, aspiration pneumonia, asthma, chronic cough, and sudden infant death syndrome.

The cough reflex, a crucial airway defensive behavior, is typically elicited by peripheral stimulation of vagal sensory nerve fibers innervating the laryngeal and tracheobronchial airways(Canning, 2006; Canning et al., 2014; Coleridge and Coleridge, 1994). Vagal nerves are heterogeneous in diameter, myelination, conduction velocity, stimuli sensitivity, and physiological functions (Mazzone and Undem, 2016). Strong evidence demonstrates that activation of either mechanical Aδ or chemical-sensitive C-fibers can trigger the cough reflex in various mammalian species(Canning et al., 2004; Widdicombe, 1995). Recent studies have further revealed distinct molecularly-defined subtypes of vagal neurons mediating different airway reflexes, including cough (Chang et al., 2015; Gannot et al., 2024; Kupari et al., 2019; Park et al., 2024; Prescott et al., 2020).

The central fibers of vagal sensory neurons may differentially terminate on second-order neurons in the nucleus of tractus solitarius (NTS) or the paratrigeminal nucleus (Pa5) (Kim et al., 2020; McGovern et al., 2015; Su et al., 2022). These second-order neurons are proposed to transmit cough signals, directly or indirectly, to the ventral brainstem respiratory network, thereby reconfiguring its eupneic function to produce cough motor patterns. The ventral brainstem respiratory network, mainly consisting of the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG), the Bötzinger complex (BötC), the pre-Bötzinger complex (preBötC), the rostral ventral respiratory group (rVRG) and the caudal ventral respiratory group (cVRG), has been extensively characterized for its role in coordinating the breathing cycles of inspiration, post-inspiration, and expiration (Del Negro et al., 2018; Krohn et al., 2023; Smith et al., 2013). This remarkably multifunctional network dynamically adapts to control respiration and many non-respiratory motor behaviors such as vocalization, swallowing, coughing, vomiting, and sneezing(Bianchi and Gestreau, 2009; Krohn *et al*., 2023; Li et al., 2021). For example, studies have shown that the cVRG plays crucial roles in many expiration-related behaviors including vocalization(Park *et al*., 2024), swallowing(Subramanian and Holstege, 2009), and coughing(Mutolo, 2017). Indeed, the cVRG has long been proposed as “the cough center” based on various evidence such that pharmacological or electrical stimulation strongly evokes cough in anesthetized cat or rabbit(Bianchi and Gestreau, 2009; Cinelli et al., 2020; Mutolo, 2017). Consistently, pharmacological blockade of glutamatergic transmission in the cVRG completely suppresses cough induced by mechanical stimulation of the tracheobronchial tree in anaesthetized rabbits(Bongianni et al., 2005). Interestingly, however, a recent study using mice as an animal model has elegantly shown that Tac1 neurons in the NTS play a central role in cough-like behaviors by coordinating distinct medullary nuclei including the cVRG to elicit the sequential motor patterns of cough (Gannot *et al*., 2024).

Despite these advances, many key questions remain unanswered: what is the exact function of ventral brainstem respiratory network in the cough reflex? How are distinct nuclei activated by second-order cough relay neurons in the NTS or Pa5, either directly or indirectly? Are there other neuronal populations controlling cough? How are these populations connected with the respiratory network to reconfigure its function? What role might they play in cough hypersensitivity? To address these questions, we utilized the mouse as a powerful model for

circuit analysis, employing whole-body plethysmography (WBP), optogenetic, chemogenetic, virus-based tracing and manipulation, and in vivo fiber photometry to explore brain mechanisms of cough and cough hypersensitivity.

Results

The SP5C nucleus is necessary for tussigen-evoked cough-like responses in mice

Extensive cough studies have utilized human and larger mammalian models like cats, dogs, rabbits, and guinea pigs, providing critical insights into the mechanisms of cough production and regulation(Bolser, 2004; Canning, 2008; Mutolo, 2017). Although the suitability of mice for cough studies remains debated (Bolser, 2004), their use has recently increased (Chen et al., 2022; Gannot *et al*., 2024; Zhang et al., 2017).

To ensure precise and consistent monitoring of cough-like behaviors in mice, we combined WBP with audio and video tracking of animal behaviors (**Figure 1A**). We nebulized several widely-used tussigens, including citric acid (CA), capsaicin or irritant gas ammonia (NH3)(Canning *et al*., 2014; Canning *et al*., 2004; Mazzone and Undem, 2016; Zhang *et al*., 2017) to induce the cough reflex. Exposure to these stimuli consistently induced marked cough-like responses in mice, identifiable by tightly-correlated sharp airflow and characteristic cough sound (**Figure 1b-e** and **Figure S1**) in wild-type (WT) mice. Cough-like events were rarely observed at baseline or after nebulizing saline (Ctrl), underscoring the specific effectiveness of these tussigens (**Figures 1B** and **1D**). All tussive stimuli reliably triggered cough-like responses in mice, though with different time course. Nebulization of CA or capsaicin rapidly triggered cough-like responses with short latency (< 1 min), which decreased and stopped shortly after nebulization. In contrast, NH3 exposure evoked cough-like responses with longer latency (> 1 min), which reached a high plateau and persisted after nebulization. Notably, NH3-evoked cough-like events were several fold higher in number than those evoked by capsaicin and CA (**Figure 1E**), likely because NH3 may activate TRPV1, TRPA1 and other target receptors on the sensory neurons(Dhaka et al., 2009). Consequently, given the robustness and specificity of NH3-evoked cough(Sun et al., 2019; Zhang *et al*., 2017), we employed NH3 stimuli for most subsequent experiments. The sneeze reflex, distinguished by unique WBP and audio signals(Chen *et al*., 2022; Gannot *et al*., 2024; Li *et al*., 2021), was occasionally observed following exposure to saline, and capsaicin, but not to NH3, and was excluded from further analysis (**Figure S1D**). Surgical ablation of bilateral anterior ethmoidal nerves (AENs) had little impact on NH3-evoked cough-like responses, further validating that NH3 exposure predominantly induced cough, rather than sneezing.

To identify brainstem nuclei mediating cough-like behaviors in mice, we genetically labeled activated neurons using TRAP2:: Ai9 mice(Allen et al., 2017; DeNardo et al., 2019) by injecting 4-hydroxytamoxifen (4-OHT) following nebulized saline or NH3 (**Figure 1F**). Compared to saline, NH3 evoked robust neuronal activation, particularly in the brainstem, including the NTS, SP5, VRG, ventrolateral periaqueductal gray (vlPAG), and the parabrachial nucleus (PBN) (**Figure S2** and **Figure 1F**).

To directly validate whether these identified nuclei are functionally correlated with cough, we performed calcium imaging using in vivo fiber photometry. We injected adeno-associated virus encoding the calcium indicator GCaMP6s (AAV-hSyn-GCamp6s) into various nuclei, including the NTS, VRG, or SP5C, and implanted optic fibers above these regions. Consistent with their established role in cough, real-time neuronal activity in the NTS and VRG were tightly cough-correlated (**Figure S2B** and **S2C**). Surprisingly, we also observed a strong correlation between SP5C neuronal activity and cough (**Figures 1G** and **1H**), suggesting that the SP5C nucleus may play an important role in mediating cough.

