Genetic identification of a hindbrain nucleus essential for innate vocalization

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Vocalization in young mice is an innate response to isolation or mechanical stimulation. Neuronal circuits that control vocalization and breathing overlap and rely on motor neurons that innervate laryngeal and expiratory muscles, but the brain center that coordinates these motor neurons has not been identified. Here, we show that the hindbrain nucleus tractus solitarius (NTS) is essential for vocalization in mice. By generating genetically modified newborn mice that specifically lack excitatory NTS neurons, we show that they are both mute and unable to produce the expiratory drive required for vocalization. Furthermore, the muteness of these newborns results in maternal neglect. We also show that neurons of the NTS directly connect to and entrain the activity of spinal (L1) and nucleus ambiguus motor pools located at positions where expiratory and laryngeal motor neurons reside. These motor neurons control expiratory pressure and laryngeal tension, respectively, thereby establishing the essential biomechanical parameters used for vocalization. In summary, our work demonstrates that the NTS is an obligatory component of the neuronal circuitry that transforms breaths into calls.

vocalization | expiration | hindbrain | premotor neurons | Olig3

Vocalization is the primary mechanism used by many vertebrate species for communication (1). Whereas adult mice call during courtship, mating, and territorial disputes, newborn mice use vocalization to communicate with their mothers (2, 3). Newborn mice, when isolated, produce ultrasonic calls (USCs) that elicit search and retrieval behavior by their mothers. Thus, vocalizations of newborn mice represent an innate behavior that is thought to rely on a genetically determined circuit. Such innate vocalizations are reminiscent of nonverbal utterances of humans like laughing, crying, sighing, and moaning.

The central circuits that control vocalization have been widely studied in adult vertebrates, where they overlap in their executive components with respiratory circuits (4). Forebrain pathways that control the frequency and sequence of ultrasounds in mice are not essential for innate vocalization (5, 6); rather, it is the periaqueductal gray in the midbrain that modulates the activity of motor neurons in the hindbrain and spinal cord to implement calls and modulate breathing (7, 8). Calls are shaped through a biomechanical process that involves variations in subglottal air pressure and laryngeal muscle tension (9, 10). Expiration is an important determinant of subglottal air pressure (11), suggesting that expiratory muscle activity and laryngeal tension are highly coordinated during vocalization. However, because expiratory and laryngeal motor neurons are located at markedly different axial levels of the nervous system, in the spinal cord (T11–L1 levels, expiratory) and hindbrain (nucleus ambiguus, laryngeal), how the activities of these motor pools are coordinated is unclear (12, 13). More importantly, the identity and location of functionally important premotor neurons for vocalization are little known.

Using mouse genetics to investigate the neuronal basis of innate vocalization, we identified the nucleus tractus solitarius (NTS) as a crucial vocal nucleus. Newborn mice that lack the NTS are mute (i.e., unable to call), and do not receive adequate maternal care; however, these mice do display other vocalization-associated behaviors, such as mouth opening and the production of clicks. We found that in these mice, muteness is accompanied by a deficit in the ability to generate the expiratory pressure necessary for vocalization. We also show that the NTS directly connects to and activates spinal (L1) and nucleus ambiguus motor neurons at positions where expiratory and laryngeal motor neurons reside (12, 13). Our data suggest that NTS neurons establish the biomechanical parameters essential for call production by controlling the activity of expiratory and laryngeal motor neurons. Thus, we identify the NTS as an obligatory component of the circuit that links breathing and vocalization.

Results

Identification of Hindbrain Neurons Associated with Vocalization. The vocalizations of young mice in the postnatal period provides a model for exploring the neuronal circuitry responsible for this behavior. When isolated, newborn mice produce USCs

Significance

Vocalization is a primary method of communication in many species and relies on coordinated muscle activity. Vocalization and breathing must be synchronized, because calls can be evoked only during expiration. How vocalization and breathing are coordinated is not well understood. Here, we show that newborn mice with impaired development of the nucleus tractus solitarius (NTS) are mute and cannot generate the expiratory pressure needed for vocalization. Furthermore, they do not receive appropriate maternal care. We demonstrate that the NTS contains premotor neurons that directly project to and entrain the activity of spinal (L1) and nucleus ambiguus motor neurons known to control expiratory pressure and laryngeal tension, respectively. We conclude that the NTS is an essential component of the vocal circuit.

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and short clicks (Fig. 1A; spectrograms illustrate sound frequencies and intensity of calls). Calls are particularly frequent during the first hours of life (Fig. S1). Newborn mice also vocalize with a combination of stereotypic broadband audible calls (ACs), USCs, and short clicks in response to mechanical stimulation (Fig. 1A).

