

Silencing preBötzing Complex somatostatin-expressing neurons induces persistent apnea in awake rat

Wenbin Tan^{1,3}, Wiktor A Janczewski^{1,3}, Paul Yang¹, Xuesi M Shao¹, Edward M Callaway² & Jack L Feldman¹

Delineating neurons that underlie complex behaviors is of fundamental interest. Using adeno-associated virus 2, we expressed the *Drosophila* allatostatin receptor in somatostatin (Sst)-expressing neurons in the preBötzing Complex (preBötC). Rapid silencing of these neurons in awake rats induced a persistent apnea without any respiratory movements to rescue their breathing. We hypothesize that breathing requires preBötC Sst neurons and that their sudden depression can lead to serious, even fatal, respiratory failure.

The preBötC in the ventrolateral medulla is hypothesized to be a kernel for the generation of respiratory rhythm *in vitro* and *in vivo*^{1–4}. Adult rats with slow (~days), toxin-induced neurodegeneration of >80% of neurokinin 1 receptor (NK1R)-expressing preBötC neurons survive with an ataxic rhythm during wakefulness and apnea during sleep^{1,5}. Whether this pathological breathing pattern is driven by neurons that normally control respiratory-related muscles, including those underlying volitional or emotional behaviors, or by a compensatory reorganization in response to the slow neurodegeneration is unknown. To eliminate adaptation resulting from slow lesions, we rapidly (~minutes) decreased excitability in a glutamatergic subpopulation of preBötC neurons that express Sst⁶. This population overlaps with neurons expressing NK1R; 28 ± 2% of preBötC Sst neurons expressed NK1R and 41 ± 1% of preBötC NK1R neurons expressed Sst (5–6-week-old rats, *n* = 3; **Supplementary Fig. 1** online), consistent with their overlap in neonatal rat preBötC⁷. We hypothesized that preBötC Sst neurons are essential for normal breathing and predicted that silencing these neurons would cause acute apnea in awake adult rats; as hypoxia and hypercapnea worsened with apnea, we presumed that other mechanisms, particularly in relation to volitional or emotional drives, would restore breathing, as appears to be the case in central congenital hypoventilation syndrome⁸. Rapid, reversible silencing of genotypic neuronal subpopulations can illuminate their role in behavior. This can be done by targeted expression of allatostatin receptor (AlstR), a G protein-coupled receptor that is neither expressed nor activated by any endogenous ligand in mammals^{9–11}. Mammalian cortical and spinal cord neurons that are made to express AlstR can

be rapidly and reversibly inactivated *in vitro*^{12,13} and *in vivo* under anesthesia¹⁰ by administration of allatostatin, which opens K⁺ channels^{9,13} to hyperpolarize them. We expressed AlstR and enhanced green fluorescent protein (EGFP) in targeted preBötC neurons and studied the effect of allatostatin application on breathing in adult rats.

To obtain stable and reliable expression of exogenous genes in adult rat preBötC neurons, we used adeno-associated virus 2 (AAV2) to ensure high infection efficiency and long-term expression with low cytotoxicity or inflammation^{10,14}. First, we describe the infection profile using AAV2 with the synapsin promoter to drive EGFP expression (Syn-EGFP; **Supplementary Methods** online). In 3 weeks after preBötC injection of Syn-EGFP¹⁰, EGFP could be detected in neuronal somas up to ~250 μm from the injection site. There were very few EGFP-positive cells in adjacent regions (for example, the Bötzing Complex; **Fig. 1a**). In contrast with the wide expression that is inherent in transgenic mouse models (for example, see ref. 12), these viral microinjections produced site-specific expression of target genes that was highly localized in three dimensions around the injection site, as we did not find any EGFP expression elsewhere in the brainstem, spinal cord or cerebellum (**Supplementary Table 1** online).

To modulate preBötC neuronal excitability *in vivo*, we made an AAV2 construct using the synapsin promoter to drive AlstR cDNA with an internal ribosome entry site linked to the EGFP gene (Syn-AlstR-EGFP)¹⁰. EGFP was detectable 4 weeks after viral injection in the preBötC. Almost all of the EGFP-positive cells (99 ± 1%, *n* = 7 rats) were immunoreactive for NeuN, a neuronal specific marker (**Fig. 1b**), and represented 64 ± 8% of all the neurons in 100 μm and 20 ± 2% of all the neurons in 250 μm of the injection site (*n* = 7). This pattern of viral infection probably depends on genotypic differences in the infectious preference of AAV2 in various subpopulations of preBötC neurons and decreased probability of infection with distance from the injection site. We found that 21 ± 4% of the preBötC neurons that expressed NK1R also expressed EGFP, and 10 ± 2% of the neurons that expressed EGFP were also immunoreactive for NK1R (*n* = 9; **Fig. 1c**). Of the preBötC neurons that expressed Sst, 20 ± 4% also expressed EGFP, and 15 ± 4% of the neurons that expressed EGF were also Sst positive (*n* = 3; **Supplementary Fig. 2** and **Supplementary Table 2** online). Neurons that were immunoreactive for tyrosine hydroxylase, adjacent to the preBötC^{2,6}, were poorly infected; only 2 ± 1% of EGFP neurons were immunoreactive for tyrosine hydroxylase and <5% of the neurons that were immunoreactive for tyrosine hydroxylase (4 ± 3%; *n* = 7) expressed EGFP (**Supplementary Fig. 2**).

The synapsin promoter is not selective for Sst neurons. To specifically target these neurons, we replaced the synapsin promoter with the mouse Sst promoter in the AAV2 vectors (**Supplementary Fig. 3** online). EGFP was exclusively expressed in 465 ± 26 preBötC neurons on each side (*n* = 6) 3 weeks postinjection of Sst-EGFP (**Fig. 1d**) or 6 weeks postinjection of Sst-AlstR-EGFP (**Supplementary Fig. 3**).