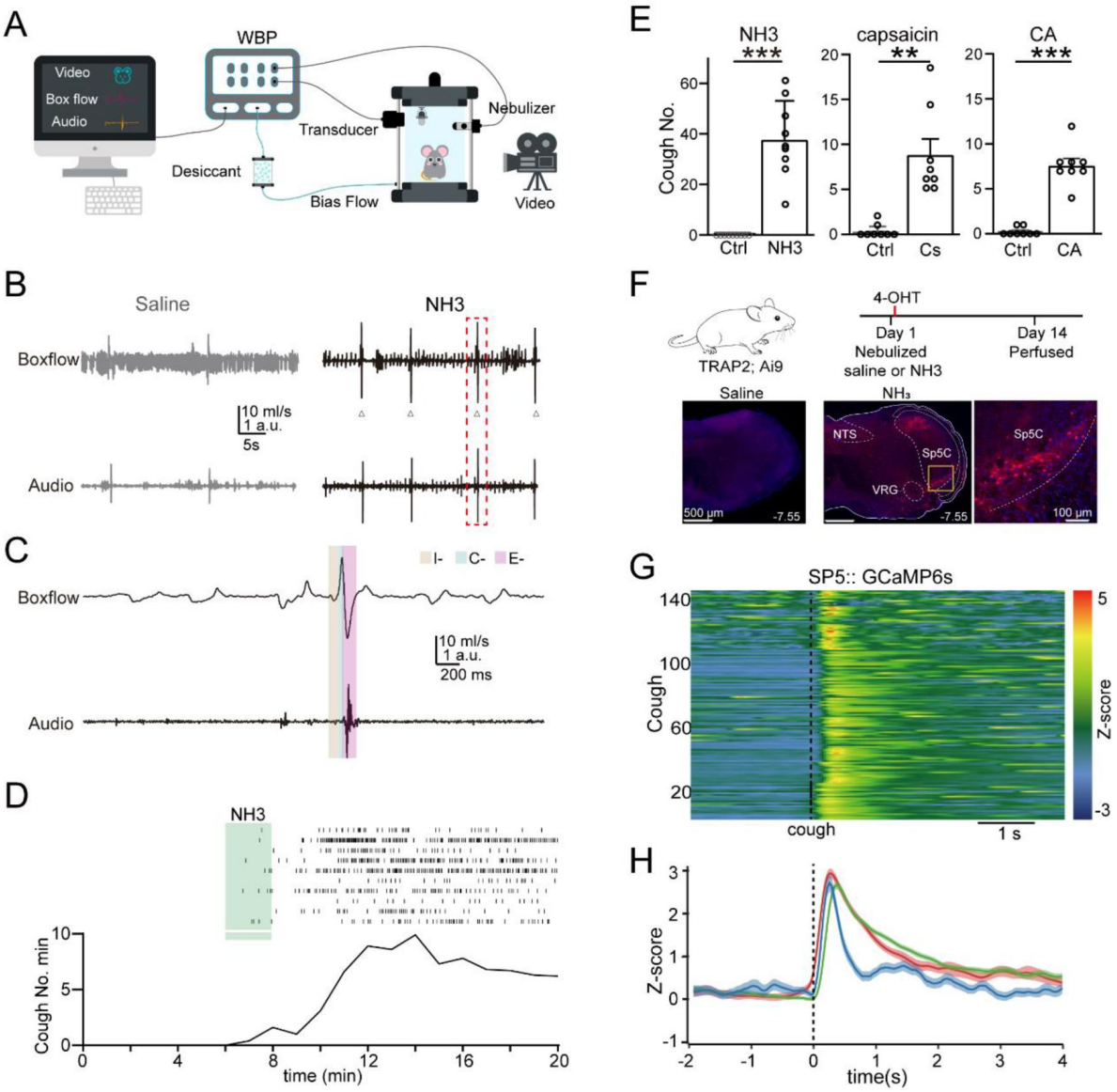


Figure 1

Activation of the SP5C is correlated with tussive-evoked cough responses in mice.

(A) Diagram illustrating the setup for cough monitoring in awake mice. (B) Representative traces of box flow and audio signals after exposure to nebulized saline (left) or 1% NH3 (right) in WT mice. Triangles indicate cough responses. (C) Expanded traces of box flow and audio signals from (B) (highlighted by red dashed rectangle), showing eupnoea and a cough along with its corresponding audio signal. I-inspiration; C-compression; E-expiration. (D) Top, raster plot of coughs of individual mice exposed to nebulized NH3 (green bar). Bottom, the number of cough per minute. n = 10 mice. (E) Summary of the number of coughs evoked by various tussigenic stimuli. (F) Experimental strategy and representative images of TRAP2 labeling brainstem neurons during NH3 or saline nebulization. (G) NH3 exposure evoked strong cough-related neuronal Ca^{2+} signals in the SP5C as measured by in vivo fiber photometry. Individual trials of Ca^{2+} signals registered to the peak airflow (time = 0, dashed line) during cough. H the average Ca^{2+} signals in the SP5C of individual mice. n = 3.

Figure 1-Figure supplement 1

Cough responses with variation in the boxflow waveforms can be observed across different tussive stimuli.
(A) Representative box flow and audio signals of two cough events evoked by nebulized CA (0.1M).
(B) Representative box flow and audio signals of two cough events evoked by nebulized capsacin (10 μM).
(C) Representative box flow and audio signals of two cough events evoked by nebulized NH3 (1%).
(D) Representative box flow and audio signals of two sneeze events evoked by nebulized capsacin (10 μM).

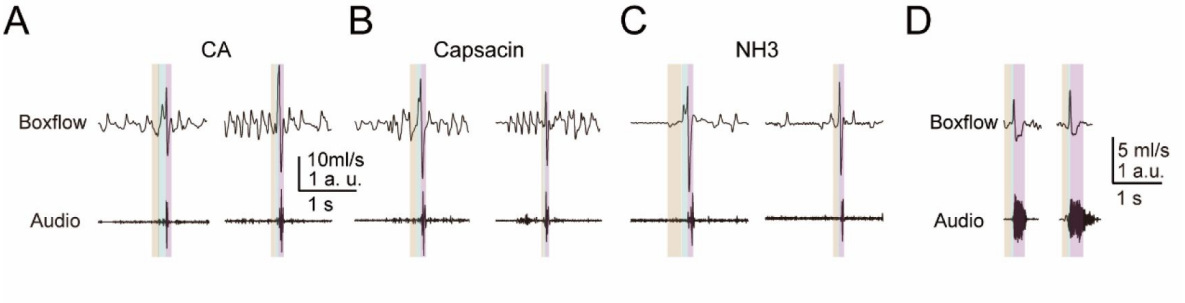
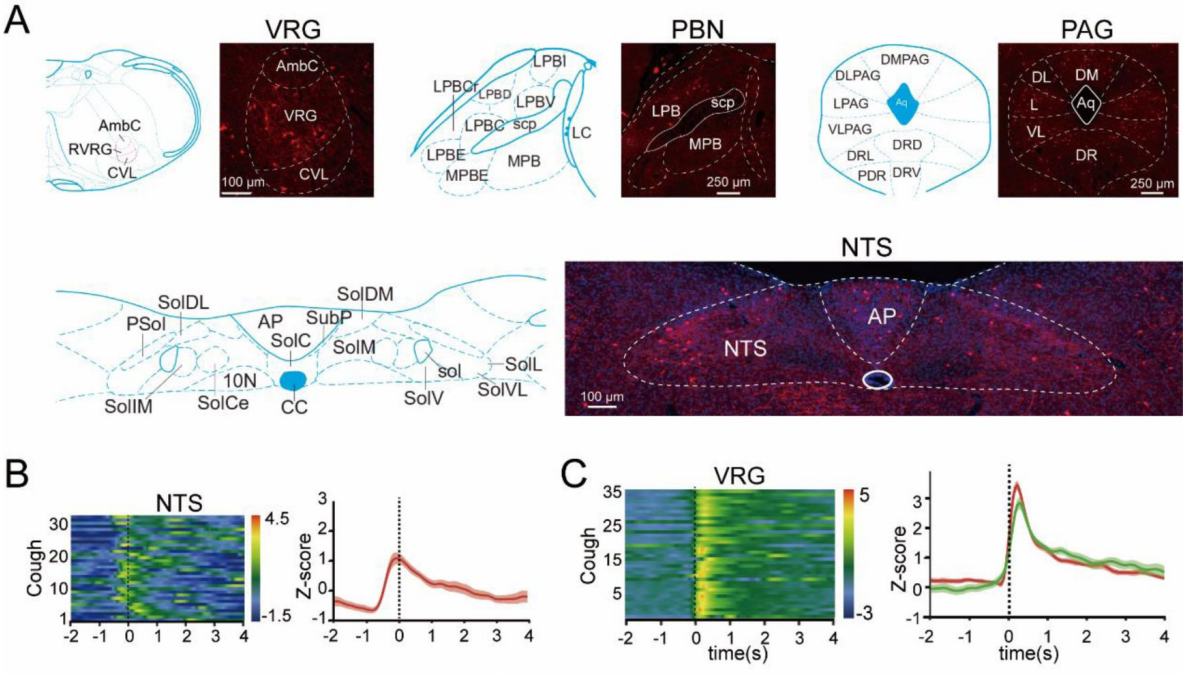


Figure 1-Figure supplement 2

The neural activity in the NTS or VRG is correlated with tussive-evoked cough responses in mices.
(A) Representative images of TRAP2 labelled neurons in the VRG, PBN, PAG, and the NTS following NH3 exposure in TRAP2::Ai9 mice.
(B) Individual (left) and average (right) cough-correltaed GCaMP6s signals in the NTS recorded by in vivo fiber photometry.
(C) Individual (left) and average (right) cough-correltaed GCaMP6s signals in the VRG recorded by in vivo fiber photometry.



To test the hypothesis that the SP5C plays a crucial role in mediating cough-like behaviors, we first investigated its necessity for triggering cough-like resonses in mice by abolishing synaptic outputs from the nucleus using AAVs overexpressing the light chain of tetanus toxin (TeTx)(Xu et al., 2012). Unexpectedly, all mice died, likely due to the disruption of the vital physiological functions governed by these nuclei. To overcome this challenge, we utilized neurexin1/2/3 conditional knockout transgenic mice (Nrxn123 cTKO)(Chen et al., 2017; Luo et al., 2020), which enables a strong but not complete blockade of synaptic outputs by Cre-recombinase. In control mice injected with AAV-EGFP in the SP5C, NH3 induced robust cough-like responses similar to wild-type animals. However, Nrxn123 cTKO mice injected with AAV-Cre-EGFP (TKO) exhibited significantly reduced cough-like responses to NH3 (**Figures 2A-C**). Using a similar strategy, we found that pan-neurexin ablation in the VRG also blocked NH3-evoked cough-like responses (Figure S3). Therefore, impairing synaptic outputs from either the SP5C or VRG markedly blocked NH3-evoked cough-like responses, suggesting their crucial roles in triggering the cough reflex.