Given the central role of hindbrain circuits in breathing and breathing-associated behaviors, we analyzed call production in mutant mouse strains that display deficits in hindbrain development. In doing so, we identified two strains, Oligodendrocyte transcription factor 3 (*Olig3*) and T-cell leukemia homeobox 3 (*Tlx3*) mutants, with severe vocalization impairment (Fig. 1A) (14, 15). Whereas *Tlx3* mutants produced weak and rare calls, *Olig3* mutant newborns produced no calls either after mechanical stimulation or when isolated from the mother (Fig. 1 B and C). Both mutants were able to generate other behaviors associated with vocalization, including mouth openings and click production (Fig. 1B). This indicates that these mice have a deficit in the neuronal circuitry that specifically controls the production of calls.

Previous studies have shown that mutations in *Olig3* and *Tlx3* affect the development of largely nonoverlapping neuronal populations in the mouse hindbrain (14, 15). The single exception is the dA3 neuronal type produced exclusively in rhombomeres 4–7, which is impaired in both mutant strains. This raises the possibility that dA3 neurons are required for call production. The Olig3 transcription factor is expressed in a dorsal hindbrain progenitor domain that generates dA1–dA4 neuronal types; among these, dA3 neurons express the Tlx3 transcription factor (Fig. 2A). dA3 neurons contribute to four distinct hindbrain centers: the NTS, area postrema, and A1/C1 and A2/C2 adrenergic groups (shown schematically in Fig. 2B) (14–17). During development, all dA3 neurons are excitatory (vGluT2$^+$) and coexpress the Paired-like homeobox 2b (Phox2b) (Fig. S2 A–K). Thus, all vGluT2$^+$ NTS neurons express Phox2b at P0, but some down-

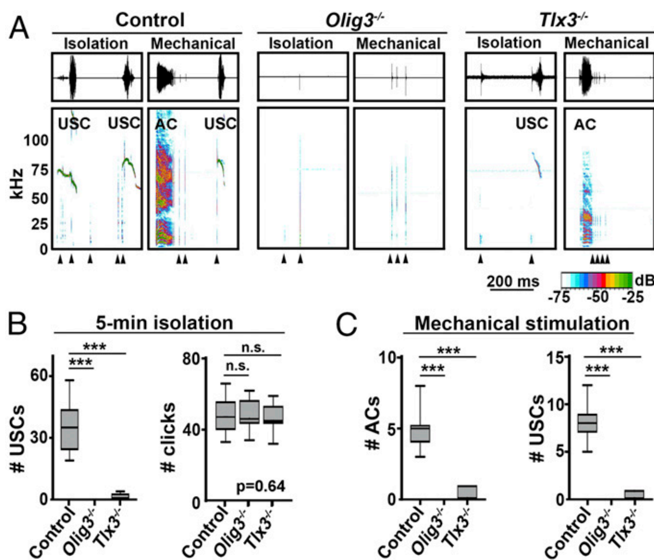

Fig. 1. Vocal impairment of *Olig3* and *Tlx3* mutant mice. (*A*) Representative waveforms (*Upper*) and spectrograms (*Lower*) illustrating ACs, USCs, and clicks (arrowheads) produced by control, *Olig3*$^{-/-}$, and *Tlx3*$^{-/-}$ newborn mice in isolation or after mechanical stimulation. Sound intensity (in dB) is color-coded. (*B*) Quantification of USCs and clicks produced by control (n = 24), *Olig3*$^{-/-}$ (n = 12), and *Tlx3*$^{-/-}$ (n = 12) mice during a 5-min isolation period. (*C*) Quantification of ACs and USCs produced by control (n = 24), *Olig3*$^{-/-}$ (n = 12), and *Tlx3*$^{-/-}$ (n = 12) mice after a single tail stimulation. The boxplots in *B* and *C* show median (black line), quartiles (boxes), and ranges (whiskers). The numbers and types of calls produced by *Olig3*$^{+/-}$ and *Tlx3*$^{+/-}$ heterozygous mice were indistinguishable from those produced by WT animals and are displayed together as controls (Fig. S1B). ***$P < 0.0001$.