¹Department of Neurobiology, Box 951763, David Geffen School of Medicine at University of California Los Angeles, 650 Charles E. Young Dr. South, Los Angeles, California 90095, USA. ²System Neurobiology Laboratories, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, California 92037, USA. ³These authors contributed equally to this work. Correspondence should be addressed to J.L.F. (feldman@ucla.edu).

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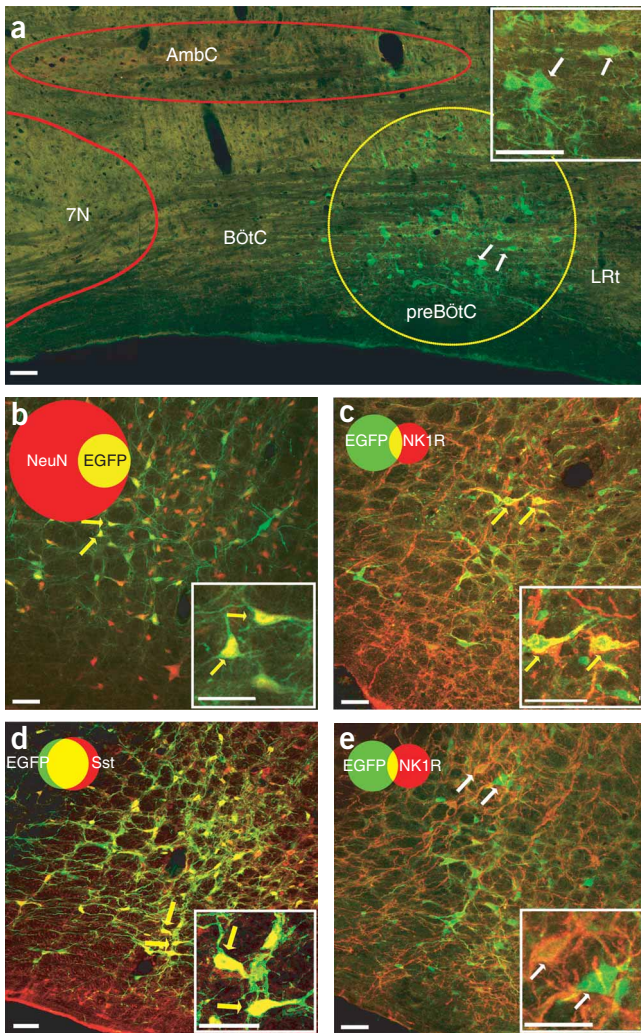


Figure 1 preBötC neurons infected by AAV2 containing a Syn or Sst promoter. (a) EGFP expression (green) in adult rat preBötC (yellow circle) 3 weeks after microinjection of Syn-EGFP AAV2. 7N, facial nucleus; AmbC, ambiguous nucleus compact; BötC, Bötzing Complex; LRt, Lateral reticular nucleus. (b) Syn-AlstR-EGFP AAV2-infected preBötC cells (green) were NeuN immunoreactive (red); that is, they were neurons. (c) Some Syn-AlstR-EGFP AAV2-infected preBötC neurons (green) were NK1R immunoreactive (red). (d) Sst-EGFP AAV2-infected preBötC neurons (green) were Sst immunoreactive (red). (e) Small portion of Sst-AlstR-EGFP AAV2-infected preBötC neurons (green) were NK1R immunoreactive (red). Images were obtained using a confocal microscope. Insets are high magnification of indicated neurons. Venn diagrams represent the relative number of neurons and the overlap of the relevant markers (b–e) normalized to NeuN immunoreactivity ($n \approx 3,000$ neurons on each side of the preBötC). Yellow arrows represent neurons coexpressing and white arrows represent neurons not coexpressing the relevant markers. Scale bars represent 50 μm .

hyperventilation in 20 s of allatostatin application ($P < 0.05$; **Supplementary Fig. 5** online). Administration of allatostatin into Syn-EGFP-injected anesthetized rats did not change their breathing patterns (**Supplementary Fig. 4**).

Could viral expression of AlstR in preBötC neurons change the breathing pattern in behaving adult rats? When we administered allatostatin to awake rats without viral injection ($n = 4$; **Supplementary Fig. 6** online) or in rats 4–6 weeks postinjection of Syn-EGFP into the preBötC ($n = 4$; **Supplementary Fig. 7** online), we did not observe a substantial change in breathing. In contrast, similar administration of allatostatin to awake rats 4–6 weeks postinjection of Syn-AlstR-EGFP into the preBötC resulted in a transient (~ 1 min) increase in 30 s of both amplitude ($214 \pm 89\%$) and frequency ($157 \pm 43\%$; **Supplementary Fig. 7**), followed by a gradual decrease of both. At 90 s, breathing was back to baseline (amplitude, $117 \pm 53\%$; frequency, $106 \pm 29\%$). Between 2 and 2.5 min, amplitude and frequency decreased to 50% of baseline. At 4.5 min, these awake rats became apneic (**Supplementary Fig. 7**). Despite being awake, the rats did not breathe, even as their uninterrupted apnea progressed to severe hypoxia (indicated by bluish skin); after 1.5 min of apnea they were mechanically ventilated to prevent asphyxiation. A lower dose of allatostatin ($0.34 \mu\text{mol}$ per kg of body weight) affected amplitude ($66 \pm 17\%$ versus $93 \pm 6\%$, $P < 0.05$, $n = 6$, **Supplementary Fig. 8** online), but had little effect on frequency ($P = 0.084$) and did not lead to apnea; lowering the dose further had no effect (**Supplementary Fig. 8**). Heart rate was elevated during the early transient hyperventilation period, but was minimally affected just before the onset of apnea (**Supplementary Fig. 9** online), indicating that induction of apnea was not secondary to cardiovascular changes. Thus, rapid silencing of a heterogeneous subset of preBötC neurons can produce persistent apnea.