To achieve more timely control of neuronal activity, we used chemogenetic approach by bilaterally injecting AAV-hM4Di-mCherry into the SP5C. Inactivation of SP5C neurons using deschloroclozapine (DCZ)(Nagai et al., 2020) strongly inhibited NH3-evoked cough-like responses in mice (**Figures 2D**-**2F**). In contrast, three control groups, either with AAV-mCherry injection or saline treatment, showed robust cough-like activities upon NH3 nebulization (**Figures 2D**-**2F**). These data collectively demonstrate the essential function of the SP5C in tussigen-evoked cough-like behaviors in mice.

Activating SP5C CaMKII$^+$ excitatory neurons is sufficient to trigger cough in mice

We then investigated whether activating the SP5C could directly trigger cough-like behaviors using an optogenetic technique. We injected AAV-ChrimsonR, a red-light-sensitive variant of channelrhodopsin(Klapoetke et al., 2014), into the SP5C and implanted an optic fiber (**Figure 3A**). Optogenetic stimulation of the SP5C caused pronounced motor behaviors that confounded cough response measurement, probably due to concurrent activation of distinct motor control circuits. To address this challenge, we used transgenic Cre-lines for more precise manipulation of specific cell types. We tested the functional impacts of activating excitatory or inhibitory neurons by selectively expressing Cre-dependent ChrimsonR in the SP5C of VGluT2-IRES-Cre or GAD2-IRES-Cre mice. Optogenetic stimulation of VGluT2$^+$ or GAD2$^+$ neurons caused a remarkable increase or decrease in the respiration rate of mice, respectively (**Figures 3B** and **3C**). Surprisingly, however, activation of neither VGluT2$^+$ excitatory neurons nor GAD2$^+$ inhibitory neurons triggered cough (**Figures 3B** and **3C**).

Interestingly, when we injected AAV-CaMKII-ChrimsonR into the SP5C, we found that optogenetic stimulation of CaMKII$^+$ neurons induced strong cough-like activity in the absence of tussive stimuli (**Figures 3D-F**). These data suggest that a specific population of excitatory neurons in the SP5C, although their precise identity remains to be further characterized, plays a crucial and sufficient role in triggering cough-like behaviors in mice.

Activating the SP5C projections to the VRG triggers cough in mice

Next, we investigated how the SP5C neurons connect with the respiratory network to produce cough motor behavior. Given that the VRG is well-known as the cough center (Bolser and Davenport, 2002) and its critical roles in NH3-evoked cough-like responses we observed (**Figures S2** and S3), we hypothesized that the SP5C may project to the VRG to induce cough. To test this hypothesis, we characterized the synaptic connectivity between the SP5C and the VRG using anterograde and retrograde transynaptic tracing techniques.

Figure 2

The SP5C is necessary for tussive-evoked cough responses in mice.

(A) Experimental strategy, combining stereotactic injection of AAV overexpressing Cre with transgenic mice of neurexin1/2/3 conditional knockout mice, to impair synaptic outputs of the SP5C. (B) Representative traces of box flow signals following exposure to 1% NH3 in mice with or without pan-neurexin deletion in the SP5C. (C) Summary of NH3-evoked cough responses in Ctrl and Nrxn123 TKO in the SP5C. (D-E) Representative traces of box flow signals following exposure to 1% NH3 in mice with EGFP or hM4Di expression in the SP5C when saline (D) or DCZ (E) was injected intraperitoneally. (F) Summary of NH3-evoked cough with and without chemogenetic inhibition of the SP5C.
Data are means ± SEM. Statistical difference was assessed by Student's t-test. (***P < 0.001).

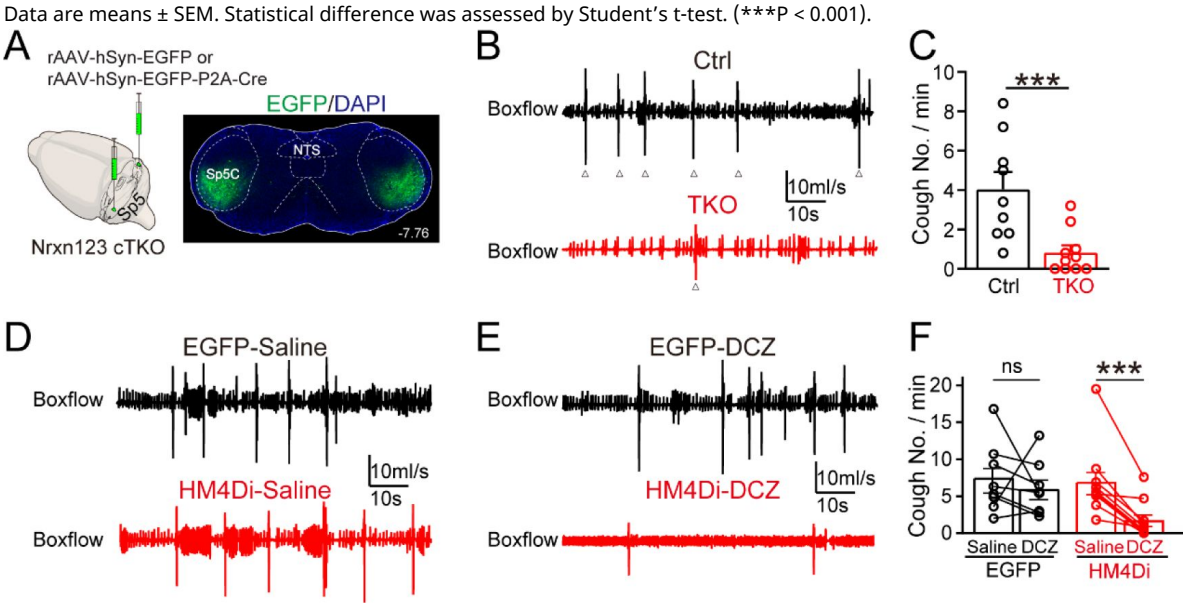
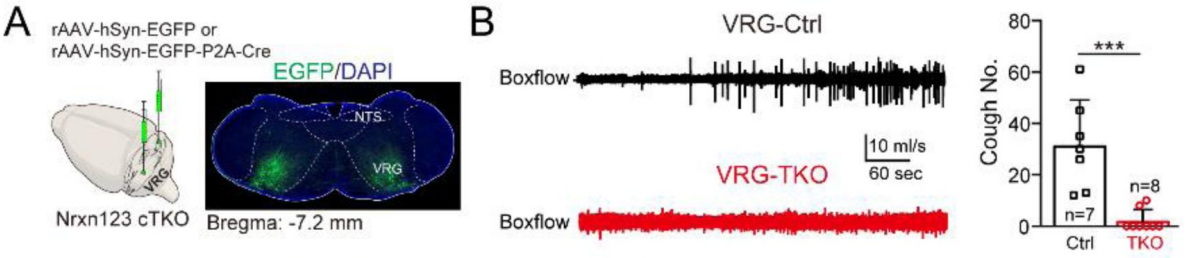


Figure 2-Figure supplement 1

The VRG is necessary for tussive-evoked cough responses in mices.
(A)Strategy and fluorescence image showing AAV mediated pan-neurexin ablation in the VRG.
(C) Representative box flow signals of mice without or with pan-neurexin deletion in the VRG following NH3 exposure (left) and the summary of cough number (right).



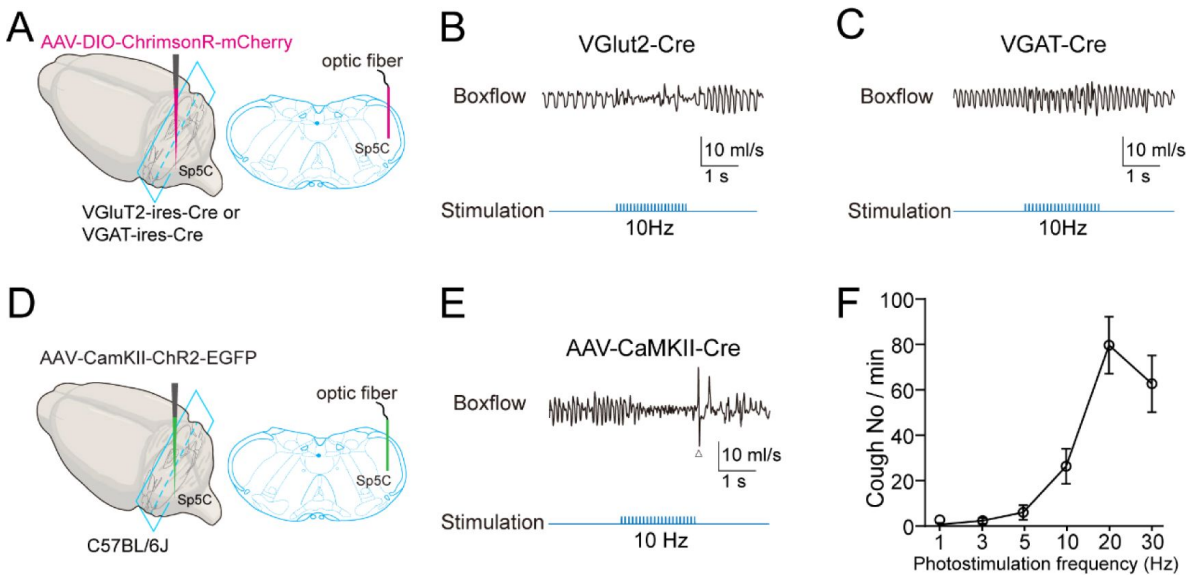


Figure 3

Optogenetic stimulation of SP5C excitatory neurons is sufficient to trigger robust cough activities in mice.

