Breathing control center neurons that promote arousal in mice

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Slow, controlled breathing has been used for centuries to promote mental calming, and it is used clinically to suppress excessive arousal such as panic attacks. However, the physiological and neural basis of the relationship between breathing and higher-order brain activity is unknown. We found a neuronal subpopulation in the mouse preBötzinger complex (preBötC), the primary breathing rhythm generator, which regulates the balance between calm and arousal behaviors. Conditional, bilateral genetic ablation of the ~175 Cdh9/Dbx1 double-positive preBötC neurons in adult mice left breathing intact but increased calm behaviors and decreased time in aroused states. These neurons project to, synapse on, and positively regulate noradrenergic neurons in the locus coeruleus, a brain center implicated in attention, arousal, and panic that projects throughout the brain.

Although breathing is commonly viewed as a simple autonomous function that sustains life, it has long been known to influence higher-order behavior and thinking (1). Slow, controlled breathing is used by practitioners of pranayama yoga and other forms of meditation to promote mental calming and contemplative states, and it is used clinically to suppress excessive arousal and stress such as certain types of panic attacks (2, 3). Although the effect of breathing on behavior and mental state could easily be indirect, there could also be more direct connections and impact of the breathing center on higher-order brain function (4), as demonstrated here.

The preBötzheimer complex (preBötC) is a cluster of several thousand neurons in the ventrolateral medulla of the murine brain that can autonomously generate respiratory rhythms in explanted brain slices (5, 6), and whose rhythmic activity in vivo initiates breathing by recurrently activating premotor and motor neurons of the respiratory muscles (5). The preBötC is not a homogeneous population of neurons but is composed of distinct, though intermingled, neuronal subpopulations (5, 7), one of which is essential for respiratory-rhythm generation (5, 7) and another for sighing (10).

To systematically explore the molecular diversity of breathing center neurons, we screened expression patterns of over 19,000 genes in the Euroexpress embryonic day 14.5 (E14.5) mouse hindbrain database (II). Cadherin-9 (Cdh9) was the gene most selectively expressed in preBötC (Fig. 1A). We constructed a bacterial artificial chromosome (BAC) transgene with the mOrange coding sequence inserted at the Cdh9 translation start codon (Fig. 1B). Cell counts in early postnatal brains detected 319 ± 130 (n = 6) Cdh9-mOrange–expressing cells in preBötC (Fig. 1C). These cells intermingled with neurons expressing canonical preBötC markers somatostatin (SST) and neurokinin 1 receptor (NKIR) (Fig. 1, D and E, and fig. S1, A to E). Few expressed the markers themselves: 0 out of 43 Cdh9-mOrange+ cells scored as SST+, and 7 out of 179 Cdh9-mOrange+ (4%) were NKIR+. All Cdh9-mOrange–expressing cells coexpressed neuronal marker NEUN (n = 61 cells, Fig. 1F). These Cdh9-mOrange–expressing neurons can be further divided into seven subtypes based on differential expression of transcription factors Pax2, Dach1, Lmo4, Evc1, and Ddx1. We focused on the ~175 neurons in each preBötC (fig. S1, F to J; ~350 neurons bilaterally) that coexpress the Ddx1-lineage marker, the major subpopulation (56%, 165 out of 292 scored Cdh9-mOrange+ cells) that we call Cdh9/Ddx1 neurons (Fig. 1, G to I).

We electrophysiologically recorded 36 mOrange–positive neurons in 15 preBötC slice preparations from Cdh9-mOrange/dbx1-LOSL-DTR;Dbx1-lacZ double-transgenic postnatal day 0 to 5 (P0 to P5) mice, then stained the neurons for β-galactosidase (LacZ) in some preparations to identify recordings of Cdh9/Ddx1 neurons (table S1). We definitively identified five Cdh9/Ddx1 neurons. One showed bursts of action potentials just before or during each preBötC inspiratory burst (Fig. 1J and fig. S2), like most other Ddx1-lineage preBötC neurons (12). Three other neurons were more broadly active with bursts during some, but not all, preBötC inspiratory bursts (42, 41, and 88% of inspiratory bursts; Fig. 1K and fig. S2), called an “inspiratory-associated” activity pattern. The other neuron showed sporadic activity with no apparent relationship to preBötC inspiratory bursts. Seven of the 15 Cdh9-mOrange neurons whose Ddx1-lacZ expression status was not determined also displayed inspiratory (four neurons) or inspiratory-associated (two neurons) patterns (table S1).