Next we tested whether silencing Sst neurons could cause apnea. We microinjected Sst-AlstR-EGFP (**Supplementary Fig. 3**) into adult rat preBötC. Subsequent (~ 6 –8 weeks) administration of allatostatin to these rats during anesthesia produced similar respiratory depression compared to Syn-AlstR-EGFP-injected anesthetized rats, but took slightly longer (~ 4 min) to progress to prolonged (~ 60 min) apnea ($n = 4$; **Fig. 2a–c**). Two anesthetized rats showed a significant transient hyperventilation in 20 s of allatostatin application ($P < 0.0001$, **Supplementary Fig. 5**). Similar administration of allatostatin to awake rats 6–8 weeks postinjection produced a transient hyperventilation (amplitude, $150 \pm 20\%$; frequency, $215 \pm 54\%$) that lasted ~ 3 min before ventilation decreased markedly, progressing at ~ 5 min to apnea ($n = 6$; **Fig. 2d–f**). Despite being awake, the rats did not breathe and they required mechanical ventilation to prevent asphyxiation. Sst-EGFP-injected anesthetized ($n = 4$) or awake ($n = 4$) rats did

We found that $66 \pm 7\%$ of neurons in 100 μm and $16 \pm 1\%$ of neurons in 250 μm of the injection site expressed EGFP ($n = 6$; **Supplementary Fig. 3**), and that $81 \pm 8\%$ of preBötC Sst neurons expressed EGFP and $80 \pm 6\%$ of preBötC EGFP neurons were Sst positive ($n = 4$; **Supplementary Fig. 3** and **Supplementary Table 2**). Some preBötC NK1R neurons ($16 \pm 5\%$, $n = 5$) were EGFP positive and $11 \pm 3\%$ ($n = 5$) of EGFP neurons were immunoreactive for NK1R (**Fig. 1e**); no neurons were immunoreactive for tyrosine hydroxylase ($n = 6$; **Supplementary Fig. 3**).

Could activation of AlstR in infected preBötC neurons change the breathing pattern in anesthetized rats where volitional and emotional drives to breathe are suppressed? We examined the effects on breathing of inactivating larger numbers of neurons (590 ± 62 per side) using Syn-AlstR-EGFP AAV2. Allatostatin was applied in anesthetized rats 4–6 weeks postinjection of Syn-AlstR-EGFP into the preBötC. Tidal volume (that is, peak inspiratory amplitude) tended to decrease in 30 s, dropping to 50% at ~ 90 s, whereas frequency tended to decrease in ~ 60 s, dropping to 50% at 2 min with apnea onset at ~ 2.5 min ($n = 5$; **Supplementary Fig. 4** online). After 1.5 min of apnea, rats were mechanically ventilated. The ventilator was stopped for 1 min (or less if cardiac arrhythmia was noticed) every 10 min to check for spontaneous breathing, but no breathing was observed before 40 min, at which point the rats started to breathe ($n = 5$; **Supplementary Fig. 4**). We observed that 3 out of 5 rats showed a significant transient

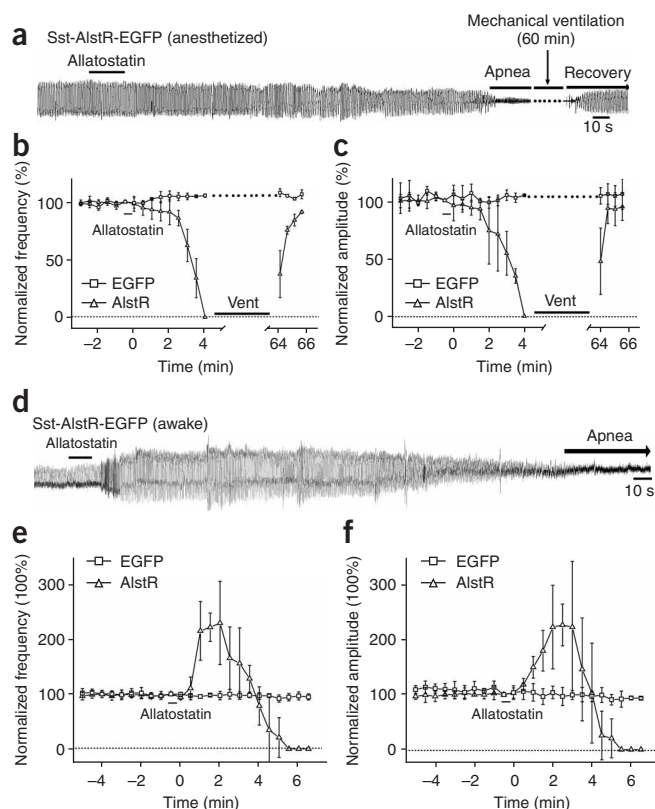


Figure 2 Rapid silencing of AlstR-expressing preBötC Sst neurons induced apnea in anesthetized and awake rats. Allatostatin was administered ($0.42 \mu\text{mol per kg}$, intracerebellomedullary cisternally) after prior (6–8 week) bilateral preBötC microinjection of Sst-AlstR-EGFP. **(a)** Changes in the breathing pattern (airflow) of anesthetized rat on allatostatin application; mechanical ventilation was used for the duration of apnea (~ 60 min). **(b,c)** Allatostatin administration induced a gradual decline of both the frequency **(b)** and tidal volume **(c)** until apnea developed at ~ 4 min post-allatostatin application. After mechanical ventilation, the rats resumed spontaneous breathing ($n = 4$). It should be noted that, in $n = 2$ out of 4 anesthetized Sst-AlstR-EGFP rats, there was a small, but significant, transient increase in ventilation in ~ 20 s after allatostatin administration (**Supplementary Fig. 5**) that is not apparent in group data **(b and c)**. **(d)** Changes in the breathing pattern (tidal volume) of awake rats on allatostatin application. **(e,f)** Changes in the normalized frequency **(e)** and amplitude **(f)** on allatostatin application were plotted. Values are mean \pm s.d. (Sst-AlstR-EGFP, $n = 6$; Sst-EGFP, $n = 4$). Vent, mechanical ventilation. Error bars represent \pm s.d.

neurons was causal to the induction of rapid onset persistent apnea and that these neurons are important for the moment-to-moment generation of respiratory rhythm underlying the regulation of blood gases and pH, in particular, in awake mammals. The inability of awake rats to ventilate at all in the face of relentlessly progressive hypoxia was notable, suggesting that other mechanisms that could drive breathing, such as may be associated with voluntary and/or emotional breathing control, were absent or blocked in these conditions.