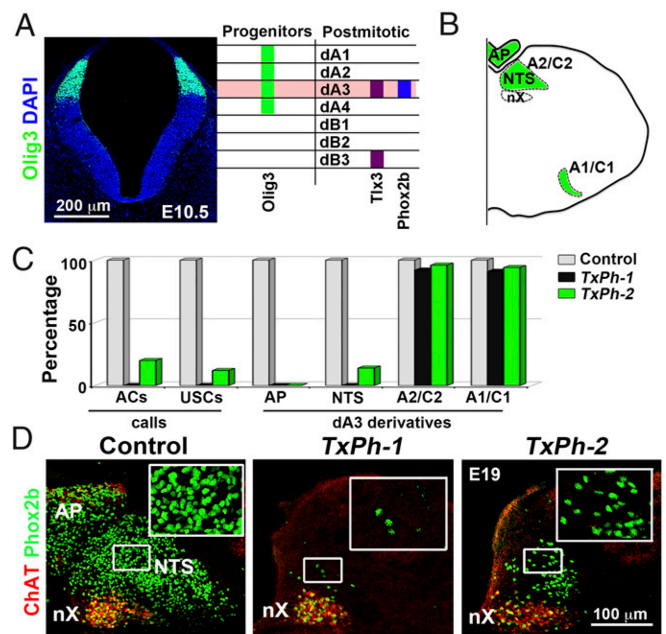

Fig. 2. NTS neurons are essential for vocalization. (*A*, *Left*) Transverse section of rhombomere 7 stained with Olig3 antibodies (green) and DAPI (blue). Olig3 is expressed in dorsal progenitor cells at E10.5. (*A*, *Right*) Scheme showing genes expressed in progenitor cells and neurons of the dorsal hindbrain. Olig3$^+$ progenitors generate dA1-4 neurons; dA3 neurons express Tlx3 and Phox2b. (*B*) Scheme of a transverse hindbrain section depicting dA3 neuronal derivatives (green): area postrema (AP), NTS, A1/C1, and A2/C2 adrenergic groups. (*C*) Column plot comparing the numbers of ACs, USCs, and dA3-derived neuronal types in control (n = 22), *TxPh-1* (n = 14), and *TxPh-2* (n = 9) animals (Table S1). (*D*) Transverse sections of control, *TxPh-1*, and *TxPh-2* mice costained with Phox2b (green) and ChAT (red) to visualize NTS neurons at E19.

regulate Phox2b subsequently. Inhibitory neurons associated with the NTS and area postrema are not derived from dA3, but emerge from dB neurons (18). A1/C1 and A2/C2 adrenergic groups express tyrosine hydroxylase, but again some neurons down-regulate Phox2b and vGlut2 in postnatal life (19, 20).

Changes in the generation of dA3 neuronal derivatives were quantified in *Olig3* and *Tlx3* mutant mice. Costaining of serial hindbrain sections with antibodies against Phox2b and choline acetyltransferase (ChAT) distinguished residual ChAT$^-$/Phox2b$^+$ NTS neurons from nearby ChAT$^+$/Phox2b$^+$ vagal motor neurons that emerge ventrally and have no history of Olig3 or Tlx3 expression. Tyrosine hydroxylase served as a marker for adrenergic neurons. In *Olig3* mutants, all dA3 derivatives (NTS, area postrema, and A1/C1 and A2/C2 neurons) were absent. In *Tlx3* mutants, the number of ChAT$^-$/Phox2b$^+$ NTS neurons was reduced by 86%, and the area postrema, A1/C1, and A2/C2 neurons were absent (Fig. S2 L and M). Thus, Phox2b$^+$ NTS neurons are impaired to different degrees in *Olig3* and *Tlx3* mutant mice. Most strikingly, the degree of vocal impairment was correlated with the reduction of Phox2b$^+$ NTS neurons; *Olig3* mutants lost all Phox2b$^+$ NTS neurons and were unable to produce calls, whereas *Tlx3* mutants displayed a severe reduction in these neurons and produced weak and infrequent calls. Although we reasoned that the Phox2b$^+$ NTS neurons play an important role in vocalization, we could not exclude the possibility that other neuronal deficits in *Olig3* and *Tlx3* mutants might contribute to their vocal impairment.

NTS Neurons Are Essential for Vocalization. To directly assess the function of NTS neurons in vocalization, we set out to specifically eliminate Phox2b$^+$ NTS neurons. Because neuronal progenitors

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commonly generate different neuronal subtypes in a defined temporal order (21), we performed a lineage trace analysis of dA3 neuronal derivatives. We found that dA3 neurons that produce the NTS and area postrema emerge after embryonic day (E) 10.5, whereas those that form adrenergic A2/C2 and A1/C1 groups arise earlier (Fig. S3 *A–C*). Therefore, we selectively ablated late-born Phox2b$^+$ neurons by combining the inducible *Olig3^{creERT2}* and conditional *Phox2b^{Flox}* alleles, and induced recombination with tamoxifen treatment at E10.5 (*Olig3$^{creERT2/+}$;Phox2b$^{Flox/Flox}$* mice, hereinafter called *TxPh* mutants; Fig. S3 *D–F*) (14, 22). All *TxPh* mutants displayed major deficits in vocalization, whereas tamoxifen-treated control littermates vocalized normally (Fig. 2*C* and Fig. S3 *G–I*). The *TxPh* mutants were classified into two subgroups according to the degree of vocal impairment: 18 of 31 pups in the *TxPh*-1 subgroup produced no calls, and 13 of 31 pups in the *TxPh*-2 subgroup produced only a few weak calls.