We used intersectional genetics (Cdh9-LOSL-DTR;Ddx1-cre) (Fig. 1, B, M, and N) to express human diphtheria toxin receptor (DTR) only in Ddx1-lineage cells that coexpress Cdh9, so that Cdh9/Ddx1 neurons could be specifically ablated by intraperitoneal injection of diphtheria toxin. We expected there would be few, if any, cells besides Cdh9/Ddx1 preBötC neurons that express both genes (11, 13). We examined this in two ways. First, we compared mOrange expression in Cdh9-LOSL-DTR and Cdh9-LOSL-DTR;Ddx1-cre transgenic mice by immunostaining serial sections of adult brains. The only regions where Ddx1-cre reduced the number of mOrange-expressing cells were preBötC and inferior colliculus (fig. S3). Co-staining with other markers showed that loss of mOrange in preBötC was specific and complete for Cdh9/Ddx1 neurons and had little or no effect on the six other (Ddx1-negative) Cdh9-positive preBötC cell types (fig. S4). Second, immunostaining of serial sections of Cdh9-LOSL-DTR;Ddx1-cre adult brains detected DTR expression only in Cdh9/Ddx1 preBötC neurons (Fig. 1M) and the inferior colliculus (fig. S3). Intraperitoneal injection of diphtheria toxin once a day for 3 days eliminated the DTR-expressing cells (Fig. 1N).

We analyzed adult mice several days after ablating Cdh9/Ddx1 neurons. We expected Cdh9/Ddx1 neurons would be essential for breathing and viability because Ddx1 neurons are essential for breathing in vivo (5, 8) and ablating just 85 random Ddx1 neurons abolishes preBötC rhythms in vitro (14). However, there was no overt effect on viability (three of three scored mice alive >1 year after ablation), breathing, or sensory and motor behaviors. Plethysmography of freely moving adult mice after ablation did not detect significant differences in inspiratory time, expiratory time, or tidal volume of standard (eupneic) breaths (figs. S5, B to E, and S6) or breaths during sleep or under hypercapnic or hypoxic conditions (figs. S5, F to H, and S6).

There was, however, a change in abundance of different breath types, first noted in respiratory-rate histograms: Cdh9/Ddx1 ablation shifted the distribution toward slower breaths (low respiratory rate) (Fig. 2B). Inspection of plethysmograph traces indicated that the shift was due to increased slow breaths (eupneic and grooming) associated with calm behaviors and reduction in rapid breaths associated with sniffling and other active behaviors (Fig. 2A and fig. S7).
The change in breathing patterns after Cdh9/Dbx1 neural ablation was accompanied by a corresponding change in behavior. Ablation reduced exploration of a new environment (97 ± 9% versus 62 ± 20% time spent in active exploration, pre- versus postablation; P = 0.02) and increased time engaged in grooming threefold (10 ± 7% versus 31 ± 16%; P = 0.02) and still-sitting twofold (3 ± 4% versus 7 ± 7%; P = 0.07), although the latter did not reach statistical significance (Fig. 2C). There was an increase in both number of calm episodes (Fig. 2D) and their duration (Fig. 2E and movie S1). Because the breathing pattern associated with each behavioral state was not detectably altered by ablation (figs. S5 and S6), the observed change in respiratory-rate distribution could be explained by the overall change in behavior (fig. S8): a shift from active toward calm behaviors. Electroencephalographic (EEG) monitoring showed an increase in slow-wave (delta, 2 to 4 Hz) brain activity after ablation (Fig. 2F) and a selective decline in time spent in an active-brain state dominated by theta activity (Fig. 2G) compared to that of littermate controls (Fig. 2, H and I). These changes could be temporarily reversed by illuminating the chamber, providing a stimulus that apparently over-rides the decrease in arousal caused by Cdh9/Dbx1 ablation (fig. S9).

To confirm that the observed behavioral and breathing changes were due to ablation of neurons in the preBötC, we restricted DTR induction and hence neural ablation to just Cdh9-expressing preBötC neurons (fig. S10, A to C). The animals displayed a diminution in active exploratory behaviors and breathing patterns and an increase in calm behaviors and breathing patterns similar to animals with Cdh9/Dbx1 neurons ablated using our intersectional genetic strategy (compare figs. S10, D and E, and Fig. 2).