Note: Supplementary information is available on the Nature Neuroscience website.

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not show any substantial respiratory depression after allatostatin application (**Fig. 2b,c,e,f**). Thus, selective and rapid silencing of preBötC Sst neurons induces a persistent apnea in awake adult rats.

The preBötC is heterogeneous, consisting of several phenotypically distinct subpopulations of neurons, some overlapping, such as NK1R- and Sst-expressing neurons, and some exclusive, such as glutamatergic and glycinergic neurons. Perturbations of either the NK1R- or Sst-expressing subpopulations substantially disrupt normal rhythmogenesis. We cannot yet say whether this is because they have particular distinct or shared functional roles, or because they are part of a larger group of preBötC neurons that cannot generate rhythm when some threshold percentage of neurons are silenced or lesioned, as suggested by results in rats infected with Syn-AlstR AAV2 (where only a small fraction of infected neurons expressed Sst or NK1R). Regardless, essential neuronal circuitry underlying rhythmogenesis is in the preBötC.

We assume that our approach was successful in silencing preBötC neurons, but confirmation of this will require electrophysiological recordings. We suggest that the direct inhibition of preBötC Sst

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Silencing of preBötzinger Complex somatostatin–expressing neurons induces persistent apnea in awake rats

Wenbin Tan^{1*}, Wiktor A. Janczewski^{1*}, Paul Yang¹, Xuesi M. Shao¹, Edward M. Callaway², and Jack L. Feldman^{1#}

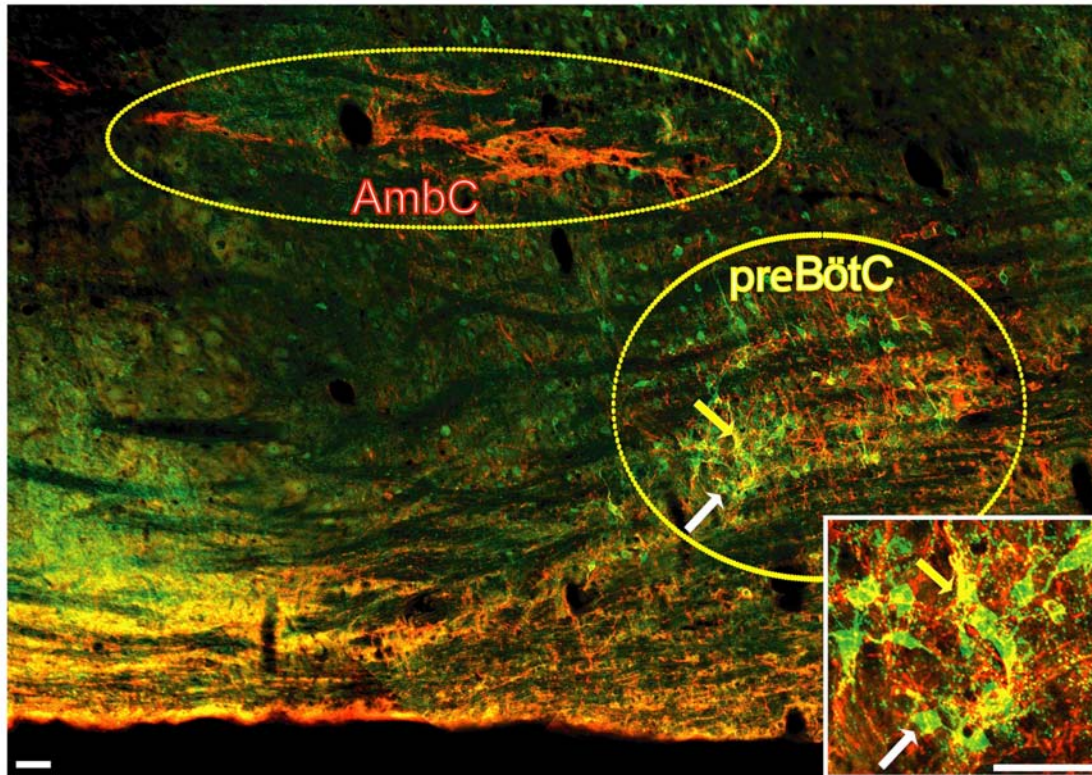
¹Department of Neurobiology, Box 951763, David Geffen School of Medicine at University of California, Los Angeles, 650 Charles Young Dr. South, Los Angeles, CA 90095, USA

²System Neurobiology Laboratories, The Salk Institute for Biology Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA

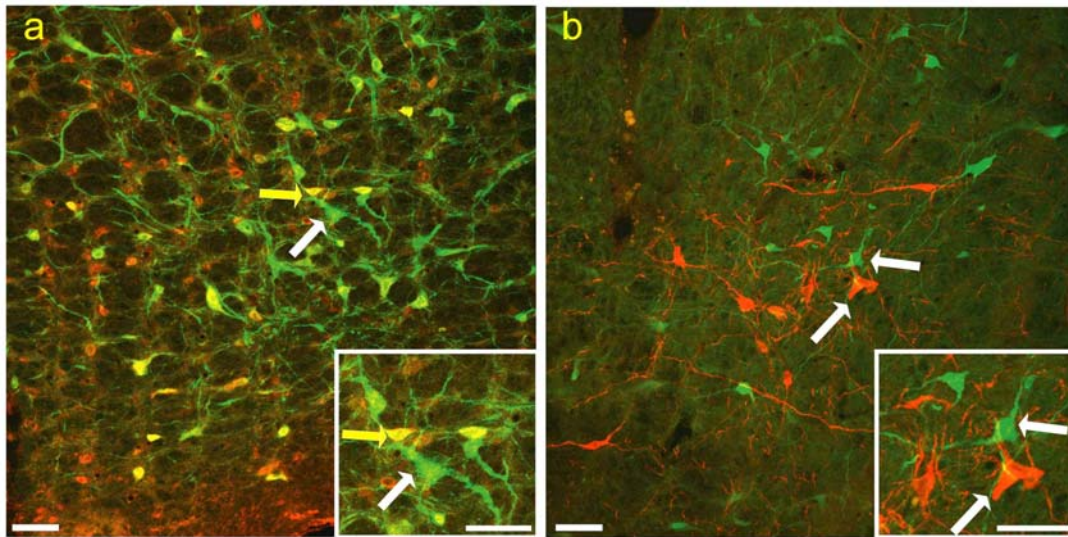
*These authors contributed equally to this work.