We next quantified Phox2b$^+$ NTS neurons, and found that >98% of these neurons were absent in the *TxPh-1* mice and 84% were lacking in the *TxPh-2* mice (Fig. 2*D* and Table S1). This finding supports a direct correlation between the depletion of NTS neurons and the inability to emit calls. In contrast, area postrema neurons were completely eliminated in all *TxPh* mutants, whereas A2/C2 and A1/C1 neurons were mostly spared (Fig. 2*C*, Fig. S3*J*, and Table S1). Therefore, *TxPh-1* and *Olig3* mutant mice are similar; they lack Phox2b$^+$ NTS neurons and are unable to vocalize. *Tlx3* and *TxPh-2* mutants also are alike, in that they lose most, but not all, Phox2b$^+$ NTS neurons and produce infrequent and weak calls. A2/C2 and A1/C1 neurons are present in *TxPh* mutant mice and absent in *Olig3* and *Tlx3* mutant mice, whereas the area postrema is lacking in all mutants. Thus, deficits in adrenergic neurons and the area postrema do not correlate with impaired vocalization. Taken together, these findings reveal that ablation of Phox2b$^+$ NTS neurons causes muteness, pointing to an essential role of NTS neurons in vocalization.

NTS Neurons Regulate Vocal Expiration. We next tested whether perturbations in the development of the NTS compromise basal breathing in early postnatal life. Basal breathing parameters (ventilatory volume/min; Ttot, which assesses the length of the breathing cycle; and tidal volume, which assesses ventilatory volume per breath) of *Olig3* mutants were normal during the first 3 postnatal hours; however, breathing maturation was disturbed thereafter because of insufficient increases in breathing frequency, tidal volume, and ventilation minute volume (Fig. S4*A*). *Olig3* mutants did not survive beyond the first 12 h (14, 17). Likewise, the basal breathing parameters of *Tlx3* and *TxPh* mice were unchanged in the first hours of life (Fig. S4*B*), but nevertheless, the *Tlx3* (15) and *TxPh* mice also failed to survive. Consequently, all experiments assessing vocalization were restricted to the first 2 postnatal hours, during which basic ventilatory parameters were normal in mutant mice and the production of calls was highest in control animals.

We next simultaneously investigated vocalization and breathing in newborn mice using a plethysmograph equipped with a microphone. In the plethysmograph, newborn control mice produced characteristic isolation-evoked calls—rhythmically repeated bouts of four to six USCs intermingled with clicks, but never with ACs. The bouts of USCs were associated with specific breathing episodes that we call “vocal breathing” (Fig. 3*A*; 5.9 ± 0.6 vocal breathing episodes were detected during a 5-min isolation period). Vocal breathing was characterized by (*i*) fast breathing, i.e., an average frequency of 3 Hz due to a reduction in the duration of expirations but not inspirations, which distinguishes it from basal breathing with an average frequency of 2 Hz; (*ii*) greater expiratory pressure in the postinspiratory phase, detected by the plethysmograph as negative signal, which were not observed during basal breathing; and (*iii*) production of USCs (Fig. 3*A* and Fig. S4 *C–E*). During vocal breathing, the expiratory pressure increased rapidly and peaked immediately before ultrasound emission. We refer to this large postinspiratory pressure change as vocal or active expiration.