The decrease in active behavior and increase in EEG delta waves observed after Cdh9/Dbx1 neuron ablation is reminiscent of changes following silencing or ablation of the locus coeruleus (LC), a noradrenergic nucleus in the pons implicated in generalized arousal, stress, and sleep-wake transitions (35). We thus microinjected two retrograde tracers into the LC and found that both labeled Cdh9-mOrange neurons in preBötC (FluorGold: fig. S11, A to C; fluorescent retrograde beads: data not shown). Most of the labeled Cdh9-mOrange preBötC neurons (85%, 23 out of 27, n = 40 sections, 3 mice) were contralateral to the injection site. The connection is selective because most labeled preBötC neurons expressed Cdh9-mOrange (72%, 13 out of 18, n = 10 sections, 4 mice), and no retrograde labeling of Cdh9-mOrange neurons was observed following tracer injection into regions surrounding the LC (n = 4 injections).
We tested whether the observed connection between the preBöC neurons and LC is direct to the noradrenergic (dopamine β-hydroxylase (Dbh)-expressing) neurons that dominate the LC (15). Injection of Cre-dependent adeno-associated virus (AAV) helper viruses (AAV-FLEXloxP-TVA; mCherry and AAV-FLEXloxP-rabies glycoprotein), which enables infection and monosynaptic spread of an envA-pseudotyped, glycoprotein-deleted, and GFP-expressing rabies virus (RVdG) (16), into the LC of Dbh-cre;Cdh9-mOrange mice resulted in specific infection of LC (Fig. 2, B and C). Distribution of respiratory rates (bin size 1 Hz) in 40-min assay of control (wild-type, Cdh9-LOSL-DTR; Dbx1-cre; black, n = 5) and experimental (Cdh9-LOSL-DTR; Dbx1-cre; red, n = 5) animals before (dashed lines) and 2 days after (solid lines) Cdh9-Dbx1 ablation. Percent of time in plethysmography chamber spent still-sitting (black), grooming (gray), or active (white) by control (n = 6) or experimental (n = 6) mice before (pre) or 2 days after (post) ablation or mock ablation. Values comparing pre- and postablation behavior: active (0.02), grooming (0.02), and still-sitting (0.07). After ablation, active episodes shortened (P = 0.005), grooming and still-sitting showed nonsignificant trend to lengthening (P = 0.24 and 0.21, respectively). ECoG power spectral analysis [average (solid lines) ± SEM] of 20-min recording of 1 Cdh9-LOSL-DTR;Dbx1-cre [(F), n = 5] or control Cdh9-LOSL-DTR [(H), n = 4] mice before (black) or 4 to 10 days after (red) ablation. Grooming events in new chamber of Cdh9-LOSL-DTR;Dbx1-cre mice before (black) or after (red) ablation. Solid lines, individual mice (n = 6); dashed lines, average. Duration of behaviors in (C) (mean ± SD, n = 6). After ablation, active episodes shortened (P = 0.005), grooming and still-sitting showed nonsignificant trend to lengthening (P = 0.24 and 0.21, respectively). (F) and (H) ECoG power spectral analysis [average (solid lines) ± SEM] of 20-min recording of 1 Cdh9-LOSL-DTR;Dbx1-cre [(F), n = 5] or control Cdh9-LOSL-DTR [(H), n = 4] mice before (black) or 4 to 10 days after (red) ablation. δ, delta wave; V. voltage. Active behavior correlates with faster breathing (fig. S15, C to E). (G and I) Time spent in active (solid black line, mean ± SEM) and calm (dashed black line) behavioral states defined by electromyography (EMG) and ECoG (fig. S15) of individual animals in (F) and (H) (gray lines) during two 20-min assays pre- and post-Cdh9-Dbx1 ablation. Note decreased active and increased calm periods following ablation in experimental animals (P = 0.001 and 0.02, respectively, paired t test) and no change in controls (P = 0.86 and 0.81, respectively).
However, 4 days after ablation, c-FOS was induced in scattered cells throughout the LC (Fig. 4, B and D, and fig. S14). Neuronal subtype in the preBötC comprising ~175 that other LC inputs and functions were intact. Stress (extreme arousal stimulus of physical-restraint of ablated animals remained responsive to the new chamber. We have identified and characterized a new neuronal subtype in the preBötC comprising ~175 neurons projecting to the LC neu-rons, which could provide excitatory input to the LC and appear to be the dominant activating input under mild arousal conditions of placement in a new chamber. We propose that Cdh9/Dbx1 preBötC neurons function as gateway neurons directly linking the preBötC to the locus coeruleus, and through it to the rest of the brain (Fig. 4G). This ascending circuit allows the respiratory center to communicate directly with and control higher-order brain structures associated with behavioral arousal. The excitatory input to the LC is presumably provided by the observed inspiratory-associated activity pat-
tterns of monosynaptic projection (red line) from Cdh9-expressing preBötC neurons (red circle) to contralateral LC, which projects there (red), Cre induces DTR expression, and DT injection induces ablation. (F and G) preBötC Cdh9-mOrange expression (white) in control uninjected ([F], mock ablation) and CAV-Cre injected ([G], ablation) Cdh9-LOSL-DTR mice 2 days after DT injection. Scale bar, 50 μm. Quantification showed 32% (mean) and 50% (maximal) reduction in mOrange neurons (n = 15 sections), close to the value expected if all Cdh9/Dbx1 preBötC neurons (50% of Cdh9-expressing neurons) project to LC. (H) Distribution of respiratory rates in 40-min assay (as in Fig. 2B) of CAV-Cre injected Cdh9-LOSL-DTR adult mice (red, n = 7) or wild-type littermates (black, n = 4) before (dashed) and 2 days after (solid) DT injection. (I) Behavioral analysis (as in Fig. 2C) of mice in (H). Pre-versus post-ablation P values: active (0.015), grooming (0.37), and still-sitting (0.015). The increased calm events in preablation experimental versus control mice was reproducible: it may be due to toxicity of DTR induced in adult neurons, which is not observed in Cdh9-LOSL-DTR;Dbh-cre mice when DTR is expressed in early development, perhaps due to developmental compensation. (Fig. 4H). This respiratory corollary signal would thus serve to coordinate the animal’s state of arousal with the breathing pattern, leaving the animal calm and relaxed when breathing is slow and regular, but promoting (or maintaining) arousal when breathing is rapid or disturbed. This circuit and corollary signal would explain why preBötC respiratory patterns have been observed in the LC and other reticular activating structures (19–21). The LC can increase respiratory rate (22), so there may also be a positive feedback loop from the LC ultimately back to Cdh9/Dbx1 preBötC neurons. Cdh9/Dbx1 circuit may have evolved as a defense response, mobilizing the animal in the face of rapid, irregular, or labored breathing. Indeed, fast or erratic breathing in humans increases alertness and can cause anxiety and even panic (5), and likewise increased preBötC activity, hyper-ventilation, and sighs appear to induce arousal during sleep (23–25). Conversely, slow and controlled breathing has long been known by practitioners of
pranayama yoga to induce relaxation, and related approaches have proven useful for anxiety syndromes and other stress disorders (1, 2). If the Cdh9 Dbx1 circuit is conserved in humans, it could provide a therapeutic target for breathing-related anxiety disorders and perhaps prevention of sudden infant death syndrome (SIDS), widely hypothesized to result from an inadequate arousal response to asphyxiation during sleep (24).