[#]To whom correspondence should be addressed: E-mail: feldman@ucla.edu

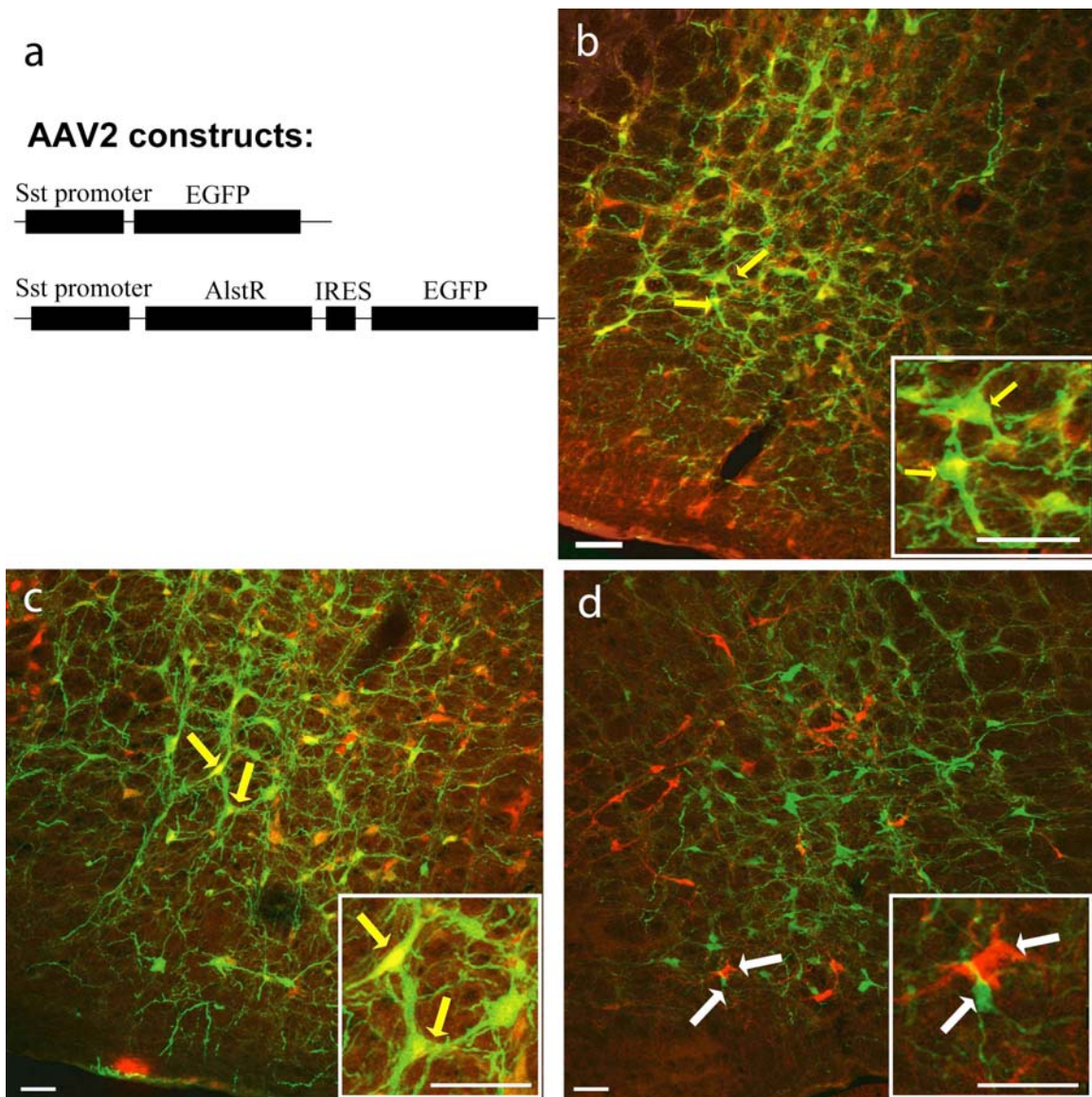
Supplementary Figures



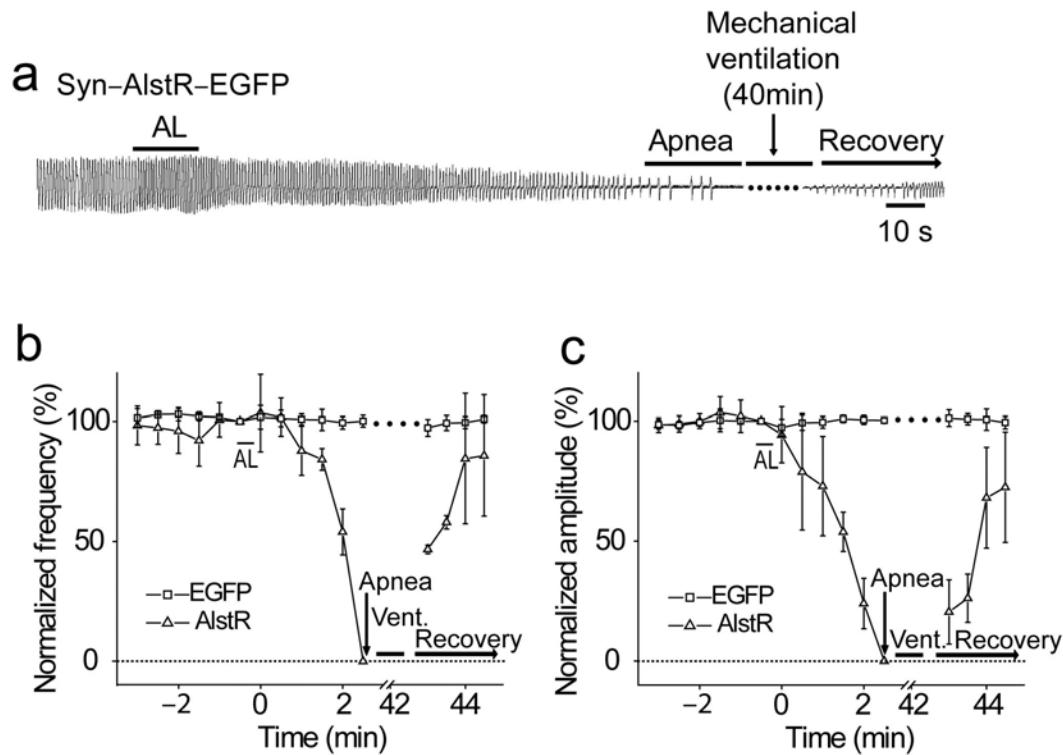
Supplementary Figure 1 Sst neurons partially overlap with NK1R neurons in preBötC in young adult (5-6 weeks) rats. Double-labeling with appropriate antibodies show NK1R-ir (red) and Sst-ir (green) neurons in sagittal section. Inset is high magnification of indicated neurons. Yellow arrow indicates neuron with colocalization of NK1R-ir and Sst-ir, whereas white arrow represents Sst-ir only. AmbC: ambiguus nucleus compact; scale bar, 50 μ m.