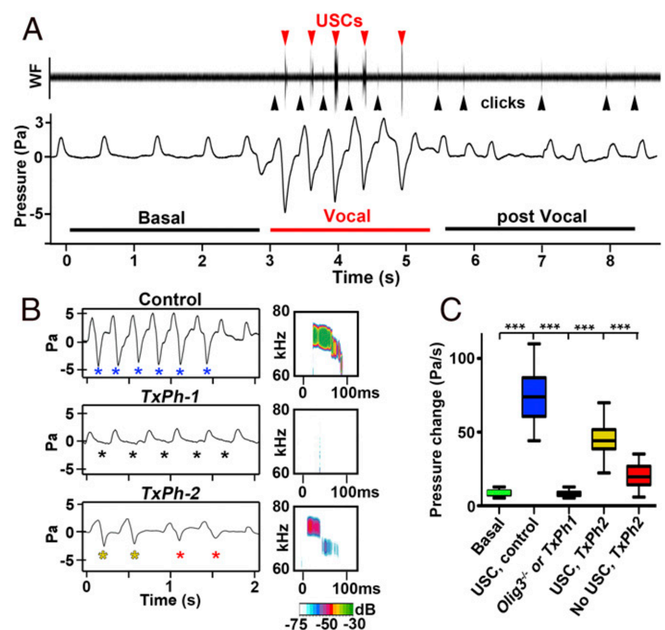

Fig. 3. Vocal breathing behavior in newborn mice. (*A*) Waveform and plethysmographic traces illustrating slow basal and fast vocal breathing episodes in control mice. Red and black arrowheads point to USCs and clicks, respectively. (*B*, *Left*) Plethysmographic recordings of fast breathing episodes in control, *TxPh-1*, and *TxPh-2* mice. Blue asterisks indicate expirations of control mice that produced USCs. Black asterisks indicate expirations of *TxPh-1* mice that produce no USCs. Red and yellow asterisks indicate expirations of TxPh-2 mice that produced no calls or weak calls, respectively. (*B*, *Right*) spectrograms from control, *TxPh-1*, and *TxPh-2* mice. Note that only clicks were observed in *TxPh-1* mice. Sound intensity (in dB) is color-coded. (*C*) Boxplots of expiratory pressure changes during vocal breathing. Pressure changes observed in basal expirations and vocal expirations of control (n = 9) mice are shown in green and blue, respectively. Pressure changes during fast breathing episodes of *Olig3$^{-/-}$* (n = 4) and *TxPh-1* (n = 14) mice are shown in black. Pressure changes that were/were not associated with USCs in *TxPh-2* mice (n = 7) are shown in yellow and red, respectively. ***P < 0.0001.

We next simultaneously recorded breathing and vocalization in *Olig3* and *TxPh-1* mutant mice whose vocal behavior was the most severely affected. These animals completely lacked active expirations but had normal incidences of fast breathing episodes (Fig. 3*B* and Fig. S4 *F* and *G*). In *Tlx3* and *TxPh-2* mutant mice that rarely vocalize, we observed fast breathing episodes associated with blunted active expirations that were characterized by smaller pressure changes (Fig. 3*B* and Fig. S4 *F* and *G*). Interestingly, only a fraction of these expirations were associated with USCs (Fig. 3*B*; expirations producing calls are indicated by yellow asterisks, and those not producing calls are shown by red asterisks), and those calls were weak (Fig. S4*H*). We used *TxPh-2* mice to estimate the rate of pressure change during expiration in which a USC was produced. This revealed that successful USCs occurred when the pressure change was >35 Pa/s (yellow and blue in Fig. 3*C*). Lower values usually were not sufficient to produce calls (red in Fig. 3*C*). We conclude that the NTS is essential for the generation of postinspiratory pressure required for USCs.

Direct Projections of Phox2b$^+$ NTS Neurons to Expiratory and Laryngeal Motor Neurons. To investigate whether the connectivity of NTS neurons is consistent with its function in vocalization, we stereotactically

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injected an adeno-associated virus (AAV) encoding a synaptophysin-GFP fusion protein (SypGFP) into the NTS of adult mice and mapped NTS synaptic connections. We observed a dense labeling of GFP^+ synapses on motor neurons at lower thoracic (T11–13) and upper lumbar (L1) levels of the spinal cord (Fig. 4 *A* and *C* and Fig. S5 *A* and *B*). We also observed synaptic contacts with other known targets of the NTS, including motor neurons of the semicompact area and loose formation of the nucleus ambiguus known to innervate the larynx (Fig. 4 *B* and *C* and Fig. S5*C*). The retrotrapezoid nucleus, caudal ventral respiratory group, A1/C1, nucleus retroambiguus, parabrachial complex, periaqueductal gray, and thalamus also were seen to be innervated by the NTS (Fig. S5 *D*–*G*). Among these, the ambiguus, retroambiguus, parabrachial, and periaqueductal gray nuclei have been implicated in controlling vocalization in mammals (4, 23).