(A) Strategy for optogenetic stimulation of specific cell types in the SP5C. (B-C) Representative box flow signals following optogenetic stimulation of VGlut2^{+} excitatory neurons, or VGAT^{+} inhibitory neurons, in the SP5C. (D) Experimental strategy of optogenetic stimulation of CaMKII^{+} excitatory neurons in the SP5C. (E) Representative box flow signals of mice during optogenetic stimulation of CaMKII^{+} neurons in the SP5C. (F) Summary of the number of coughs (converted into the rate in minute) evoked by optogenetic stimulation at various frequencies.
Data are means ± SEM. Statistical difference was assessed by Student's t-test. (**P < 0.01; ***P < 0.001).

We injected AAV-hSyn-EGFP into the SP5C of WT mice and observed widespread projection of $EGFP^+$ afferent fibers to different parts of ventral brainstem, including preBotzinger complex, rVRG, and cVRG (**Figures 4** and **S4**). To complementarily verify that SP5C and VRG neurons are monosynaptically connected, we conducted retrograde transsynaptic tracing using pseudotyped EnvA-G-deleted rabies virus (RV)(Wickersham et al., 2007). Unilateral RV injections into the VRG resulted in reliable labeling of SP5C neurons (**Figures 4A** and **4B**), suggesting monosynaptic projections originating from the SP5C.

To further confirm their direct synaptic connectivity, we used a dual virus strategy. We co-injected AAV2/1-Cre, an anterograde transsynaptic variant, and AAV2/9-DIO-Chrimson-mCherry into the SP5C to label presynaptic neurons. AAV2/9-DIO-EGFP was injected into the VRG to label postsynaptic neurons only if they were synaptically connected with AAV2/1-Cre infected SP5C neurons (**Figure 4C**). We observed prominent afferent fiber labeled by mCherry coursing from the SP5C to the VRG (**Figure 4D**). Additionally, numerous $EGFP^+$ neurons were reliably observed in the VRG. Moreover, frequent co-labeling of EGFP and mCherry was observed near the soma or proximal dendrites of VRG neurons (**Figure 4D**). Together, these data provide strong evidence for a monosynaptic connection from the SP5C to the VRG.

To test the functional impact of the SP5C-VRG pathway on the cough-like behaviors in mice, we overexpressed ChrimsonR in the SP5C and implanted the optic fiber above the VRG, where it receives synaptic input from the SP5C (**Figure 4E**). Remarkably, selective photostimulation of SP5C projections to the VRG triggered robust cough-like behaviors in mice (**Figure 4F**). Interestingly, low-frequency optogenetic stimulation barely evoked cough-like activities, suggesting that cough can only be triggered during intense stimulation of afferent fibers (**Figures 4F** and **4G**). In acute brainstem slice, we found that SP5C excitatory neurons showed relatively high membrane resistance and fired high-frequency action potentials after current injection above their threshold (**Figure S5**). In addition, optogenetic stimulations reliably induced action potential firing of SP5C neurons at a wide range of frequencies from 1 Hz to as high as 50 Hz (**Figure S5**).

In some animals, we injected AAV2/9-DIO-GCaMP6s into the VRG in conjunction with injection of AAV2/1-Cre and AAV2/9-DIO-Chrimson-mCherry into the SP5C while implanting an optic fiber above the VRG. Optogenetic stimulation of the SP5C-VRG projection induced remarkable activity-dependent Ca^{2+} signals in the VRG (**Figure 4H**). Together, these results demonstrate that specific activation of the SP5C-VRG circuitry is sufficient to trigger robust cough-like activities in mice without tussive stimuli.

Tonic excitation of the SP5C chronically induces spontaneous cough

Thus far, our findings underscore the critical role of the SP5C in cough production, primarily through $CaMKII^+$ excitatory neurons projecting to the VRG. We next investigated whether neural activity in the SP5C contributes to cough hypersensitivity, a hallmark feature manifesting in chronic cough(Chung et al., 2022). To explore this, we elevated SP5C neuronal excitability by overexpressing NaChBac (**Figure 5A**), a bacterial voltage-gated Na^+ channel(Ren et al., 2001; Xue et al., 2014).

In control mice injected with AAV-EGFP in the SP5C, spontaneous cough-like activity was barely observed (**Figure 5B**). In contrast, mice injected with AAV-NaChBac exhibited spontaneous cough-like behavior (**Figure 5C**). We chronically monitored this spontaneous behavior after virus injection. Mice began to display spontaneous cough-like behaviors approximately 3-4 days after AAV inoculation. The frequency increased with prolonged expression of NaChBac, peaked roughly 2-3 days later, and then declined, likely due to decreased mobility and health of mice. When mice began to display spontaneous cough-like behaviors, we exposed them to NH3. Both

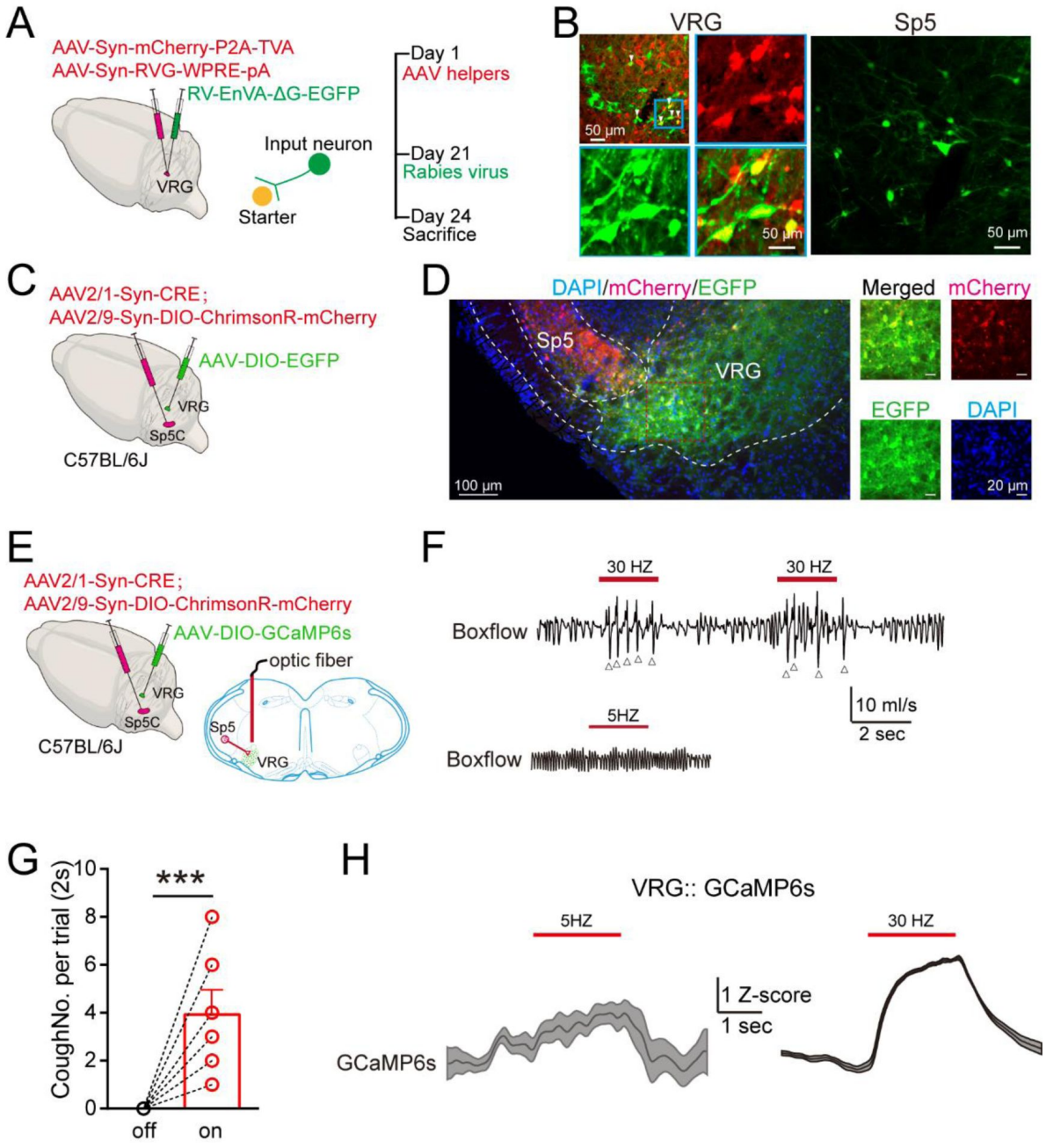


Figure 4

The SP5C projects to the VRG to trigger cough in mice.