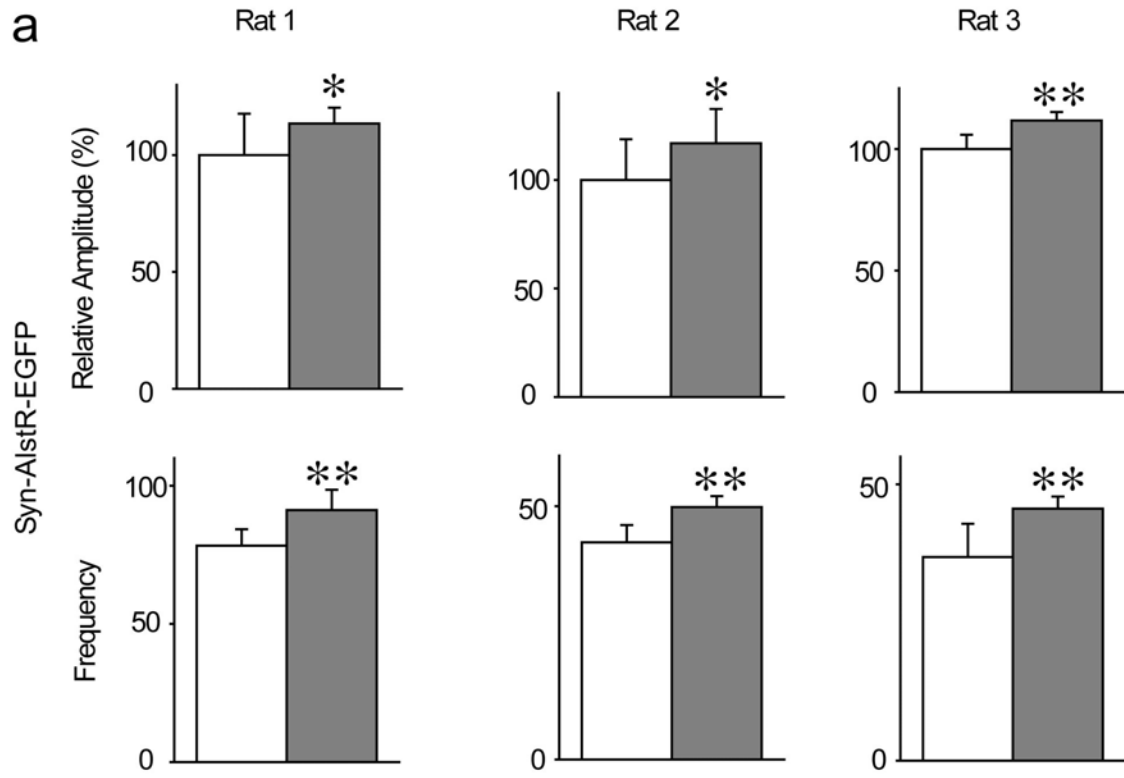
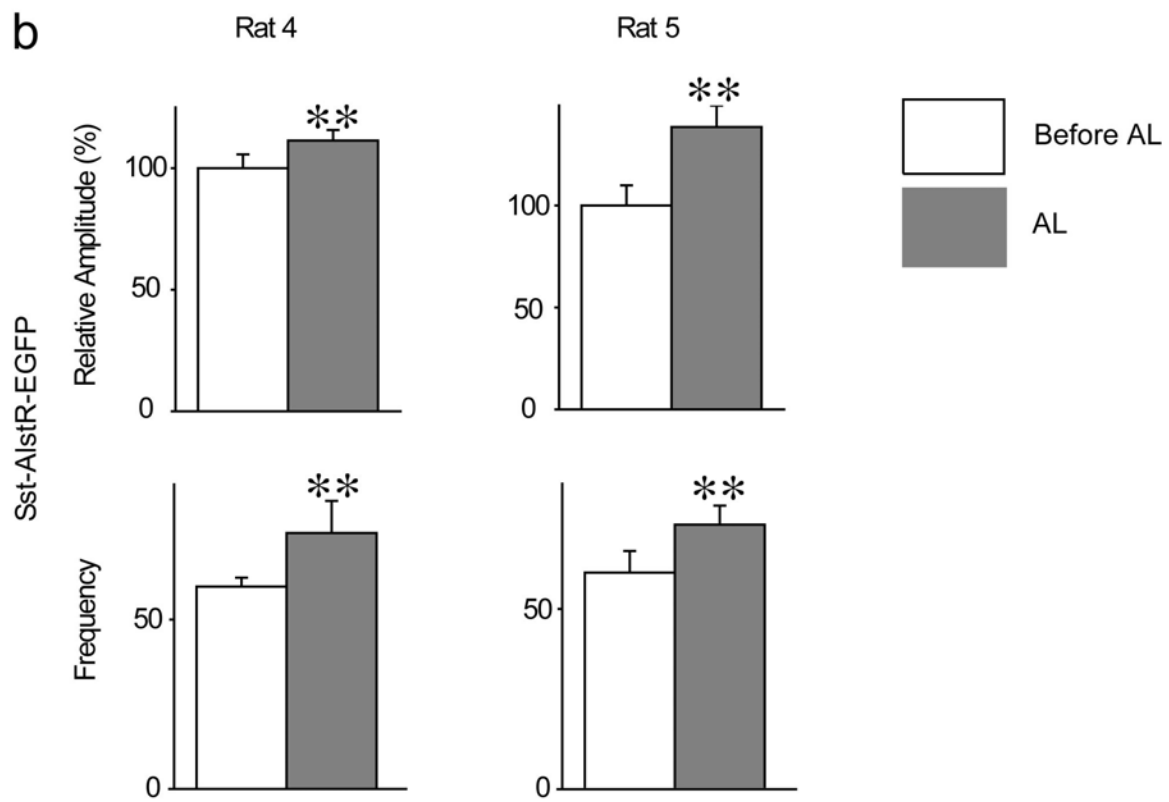
Supplementary Figure 2 Characterization of preBötC neurons infected by AAV2-Syn-AlstR-EGFP. **(a)** Double-labeling showing EGFP-ir (green) and Sst-ir (red) neurons; **(b)** Double-labeling showing EGFP (green) and tyrosine hydroxylase-ir (TH, red) neurons. All images were obtained by confocal microscopy. Insets are high magnification of indicated neurons. Yellow arrows represent neurons coexpressing relevant markers; white arrows represent neurons not coexpressing. Scale bar, 50 μ m.