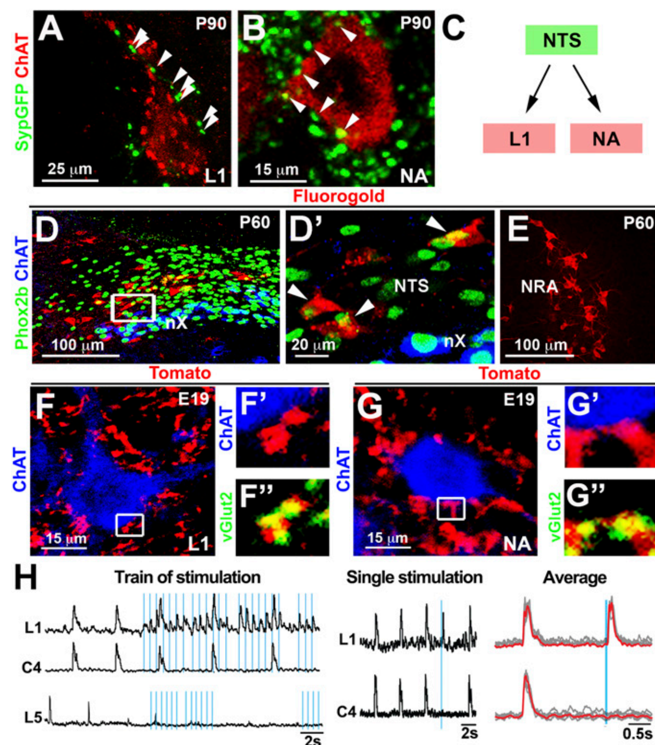

Fig. 4. NTS neurons innervate expiratory and laryngeal motor neurons in newborn mice. (*A* and *B*) Injection of AAV encoding SypGFP into the NTS ($n = 3$) at P60 demonstrating the presence of GFP^+ synaptic boutons (arrowheads) on L1 spinal (*A*) and nucleus ambiguus (NA) (*B*) motor neurons ($ChAT^+$; red; single motor neurons are shown (Fig. S5 *B* and *C*). (*C*) Scheme illustrating that premotor neurons of the NTS connect to laryngeal and expiratory motor neurons. (*D* and *D′*) After injection of Fluorogold into the ventral L1 spinal cord, $Fluorogold^+$ (red; arrowheads) neurons were present in the caudal NTS. Phox2b (green) and ChAT (blue) were used to distinguish $Phox2b^+$ NTS neurons from $Phox2b^+/ChAT^+$ vagal motor nucleus (nX) on transverse sections. *D′* shows a magnification of the boxed area in D. (*E*) $Fluorogold^+$ cells (red) in the nucleus retroambiguus. (*F* and *G*) Intersectional strategy ($Olig3^{creERT2/+};Phox2b^{Flpo/+};Ai65^{-/+}$ mice) to specifically label NTS axons and their synaptic terminals with Tomato fluorescent protein (Fig. S6). $ChAT^+$ (blue) motor neurons at L1 spinal cord levels (*F*) and in the nucleus ambiguus (*G*) display numerous $Tomato^+$ (red)/$vGluT2^+$ (green) contacts. *F′* and *F″* and *G′* and *G″* show magnifications of the boxed areas in *F* and *G*, respectively. Single motor neurons are shown in *F* and *G* (overview provided in Fig. S6 *C* and *D*). (*H*, *Left*) Recordings of L1 (expiratory), C4 (inspiratory), and L5 (locomotion) motor roots in hindbrain-spinal cord preparations ($n = 4$) after trains of light stimulation (blue lines) on $channelrhodopsin^+$ NTS cells. Light triggered only L1 responses, and not C4 or L5 responses. Note that inspiratory (C4) and expiratory motor roots (L1) are rhythmically active and fire synchronously in such preparations. (*H*, *Middle*) An evoked burst of L1 but not C4 motor root activity after a single light stimulation of the NTS. (*H*, *Right*) Superimposition of individual (gray) and average (red) traces of L1 and C4 recordings.

To confirm direct projections of NTS neurons to L1 motor neurons, we injected the retrograde tracer Fluorogold into the ventral L1 spinal cord of adult mice. $Fluorogold^+$ neurons were observed in the caudal part of the NTS (121 ± 7 $Fluorogold^+$ neurons/NTS; $n = 3$), as well as in the nucleus retroambiguus (Fig. 4 *D* and *E*). The majority of $Fluorogold^+$ cells in the NTS were $Phox2b^+$ (76 ± 3.5%; $n = 3$). In contrast, injections at L3 or L5 levels did not label neurons in the NTS. We next tested whether projections of $Phox2b^+$ NTS neurons to T11–L1 motor neurons exist in newborn mice. To achieve this, we used the Ai65 reporter mice, which conditionally express Tomato fluorescent protein on the removal of two stop cassettes flanked by either FRT or LoxP sites (24). The stop cassettes were removed by *cre* recombinase under the control of the *Olig3* (*$Olig3^{creERT2}$*; tamoxifen induction at E10.5) and through expression of *FLPo* recombinase under the control of *Phox2b* (*$Phox2^{FlpO}$*; Fig. S6 *A* and *B*) (25). This demonstrated direct excitatory ($vGluT2^+$) innervation of $Phox2b^+$ NTS neurons to T11–L1 motor neurons (Fig. 4*F* and Fig. S6*C*). Injection of fluorescent cholera toxin subunit B (CTB) into the oblique muscles of newborn mice confirmed that motor neurons controlling abdominal expiratory muscles reside at similar positions (Fig. S6*D*). In addition, excitatory $Tomato^+$ synaptic terminals were observed on motor neurons of the semicompact area and loose formation of the nucleus ambiguus (Fig. 4*G*). Among the additional NTS targets identified in adults, only projections to the thalamus were not present in newborn mice (Fig. S6 *E*–*H*). Thus, NTS projections to brainstem and spinal cord motor neurons are established at birth, whereas projections to the thalamus form postnatally. We conclude that $Phox2b^+$ NTS neurons innervate both spinal motoneurons at T11–L1 levels that control abdominal muscles needed for active expiration and motor neurons of the semicompact area and loose formation of the nucleus ambiguus that innervate the larynx.