(A) Schematic and time course of the retrograde tracing strategy. (B) RV labeling of VRG neurons receiving monosynaptic inputs directly from the SP5C. (C) Schematic of the anterograde tracing strategy. (D) AAV2/1 labeling of the SP5C afferent terminals innervating VRG neurons. (E) Strategy of optogenetic stimulation of SP5C projections to the VRG and simultaneous recording of Ca^{2+} signals from VRG neurons. (F) Representative box flow signals during optogenetic stimulation, at either high or low frequency, of the SP5C projections to the VRG. (G) Summary of the number of coughs evoked by optogenetic stimulation. (H) Summary of the GCaMP6s signals evoked by optogenetic stimulation of the SP5C projections to the VRG. Data are means ± SEM. Statistical difference was assessed by Student's t-test. (***P < 0.001).

Figure 4-Figure supplement 1

The SP5C neurons project their terminals to the respiratory network, including the cVRG (A), rVRG (B) and preBotzinger complex (PrBo) (C).

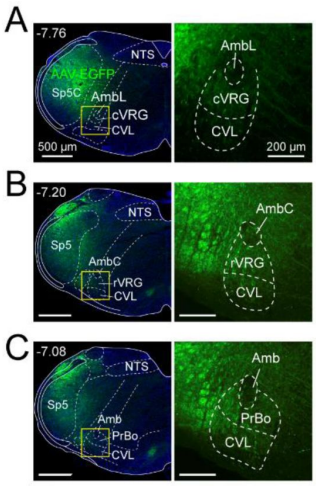


Figure 5

Elevating SP5C neuronal excitability induces spontaneous cough and cough hypersensitivity in mice.

(A) Experimental strategy (left) and fluorescence image illustrating overexpression of NaChBac in the SP5C. (B-C) Representative traces of box flow and audio signals before and after exposure to NH3 in mice overexpressing EGFP as control (B) or NaChBac (C) in the SP5C. (D) Summary of the number of coughs per minute before, during and after NH3 exposure. (E) Summary of the cough latency (the time of first cough recorded after NH3 nebulization). (F) Representative traces of box flow signals before and after injection of DCZ in mice overexpressing NaChBac in the SP5C and HM4Di in the cVRG. (G) Summary of the number of spontaneous cough events before and after chemogenetic inhibition of the cVRG.
Data are means ± SEM. Statistical difference was assessed by Student's t-test. (*P < 0.05; ***P < 0.001).

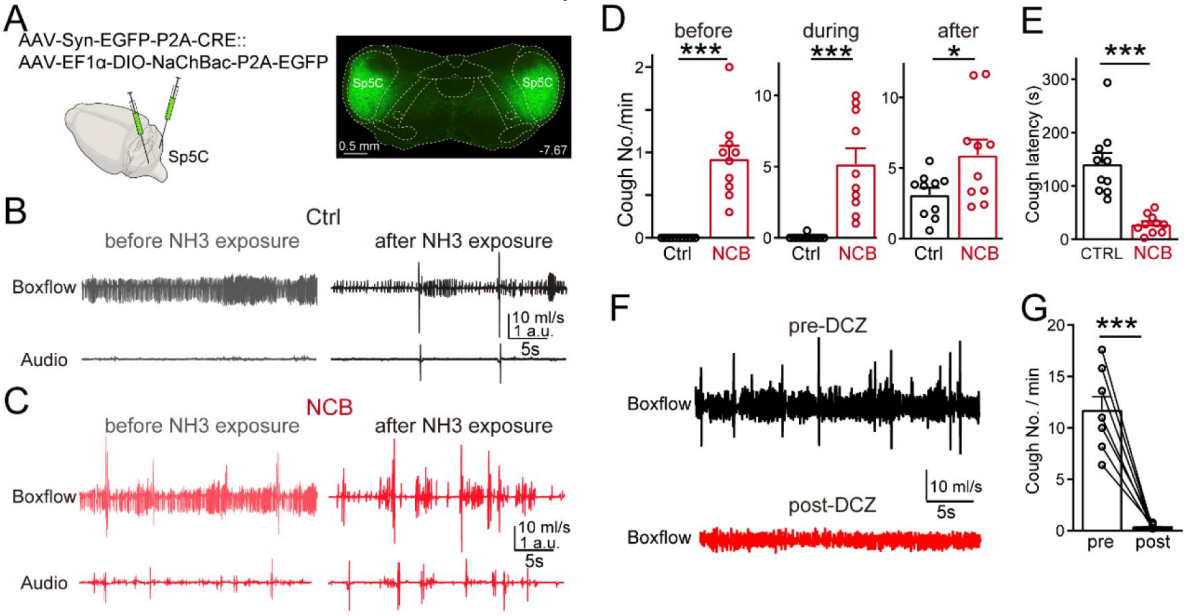


Figure 5-Figure supplement 1

Functional properties of excitatory neurons in the SP5C.
(A) Representative action potenial (AP) traces of Sp5 neurons evoked by current injection at increasing intensity, showing the neuron's capability for firing APs at high frequencies.
(B) Summary of AP firing frequency of SP5 neruons by injecting currents of increasing intensity.
(C) Summary of cell membrane resistance (left) and minimum currents required for evoking AP for SP5 neurons (right).
(D) Representative AP traces of Sp5 neurons stimulated by 1 Hz 470 nm light stimulation.
(E) Representative AP traces of Sp5 neurons stimulated by 20 Hz (left) and 50 Hz (right) 470 nm light pulses, indicating the reliability of neuronal firing in response to high-frequency optogenenetic stimulation.

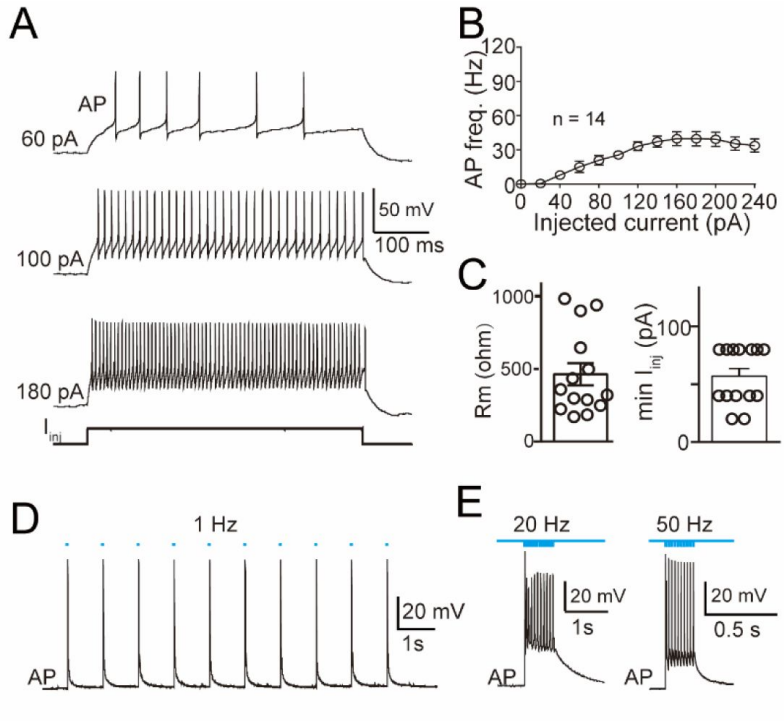
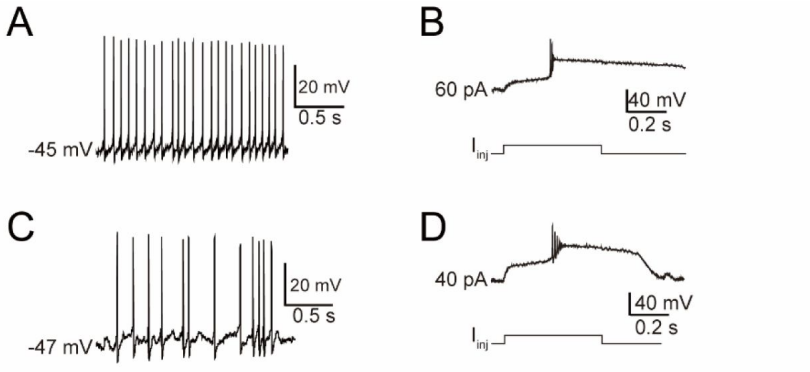


Figure 5-Figure supplement 2

NaChB-overexpressing neurons exhibit spontaneous action potentials and sustained depolarization to injected currents.
(A-B) Representative SP5 neuron overexpressing NaChBac fired spontaneous APs at high frequency (A) and evoked APs by injected current at the threshold level(B).
(C-D) Another SP5 neuron overexpressing NaChBac fired spontaneous APs but at a revlatively low frequency (C) and evoked APs by injected current at the threshold level (D).