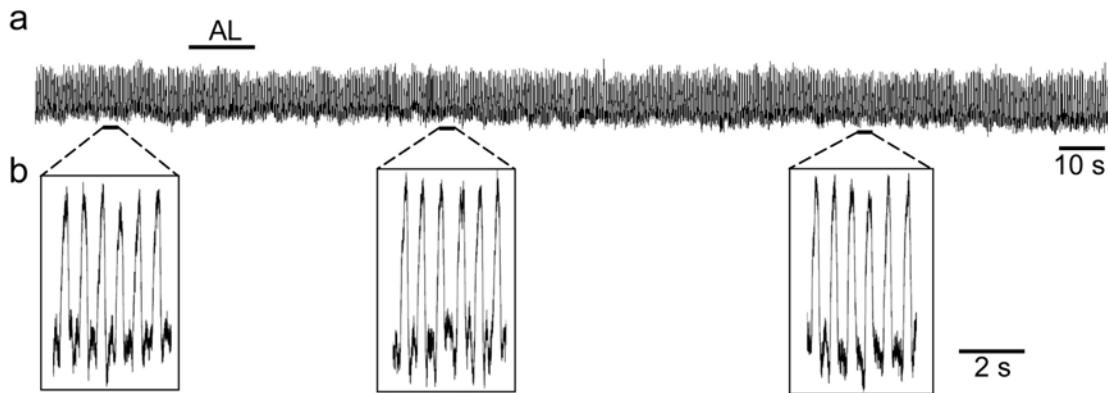
Supplementary Figure 3 Characterization of preBötC neurons infected by AAV2 containing the Sst promoter. **(a)** Schematic representation of AAV2 viral vectors with Sst promoter. **(b)** Sst-AlstR-EGFP AAV2 specifically targeted preBötC Sst neurons. EGFP-ir (green) and SST-ir (red). **(c)** Sst-AlstR-EGFP infected cells are preBötC neurons. Double staining with NeuN-ir (red) and EGFP-ir (green). **(d)** Sst-AlstR-EGFP did not infect TH-ir neurons. Double staining with TH-ir (red) and EGFP-ir (green). All images were obtained by confocal microscopy. Insets are high magnification of indicated neurons. Yellow arrows represent neurons coexpressing relevant markers; white arrows represent neurons not coexpressing. Scale bar, 50 μ m.