Activation of NTS Neurons Suffices to Drive Expiratory and Laryngeal Motor Activity. We next tested whether activation of $Phox2b^+$ NTS neurons entrains expiratory-like activity by targeting channelrhodopsin expression to these neurons (*$Olig3^{creERT2}$;Ai32* mice; tamoxifen treatment at E10.5). Using hindbrain-spinal cord preparations from these mice, recordings were made from L1 and C4 ventral roots, which contain axonal projections of expiratory and inspiratory motor neurons, respectively, or from L5 ventral roots, which contain projections from locomotor neurons. In accordance with previous studies, L1 and C4 roots were coactive in the absence of light in such preparations (Fig. 4*H*) (26). Light-dependent activation of NTS neurons selectively recruited activity of L1, but not of C4 or L5 motor roots (Fig. 4*H*; Fig. S7 *A* and *B* quantifies the latency of the L1 response). This result shows that NTS neurons control the activity of L1 motor neurons already at birth. In addition, we electrically stimulated the solitary tract that innervates NTS neurons and patch-clamped neurons of the nucleus ambiguus in transverse slice preparations (27). In control slices, 28 of 28 motor neurons in the semicompact formation of the nucleus ambiguus responded to a stimulus of the solitary tract with a short latency of excitatory postsynaptic inward current, which is indicative of a direct connection between the NTS and the motor pool (Fig. S7 *C*–*E*). In contrast, the same stimulus failed to evoke synaptic responses in nucleus ambiguus motor neurons (9 of 9) from *Olig3* mutant preparations. Taken together, these findings demonstrate that the NTS contains functional premotor neurons to spinal T11–L1 and nucleus ambiguus motor neurons.

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Lack of Vocalization Impairs Mother–Offspring Interactions. Vocalization of newborn mice elicits search and retrieval behaviors and thus is an important cue for mother–offspring interactions (2). *TxPh-1* and *Olig3* mutants are mute mice, yielding a model for directly testing the function of vocalization in maternal care. Examination of postpartum behaviors showed that vocally impaired (*Olig3*, *Tlx3*, and *TxPh* mutants) mice were licked after birth like control littermates, indicating acceptance by the mothers, but were frequently found outside of the nest. We assessed two behaviors of mother–offspring interaction, search and retrieval. In the first test, we covered individual pups with bedding material from their cages and observed that mothers found vocalizing pups faster than vocally impaired pups, and that many vocally impaired pups were not found within a 30-min period (Fig. S8). In the second test, we placed mixed litters containing vocalizing and nonvocalizing pups outside of the nest, and allowed the mothers to freely interact with the pups. In all cases, the mothers retrieved vocalizing pups and returned them to the nest. In contrast, *Olig3* and *TxPh-1* mutants that did not vocalize were not retrieved, whereas *TxPh-2* and *Tlx3* mutant pups that produced only a few calls were rarely retrieved (Fig. 5). We conclude that USCs in newborn mice are an important cue that elicits maternal care.

Discussion

In this study, we show that the NTS is essential for innate vocalization in mice. When an intersectional genetic strategy was used to prevent the development of Phox2b^{+} NTS neurons, newborn mice were found to be mute, although other vocalization-associated behaviors— mouth openings and click production—were maintained. This mutism has severe consequences for newborn pups, interfering with maternal care. Vocalization relies on coordinated expiratory and laryngeal activity. We show that Phox2b^{+} NTS neurons form functional connections with L1 and nucleus ambiguus motor pools at positions where expiratory and laryngeal motor neurons reside. When Phox2b^{+} NTS neurons are absent, pups can breathe but fail to produce the postinspiratory airway pressure required to elicit calls.

The NTS Is a Vocal Nucleus at Birth. The NTS is one of few sites in the hindbrain containing neurons that have vocalization-correlated activity (28). Our work provides an animal model that selectively lacks Phox2b^{+} NTS neurons and a direct experimental route to assess the consequences of NTS disruption. As expected, ablation of NTS neurons is incompatible with homeostasis in postnatal life, owing to a loss of chemosensory and viscerosensory reflexes linked to breathing and cardiovascular functions (29). For this reason, we examined mutants in the first hours of life, a period when wild-type (WT) pups vocalize most frequently and when ventilation parameters of WT and mutant pups are similar. Our major finding is that complete ablation of Phox2b^{+} NTS neurons causes the elimination of both USCs and ACs.

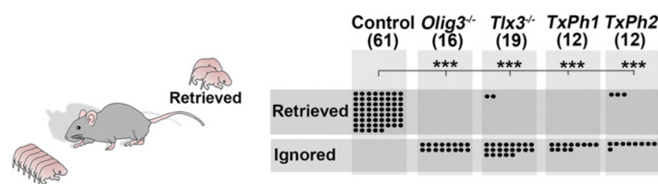

Fig. 5. Vocally impaired pups are neglected by their mothers. (*Left*) Schematic display of retrieval behavior. Control and mutant newborns were placed together outside of the nest; the mother was allowed to move freely in the cage to retrieve the pups. (*Right*) Quantification of retrieved/ignored pups with indicated genotypes. Dots represent individual pups. The number of newborns of each genotype is indicated in brackets. ***$P < 0.0001$.