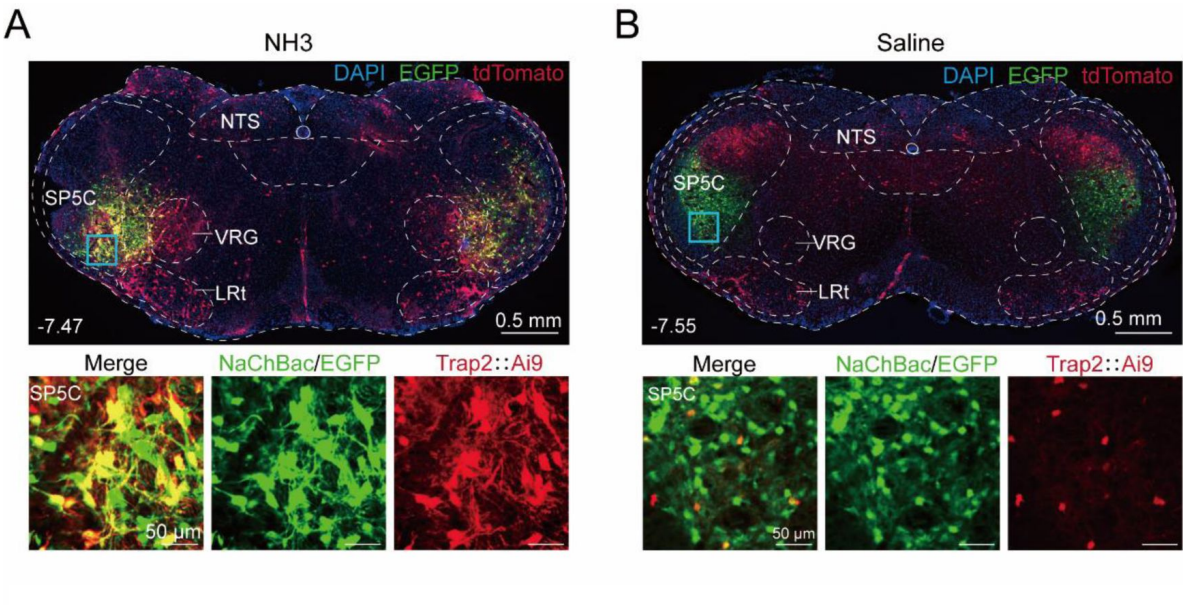


Figure 5-Figure supplement 3

Overexpression of NaChBac enhances neuronal activity in the SP5C following NH3 nebulization.
(A) Top, representative image showing TRAP2-labelled neurons after nebulized NH3 in TRAP2::Ai9 mice overexpressing NaChBac in the SP5C. Bottom, Enlarged region of interest highlighted by the blue rectangle in the Top image.
(B) Top, representative image showing TRAP2-labelled neurons after nebulized saline in TRAP2::Ai9 mice overexpressing NaChBac in the SP5C. Bottom, Enlarged region of interest highlighted by the blue rectangle in the Top image.

control and NaChBac-overexpressing mice showed robust cough-like responses to NH3 (**Figures 5B**-**5D**). However, the number of NH3-evoked cough-like responses was significantly higher in mice overexpressing NaChBac than in control mice (**Figures 5B**-**5D**). Additionally, the ability to suppress cough-like responses in mice with NaChBac overexpression appeared to decrease, as evidenced by the remarkably decreased latency of the first occurrence of cough-like responses during nebulization, while the number of cough-like responses increased compared to control (**Figure 5E**). To verify that overexpression of NaChBac indeed increases the excitability of SP5C neurons, we recorded their spiking activity in acute brainstem slice using whole-cell patch clamp recording. Indeed, compared to control, NaChBac-overexpressing neurons fired spontaneously (Figure S6). Further, injected currents caused action potential firings at higher frequency and sustained depolarization long after the end of current injection. Interestingly, when the same virus was injected into the Sp5C of TRAP2 mice, NH3 induced more abundant TRAP2-labeled neurons, most of which were overlapped with NaChBac-EGFP, suggesting an enhanced activation of SP5C neurons (Figure S7).

Finally, we examined whether the spontaneous cough-like behaviors observed in NaChBac-overexpressing mice requires the intact function of the VRG. We injected bilaterally AAV-hM4Di-mCherry into the VRG followed by injecting AAV-NaChBac into the SP5C. Inactivation of VRG neurons by injecting DCZ nearly abolished all spontaneous cough-like behaviors (**Figure 5F** and **5G**). Together, these findings demonstrate that elevating neuronal excitability in the SP5C remarkably enhances the cough sensitivity of mice, which can last as long as 48-72 hours. Moreover, albeit through a non-physiological manipulation, we show for the first time that spontaneous cough-like behaviors can occur in an animal model, warranting future study of its mechanism and pharmacological regulation.

Discussion

Consistent with recent studies(Chen *et al*., 2022; Gannot *et al*., 2024), our present findings provide multiple lines of evidence that mice are a suitable model for studying the central mechanisms of cough behavior, even in the conscious state. Several key observations support this assertion. First, various tussive agents reliably elicit characteristic airflow changes and cough sounds, which can be consistently monitored (**Figure 1**). Second, tussive stimuli evoke robust neural activity in multiple brain regions tightly correlated with cough in awake animals. Because the physiological states of the animal, i.e. anesthetized *vs.* awake, significantly impact the regulation of the cough reflex, measuring the cough responses in awake animals is crucial, especially for evaluating potential antitussive agents. Third, impairing synaptic outputs or neuronal activity from these nuclei effectively blocks tussigen-evoked cough (**Figure 1**). Finally, selective activation of these nuclei or pathways can reliably induce cough in the absence of tussigen (**Figures 2**-**4**). Consequently, transgenic mouse models hold great promise for advancing the study of genetic and neural mechanisms of cough and cough hypersensitivity.

Utilizing transgenic mice and various circuit-based tools, we have identified a novel brainstem circuit necessary and sufficient for triggering cough in mice. Remarkably, our findings demonstrate that bi-directional regulations of SP5C neuronal activity exert prominent but opposite impacts on cough activity, underscoring its central role in cough production in mice (**Figures 1**, **2**, **3**). Accumulating studies have shown that the SP5C may exert diverse physiological roles in the sneezing reflex(Li *et al*., 2021), self-grooming(Xie et al., 2022) and craniofacial nociception(Xue et al., 2024), probably via different neuronal subtypes located in distinct subregions or circuitry of the SP5C. For example, $NMDR^{+}$ neurons are specifically involved in sneezing reflex(Li *et al*., 2021) while $Clbn2^{+}$ neurons are involved in orofacial self-grooming(Xie *et al*., 2022). We have shown that $CaMKII^{+}$ excitatory neurons play an essential role in mediating cough responses (**Figure 3**), but their precise molecular identity remains to be further characterized.

Our preliminary data suggest that the SP5C does not receive direct synaptic projection from the NTS, but rather from the Pa5, the nucleus known to be specifically activated by jugular sensory neurons. However, we do not yet have conclusive evidence regarding which vagal sensory neurons project tussive information to the Pa5. Strong evidence has recently shown that $Tac1^+$ neurons in the NTS directly project to the cVRG and contribute to the cough-like behavior in mice (Gannot *et al*., 2024). It is likely that multiple neuronal pathways are involved in eliciting the cough reflex. Future studies are needed to explore whether these pathways function independently or interactively in cough control. Our data also suggest that the SP5C does not receive direct synaptic projection from the NTS, but rather from the Pa5 and other nuclei and cortical areas, such as the red nucleus, S1, and M1 cortex. It is crucial to investigate the specific contributions of distinct circuits to cough, particularly cough hypersensitivity.

Recent studies have revealed a high prevalence of chronic cough associated with RFC1 polymorphism of nucleotide repeat expansion in CANVAS patients(Cortese et al., 2019). This polymorphism has been identified in up to 25% of patients with unexplained or refractory chronic cough (UCC/RCC)(Guilleminault et al., 2023; Turner et al., 2023), indicating a strong genetic link with UCC/RCC. Therefore, it is of great interest to exploit the myriad advantages of transgenic mice to study the genetic and neural mechanisms of UCC/RCC in the future.

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Additional information

Author contributions

X. X., X.N., F.L. conceived the project. X. X., X.N., T.W., W.Z., H.J., B.L., Y.R. collected the data and performed the experiments. X. X., X.N., T.W., W.Z., H.J., F.L. analyzed the data. F.L. wrote the original draft. X.X. and F.L. revised, edited and approved the final version. F. L. supervised the project.

Declaration of interests

The authors declare no competing interests.

Methods

Animals

All experiments were conducted in accordance with the guidelines established by the Institutional Animal Care and Use Committee at Guangzhou National Laboratory. Male mice were utilized for most experiments except specified otherwise. The mice were housed under standard laboratory conditions, with a temperature range of room temperature and 40-60% humidity maintained, and subjected to a 12-hour light-dark cycle (lights on from 7:00 to 19:00) with food and water freely available. No statistical tests were used to predetermine the sample size. All experiments were conducted blindly, with the experimenter unaware of the mouse genotypes or adeno-associated viral vectors (AAVs) used. The experiments were conducted using mice aged between two to three months. C57BL/6J wild-type mice were used. All transgenic mouse lines, including Nrxn123

cKO(Chen *et al*., 2017), VGluT2-IRES-Cre (JAX#: 028863)(Vong et al., 2011), VGAT-IRES-Cre (JAX#: 028862)(Vong *et al*., 2011), and FosTRAP2(JAX#: 030323)(Allen *et al*., 2017) were previously reported.

Virus vectors

All AAV vectors were purchased from Brain VTA (Wuhan, China). The final titers of viral vectors were diluted specifically in the range from 3–8×10^{12} particles/ml before use. Rabies viruses were purchased from Brain VTA (Wuhan, China). Pseudo rabies viruses were purchased from Brain Case (Wuhan, China).