Notably, panic attacks triggered by respiratory symptoms are specifically responsive to clonidine, an α2-adrenergic agonist that silences LC (25).

Although breathing is generally thought of as an autonomic behavior, higher-order brain functions can exert exquisite control over breathing. Our results show, conversely, that the breathing center has a direct and powerful influence on higher-order brain function. It will thus be important to map the full range of behaviors and functions the breathing center controls.

**REFERENCES AND NOTES**


**ACKNOWLEDGMENTS**

K.Y. performed in situ screens; generated and characterized Cdh9 transgene; characterized Cdh9 Dbx1 neuronal ablation; and performed AAV-Cre, FloreGold, and retrograde bead injections and c-Fos experiments. I.A.S. and L.L. provided the reagents and i.A.S. injected rabies and AAV-Cre viruses; K.K. and L.L. provided the reagents and K.K. performed and analyzed slice electrophysiology. J.M.S. and J.R.H. provided the reagents and J.M.S. performed and analyzed ECoG recording. K.Y. analyzed all data. K.Y. and M.A.K. conceived experiments, interpreted data, and wrote the manuscript. All authors edited the manuscript. We thank X. Chen and G. Nachtrieb for assistance and reagents for AAV-Cre injection. J. Zeltzer for assistance with ECoG analysis, and members of the Krasnow lab for helpful comments. This work was supported by the Howard Hughes Medical Institute (M.A.K. and L.L.), NIH grants HL70029 and HL04995 (J.L.F.), and the NIH Medical Scientist Training Program (K.Y.). M.A.K. and L.L. are investigators of the Howard Hughes Medical Institute. Data are curated and stored in the Krasnow lab at the Howard Hughes Medical Institute, Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305, USA.

**SUPPLEMENTARY MATERIALS**

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**REFERENCES**


**MOVIE S1**

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Science 355 (6332), 1411-1415. [doi: 10.1126/science.aai7984]

Editor's Summary

The calming effect of breathing

The rhythmic activity of a cluster of neurons in the brainstem initiates breathing. This cluster is composed of distinct, though intermingled, subgroups of neurons. Yackle et al. found a small, molecularly defined neuronal subpopulation in this breathing rhythm generator that directly projects to a brain center that plays a key role in generalized alertness, attention, and stress (see the Perspective by Sheikbahaei and Smith). Removal of these cells did not affect normal breathing but left the animals unusually calm. The breathing center thus has a direct and dramatic influence on higher-order brain function.

Science, this issue p. 1411; see also p. 1370