The NTS receives projections from and projects to the periaqueductal gray, a region in the midbrain in which stimulation elicits calls in many species (30). The periaqueductal gray has been discussed as a gating center for vocal behavior but has no direct connections with motor neurons (7), and as such it depends on other premotor neurons to control vocalization. The nucleus retroambiguus, a loose neuronal cluster located posterior to the nucleus ambiguus, has been proposed to serve this role (23). Our work reveals that the NTS, like the nucleus retroambiguus with which it is reciprocally connected, projects to and entrains expiratory and laryngeal motor neurons. Therefore, the expiratory force and the degree of vocal fold contraction, which determine the subglottal pressure critical for call production, appear to be controlled by a parallel and convergent premotor drive from the NTS and the retroambiguus nucleus. The mutations analyzed in this study disrupt Phox2b^{+} NTS neurons, but not neurons of the nucleus retroambiguus that have no history of *Olig3* or of *Phox2b* expression. Thus, in the absence of the NTS, the nucleus retroambiguus does not suffice for vocalization.

The NTS at the Interface of Breathing and Vocalization Circuits. Our finding that call production and vocal expirations, but not basal expiration, depend on the integrity of the NTS is accounted for by the fact that basal breathing is accompanied by passive expiration. Basal expirations rely on lung recoil after inspiration, whereas vocal breathing relies on active expiration. Innate vocalization and vocal NTS activity are integrated into the respiratory cycle and produced strictly during postinspiratory phases. The parabrachial complex or the postinspiratory complex might provide postinspiratory drive to the NTS (31–33). Vocalization and breathing rely on viscerosensory feedback; in particular, information on airflow, pressure, and expansion of the lungs as well as laryngeal muscle activity is conveyed to the NTS by pulmonary and upper airway afferents, respectively (34, 35). Such feedback modifies vocal behavior and is required for the breathing phase-dependent Hering–Breuer reflexes (36). Thus, the NTS as a nucleus that integrates viscerosensory information and vocal commands is particularly well suited to coordinate vocalization with respiration. We propose that the NTS is an essential component of the vocal circuit that depends on viscerosensory feedback to modulate vocal and respiratory neurons.

Vocalization is used in distinct behavioral contexts in young and adult mice that also differ in their “readiness” to vocalize. In the monkey, two descending pathways control vocalization, one originating in the motor cortex that is used for learned vocalization and the other from the anterior cingulate cortex gating vocalization that acts via the periaqueductal gray (7). Cortical pathways are dispensable for innate vocalizations, however (5, 6). In contrast, the NTS is essential, thus substantiating and refining the notion that vocalization in newborn mice is supported by a “hardwired” circuitry in the brainstem. Elements of this circuitry likely are shared among multiple postinspiratory behaviors, such as swallowing, coughing, and sneezing, that rely on glottis closure (31). In addition, innate vocalizations have been related to nonverbal utterances in humans like laughing, crying, sighing, and moaning, which represent postinspiratory behaviors (37, 38).

Communication Behavior. The USCs of young mice are known to elicit approach and retrieval behavior of their mothers, and even the playback of such calls suffices to trigger an approach (2). We show here that mute newborn mice are not retrieved when placed outside of the nest. It should be noted, however, that deaf mothers are able to provide their pups with maternal care, and thus auditory cues can be replaced by other signals recognized by a deaf mother (39). In the retrieval tests used here, the entire litter of vocalizing and nonvocalizing pups was placed together outside the nest, and the calls of vocalizing littermates provided guidance cues. Thus, the inability of dams to locate nonvocalizing pups is not the

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sole factor responsible for their neglect. We suggest that not only does innate vocalization serve as a guidance cue during searches, but also that calls are mandatory for pup retrieval, indicating that vocalization represents one parameter by which mice assess the fitness of their offspring.

Speech disorders are frequently observed in patients with various neurodegenerative conditions, autistic spectrum disorder, and in rare cases of neurogenic mutism caused by developmental or acquired nervous system damage (40, 41). The identity of the affected brain area(s) or neuronal cell types often remains undefined, however. Our data indicate that damage to the NTS should be considered as a potential cause of speech pathologies.

Materials and Methods

Animals. Experimental procedures and animal handling were conducted according to institutional protocols and guidance approved by the Max Delbrueck Center (Berlin), CNRS (Gif sur Yvette), and Columbia University (New York). Details on mouse strains and plethysmographic, audio, and behavioral analyses are provided in *SI Materials and Methods*.

Histology. The development of dA3 neuronal derivatives and connectivity of NTS neurons were assessed in 20-μm transverse hindbrain and spinal cord sections from control and mutant mice. Details on antibodies, viruses, and retrograde tracers are provided in *SI Materials and Methods*.

Electrophysiology. Patch-clamp and optogenetic studies were performed using 450-μm transverse sections and hindbrain-spinal cord preparations, respectively. The electrophysiological experiments are described in detail in *SI Materials and Methods*.

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