Stereotactic injections of AAVs

Mice were anesthetized via intraperitoneal injection of chloral hydrate (0.5 mg/kg) (Sangon Biotech, Shanghai, China). Following anesthesia, standard surgical techniques were employed to expose the brain areas above the brainstem. Coordinates for virus injections were determined relative to the Bregma. For the NTS, the coordinates were set at Anteroposterior (AP): −7.48 mm, Mediolateral (ML): ± 0.45 mm, and Dorsoventral (DV): −4.4 mm. For the SP5C, the coordinates were AP: −7.83 mm, ML: ± 1.85 mm, DV: −5.4 mm. For the VRG, the coordinates were AP: −7.48 mm, ML: ± 1.25 mm, DV: −5.5 mm. The AAVs were injected into the designated brainstem nuclei using a glass pipette connected to a microinjector system Nanoject III (Drummond Scientific Company, Pennsylvania, USA). The injection was performed at a controlled flow rate of 60 nl/min. The volume of AAV injected was determined based on experimental requirements and viral titration. The injected volume was carefully calibrated to ensure precise delivery into the target nuclei. Experiments were conducted at least 2 weeks after virus injections to allow for sufficient viral expression and integration into the target cells.

Optogenetic and in vivo fiber photometry

After viral injections, a ceramic ferrule equipped with an optic fiber (200 μm diameter and numerical aperture (N.A.) of 0.37 for optogenetics and fiber photometry) was implanted at the target brain regions, namely the NTS, SP5C, and VRG. The fiber tip was meticulously positioned over the target nuclei, ensuring precise placement. Secure fixation of the ferrule to the skull was achieved using dental cement, ensuring stability throughout the experiment. Following implantation, the surgical site was sutured, and topical antibiotics were administered to minimize the risk of inflammation and infection.

Optogenetic and fiber photometry experiments were conducted at least 3 weeks after fiber implantation to allow for adequate viral expression and integration. After experiment, each animal underwent histological analysis to verify virus expression and fiber localization. Only mice demonstrating appropriate viral infection and accurate fiber implantation at the target nuclei were included in subsequent data analysis.

Chemogenetic inhibition

To chemogenetically silence the SP5C nucleus, AAV-hM4Di-mCherry was bilaterally injected into the SP5C of WT mice. After 3 weeks of viral injection, DCZ solution (0.1 mg/ml in saline, 0.1mg/Kg) was intraperitoneally injected into mice. Saline was used as a control. Cough tests were performed after 30 mins.

Breathing and cough measurement

After a recovery period of 3 weeks following surgery, mice were acclimated for three consecutive days being handled daily by experimenters to minimize stress. On the day of the experiment, mice were transferred to the testing room and habituated to the experimental chamber for 1 hour before the commencement of tests.

Whole-body plethysmography (TOW Tech, Shanghai, China) was utilized to monitor animal breathing and cough responses. This technique enabled continuing measurement of respiratory parameters, including the timing and magnitude of inspiratory and expiratory efforts. Throughout the experiments, mice were awake and freely moving within the recording chamber.

High-resolution audio and video recordings of the animals' movements were captured using a microphone (Boya, Shenzhen, China) and video camera (Logitech, Shanghai, China). Pressure changes induced by respiration within the recording chamber were continuously monitored before and after the administration of tussive stimuli.

A nebulized solution (1 mL) containing capsaicin (10 μM), CA (0.1 M), NH3 (0.1%), or saline was introduced into the recording chamber at a constant speed of 0.5 ml/min. All signals were simultaneously recorded for 5 min before, 2 min during, and 10 min after each nebulization event. A minimum interval of 30 min was allowed for full recovery between consecutive challenges.

Cough responses were characterized by rapid timing (<0.1 s for the entire maneuver) and a significant magnitude of inspiratory and expiratory efforts (>500% of expiratory pressure during tidal breathing). This comprehensive approach allowed for the accurate assessment and analysis of cough responses in awake mice.

Fiber photometry recording

An AAV expressing the genetically encoded calcium indicator GCaMP6s was injected into the targeted nucleus using stereotactic techniques. Subsequently, a fiber optic was implanted 100 μm above the injected region to facilitate optical recordings. A fiber photometry recording system (Inper, Hangzhou, China) was utilized to both activate and record calcium-related fluorescence transients from different nuclei in freely moving mice. Prior to each experiment, the light intensity of the optic fiber was carefully adjusted to ensure optimal fluorescence signal detection, typically within a range of 40 μW. Following exposure to tussive stimuli or optogenetic activation of presynaptic neurons, cough-associated fluorescence signals were acquired in real time from awake mice. Data acquisition was synchronized with coughing episodes to precisely capture neural activity changes associated with cough events. Post hoc analysis of the recorded fluorescence data was conducted to extract calcium transients corresponding to neural activity changes in the target brain regions. Signals were normalized and expressed in Z-score to facilitate comparison across experimental conditions and between animals.

Acute brain slice for electrophysiology

Coronal brainstem slices were prepared similarly as described previously (Luo *et al*., 2020). In brief, mice of 8-12 weeks were decapitated; brains were rapidly isolated and fixed on the cutting platform a vibratome (VT1200s; Leica, USA), which was immersed in oxygenated cold ACSF containing (in mM): 119 NaCl, 26 NaHCO3, 10 glucose, 1.25 NaH2PO4, 2.5 KCl, 0.05 CaCl2, 3 MgCl2, 2 Na-pyruvate, and 0.5 ascorbic acid, pH 7.4. Transverse slices of 250 μm were sectioned and transferred into a beaker with bubbled ACSF containing (in mM): 119 NaCl, 26 NaHCO3, 10 glucose, 1.25 NaH2PO4, 2.5 KCl, 2 CaCl2, 1 MgCl2, 2 Na-pyruvate, and 0.5 ascorbic acid, pH 7.4. Slices were recovered at 35°C for 30, and stored at room temperature (~21-23°C) for experiments.

Whole-cell patch-clamp recordings

Patch-clamp recordings were made from visually identified SP5C neurons using an Axopatch 700B amplifier (Molecular Devices, USA) and pClamp 11 software (Molecular Devices, USA). Patch pipettes were pulled from borosilicate glass capillaries using a two-stage vertical puller (PC-100, Narashige, Japan). Pipette resistance was 4-6 MΩ when filled with an internal solution containing (in mM): 145 K-gluconate, 6 KCl, 10 HEPES, 3 Na2-phosphocreatine, 4 Mg-ATP, 0.3 Na2-GTP, 0.2 EGTA, 2 Qx-314; pH 7.2 with KOH. For optogenetics, a light simulation of 5 ms was delivered by

LED (pE-300 white, CoolLED, UK). Action potentials of SP5C neurons were recorded by holding the membrane potential at approximately −70 mV. For optogenetics, a light simulation of 5 ms was delivered by LED (pE-300 white, CoolLED, UK).

Histology and immunohistochemistry

Mice were anesthetized and perfused at speed of 1ml/min with 1×PBS for 10 min followed by 4% paraformaldehyde (PFA) for 10 min. Brains were removed and stored in 4% PFA overnight at 4C. Brain tissues were cryo-protected with 50% sucrose for 48 hours and then embedded in OCT (Cat#: 14020108926, Leica) and rapidly frozen on dry ice. Transverse sections at 30 μm were cut at −20 °C using a cryostat (CM3050-S, Leica) and mounted directly on Superfrost Plus slides (Cat#: PRO-04, Matsunami Platinum Pro). Samples were mounted with a Vectashield hard-set antifade mounting medium (Cat#: H-1500, Vector Laboratories). Images were acquired using Olympus slide scanner or Zeiss LSM800 confocal microscopy with a 63x oil-immersion objective (1.4 numerical aperture) and analyzed in Zeiss 2.6 and Image J software.

Statistical analysis

Statistical analyses are described in each corresponding experimental figures. The number of samples is indicated in figures and figure legends.

Data availability

All data supporting the findings of this study are available from the corresponding author (F.L.).

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Reviewer #1 (Public review):

Summary:

The study by Xu and colleagues provides a useful study of brainstem circuits involved in evoked respiratory reflexes that they define to be cough or cough-like in nature. The study is conducted in mice which has the benefit of allowing for the use of modern transgenic tools, although many of the experiments end up using viral vector-based approaches that could be deployed in any species. The disadvantage of the mouse model is understanding the true identity of the respiratory event that is defined as cough. This limitation requires careful interrogation in order to understand the biology of the circuit under investigation. In this respect, the authors provide an incomplete description of a putative cough pathway linking the caudal spinal trigeminal nucleus with the ventral respiratory group. Neurons assigned as CaMKII+ with putative inputs from the paratrigeminal nucleus are central to this circuit, although the evidence for each of these claims is relatively weak or non-existent. Overall, the study employs interesting methods but limitations in methods and details of methods reduce interpretation of the study outcomes.

Strengths:

The use of modern methods to investigate brainstem circuits involved in an essential respiratory reflex.

Weaknesses:

(1) The most significant issue that needs careful consideration is the exact respiratory response, which is called a cough. The authors show a trace from their plethysmography recordings and superimpose the 3 phases of cough (inspiration, compression, expiration) with confidence, yet the parameters used to delineate these phases are unclear. Of more concern, an identical respiratory trace was reported recently as a sneeze in Jiang et al Cell 2024 (PMID 39243765). Comparing Figure 1 in the Xu study with Figure 5 in the Jiang study, it is impossible to see any difference in the respiratory trace that would allow the assignment of one as cough and the other as sneeze. The audio signals also look remarkably similar and the purported cough signal in the Jiang study is quite different. Gannot et al Nat Neurosci 2024 (PMID 38977887) seems to agree with Xu in the identity of a cough signal, but Li et al Cell 2021 (PMID 34133943) again labels these as sneezes. One of the older studies that tried to classify respiratory signals in mice (Chen et al PlosONE 2013) labeled the Jiang cough trace as a deep inspiration, while sneeze looks different again. To add further confusion, Zhang et al AJP 2017 (PMID 28228416) provide yet another respiratory plethysmography trace that they define as a cough, and label responses discussed above as expiration reflexes. This begs the question - who, if anyone, is correct? Interpreting the circuits underlying these peculiar mouse responses depends on accuracy in defining the response in the first instance.

(2) The involvement of the causal nSp5 in cough is an unexpected finding. Some understanding of if and how vagal afferent inputs reach this location would help strengthen the manuscript. The authors claim in the discussion that the nucleus of the solitary tract is not the source of inputs, but rather they may arise from the paratrigeminal nucleus (although no data is presented to support this claim). This could fit with the known jugular vagal afferent pathway, which is embryologically distinct and terminates in trigeminal regions, rather than the NTS. But if this is correct, what does this finding then say about the purported involvement of NTS neurons in cough in mice, for example, the recent study by Gannot et al Nat Neurosci where Tac1-expressing NTS neurons were integral for what they call cough in mice? Xu and colleagues are encouraged to resolve their input circuitry so that we can better understand the pathway under investigation and how it relates to the NTS pathway. Related to this, and the issues differentiating cough-like responses from sneeze, the authors will need to consider how to differentiate their cough-like circuitry from the sneeze pathway from the caudal nSp5 to the cVRG as reported by Li et al Cell 2021. It seems highly possible that the two groups are studying the same circuitry, yet the interpretation is confounded by an inability to agree on the identity of the evoked response.

(3) Injection volumes and titres for AAV transductions are not stated anywhere. The methods (line 484) indicate that different volumes were used for different purposes, but nowhere is this information stated properly. Looking at representative images suggests that volumes were very large, with most of the brainstem often transduced. As single slices are only ever shown it becomes a concern as to how extensive transductions truly are. The authors need to provide complete maps of viral transduction so that readers can understand exactly what regions could contribute to responses, thereby confounding interpretation.

(4) The authors do not provide any data to explore the impacts of manipulations on basal breathing. This is important as impacts on the respiratory patterning will likely have profound effects on evoked responses that are not related to the specific pathway under investigation. For example, in Figure 2b. breathing looks to be severely compromised in the TKO animals and disrupted in the M4 DREADD animals. Figure 3 also shows the effects of optical stimulation on breathing patterns, which appear like apnea with several breakthrough augmented breaths (some labeled as cough?), although hard to see properly in the traces provided. Figure 5, one would expect VRG inhibition to have impacts on breathing, and the traces supplied appear to suggest this is the case. Please include data showing breathing effects and consider how these may confound your study interpretation.

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Reviewer #2 (Public review):

Summary:

This study employs a combination of state-of-the-art experimental approaches in mice to identify components of the brainstem circuits involved in the cough reflex in a freely behaving mouse model. The cough reflex is an important respiratory airway defense mechanism, and there has been longstanding interest in defining the neural circuits involved in the mammalian brainstem. Consistent with other recent studies, the present results provide multiple lines of evidence indicating that mice are a suitable model for studying neural mechanisms generating cough behavior. The main novel finding of this study is the authors' results indicating that the caudal spinal trigeminal nucleus (SP5C) nucleus plays a role in generating cough-like behaviors in response to inhaled tussigen. The supporting data presented for this role includes the authors' findings that: (1) neural activity in the SP5C is strongly correlated with tussigen-evoked cough-like behaviors, (2) impairing synaptic outputs or chemogenetic inhibition of SP5C neurons effectively abolished these cough-like reflexes, (3) optogenetically activating a specific subpopulation of excitatory neurons in the SP5C triggers cough-like behaviors, (4) SP5C neurons project monosynaptically to ventral medullary regions containing respiratory circuits that exhibit cough-related neural activity, and (5) specific activation of the SP5C-ventral respiratory circuitry induces robust cough-like behavior without tussive stimuli. This study will be valuable to respiratory neurobiologists studying mechanosensory control of breathing in mammals.

Strengths:

(1) The authors developed an experimental paradigm in mice that combines whole-body plethysmography (WBP), audio, and video tracking to assess breathing and putative cough-like behaviors in conscious animals.

(2) The mouse model enables optogenetic, chemogenetic, virus-based circuit tracing and manipulation, and in vivo fiber photometry to analyze neural activity and define circuity in the medulla-producing cough-like behavior.

(3) Multiple lines of evidence from these experimental approaches support the conclusion that the SP5C nucleus plays a role in the respiratory reflex behaviors studied in mice, but there is uncertainty that these behaviors are definitively cough.

Weaknesses:

(1) This paper lacks essential quantitative details about the number of animals studied explicitly for many of the experimental paradigms presented and for statistical analyses as well as to verify replication of the neuroanatomical data presented.

(2) The authors' evidence is incomplete that the reflex behavior produced in their mouse model is definitively cough, limiting functional interpretation of the putative circuit identified and requiring more thorough experimental interrogation of the behavior studied.

(3) The medullary circuit described conveys afferent sensorimotor signals to downstream respiratory circuits to coordinate cough-like motor behavior, but how the circuits that typically mediate the cough reflex, which involve airway-related vagal sensory neurons, operate in conjunction or parallel with the SP5C circuit described has not been determined, which is a significant gap in understanding how the present results fit into the neural control of the cough reflex.

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Reviewer #3 (Public review):

Summary:

The authors have submitted a comprehensive manuscript on the production and central pathways that they propose mediate cough-like behaviors in a TRAP2 transgenic mouse model. While the central pathway data are good, there is significant uncertainty regarding the identity of the presumptive cough-like behavior that has been produced in their model which reduces enthusiasm for the manuscript.

Strengths:

(1) The use of the TRAP2 model in the investigation of coughing is strong.

(2) The implication of SP5 in the production of coughing in response to ammonia inhalation is a novel finding. Further, this area can be activated by AAV-CaMKII to induce coughing in the absence of coincident afferent activation is an important observation.

Weaknesses:

(1) A fundamental aspect of this investigation is the unequivocal identification of the behavior that has been evoked. In this case, the authors have not established that they are actually studying cough. The evidence that they present (especially Figure 1 - Supplement 1) clearly shows that the citric acid (2nd example), capsaicin (2nd example), and ammonia (2nd example) box flows lack a large inspiratory component which is a requirement of cough. The referenced behaviors appear to be expulsion/inspiration which is not cough. The only way these behaviors could be cough is if the conventional polarity of presentation of the flow signals are reversed. However, inspection of the flows during breathing strongly indicates that inspiration is down in your records. Again, this makes these behaviors expulsion/inspiration.

An additional issue is that there are compression phases marked when the flow is occurring. The compression phase is a period of no flow so this is not accurate. There also is no evidence that the mouse has a compression phase at all. In cough flow records in humans, the compression phase can clearly be seen when it happens but not all coughs have one. You must show that a compression phase happens according to the actual description of what this segment of cough actually is.

It may be that you are evoking behaviors that primarily occur in the mouse. As such, they would be novel airway protective behaviors that are worthy of description and study. Ironically, another manuscript in the journal Cell (Jiang et al, 2024, Cell 187:5981-5997) shows similar box flow polarities as your own and clear cough airflows (Fig. 5B). However, they also show other airflow patterns that resemble what you call cough (Figure 5A) but they call them sneeze. Those airflows are expulsion/inspiration and are clearly not sneezing as the expulsion in this behavior also is preceded not followed by inspiration.

The definitive manuscript on cough in the mouse is Zhang et al Am J Physiol Reg Integr Comp 312:R718-R726, 2017. In this manuscript, Figure 2 clearly shows both box pressures and intrapleural pressures during airway protective behaviors in the awake mouse. Note that both cough and a behavior known as expiration reflex were recorded. The key element here is that the cough elicited a tri-phasic box flow. The last excursion was associated with a sound. When compared to the pressure it is clear that this last flow excursion is mechanical chest wall recoil from residual volume. The fact that this segment of the flow record was associated with sound strongly suggests that the vocal folds were adducting at the time to

"brake" the chest wall recoil. In other words, the airway resistance went up to slow inspiratory airflow as the chest returned to its resting position. As such, this observation suggests that the chest wall mechanics of cough in the mouse are different than that of larger animals.

(2) Roger Shannon and coworkers have published a number of papers on the detailed brainstem circuits that are responsible for coughing. I recommend that the authors assimilate this knowledge in the context of their results.

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