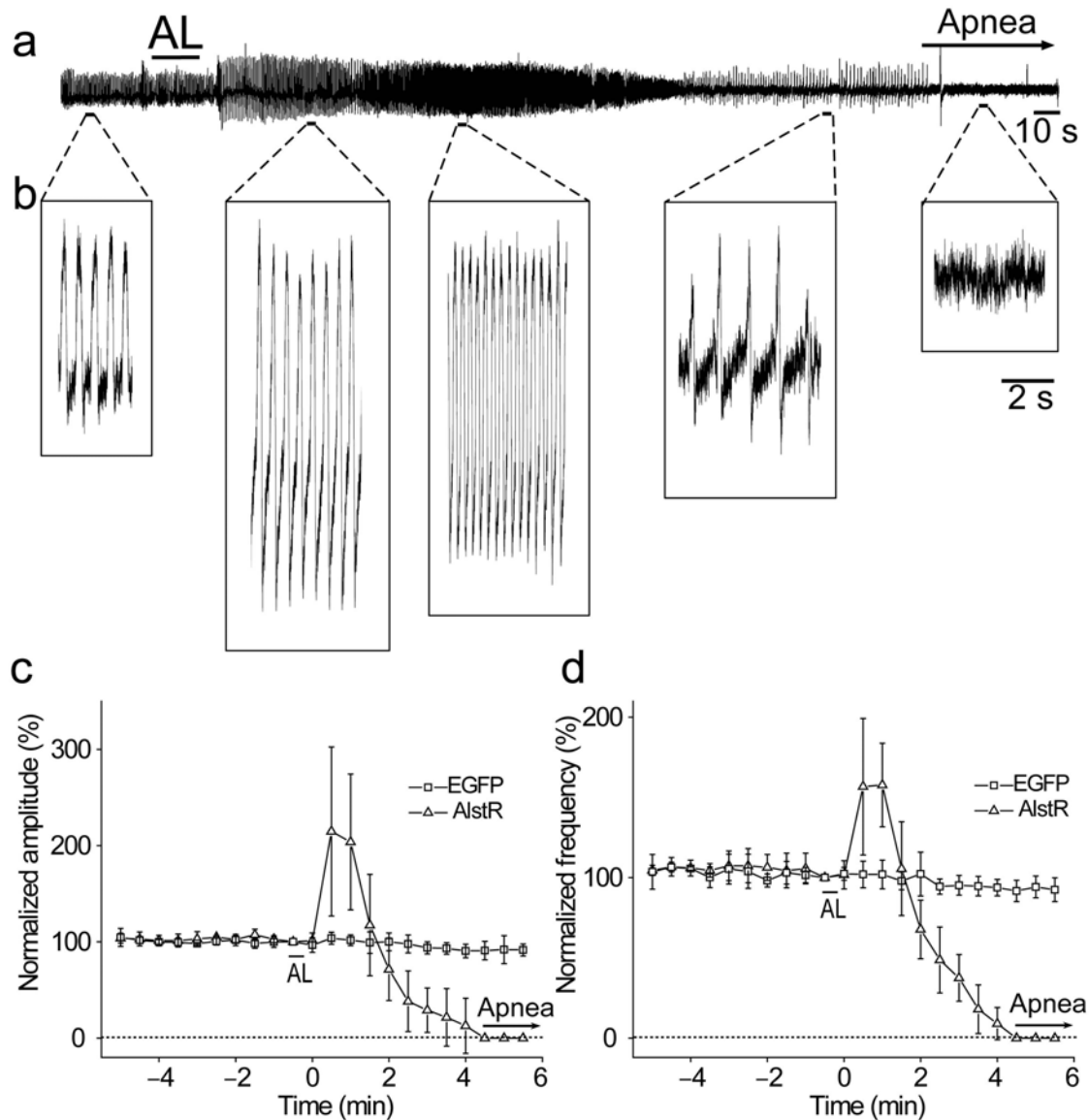
Supplementary Figure 4 Rapid silencing of preBötC AlstR-expressing neurons induces apnea in anesthetized rats. AL was administered ($0.42 \mu\text{mole kg}^{-1}$, intracerebellomedullary cisternally (*icc*)) 4-6 weeks after bilateral preBötC microinjection of Syn-AlstR-EGFP. **(a)** Changes of breathing pattern of anesthetized rat upon AL application; mechanical ventilation for duration of apnea (~40 min). (Signal represents respiratory airflow). **(b)**, **(c)**, AL administration induced a gradual decline of both frequency (b) and tidal volume (c) until apnea developed at 2.5 min post-AL application. After mechanical ventilation, the rats resumed spontaneous breathing. Vent., ventilation; Values are group mean \pm s.d. ($n=5$). Note: in $n=3$ out of 5 rats, there was a significant increase in ventilation ~20 sec after administration of AL (Supplementary Fig 5).

a**b**

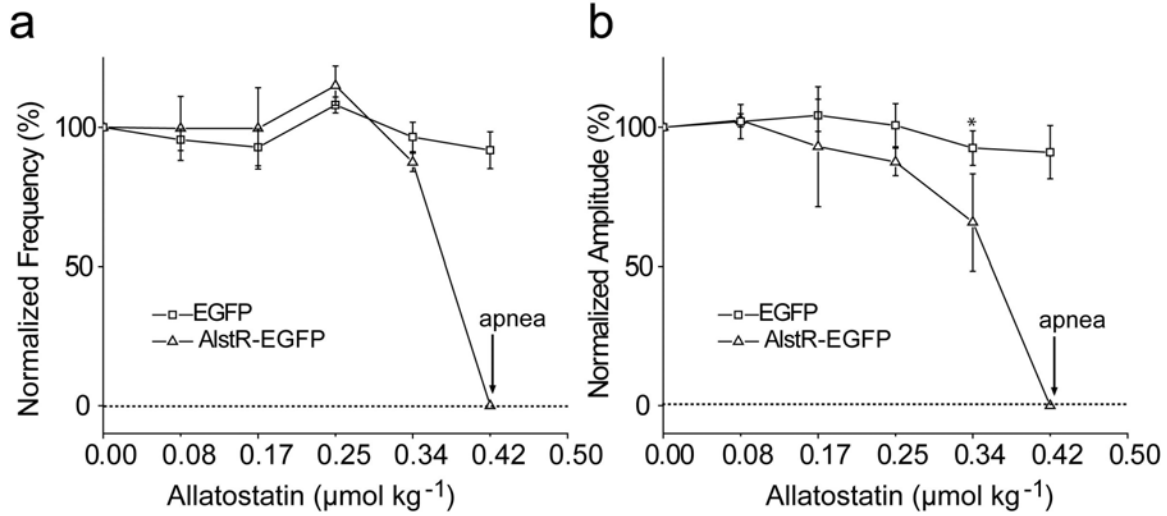
Supplementary Figure 5 AL induced transient hyperventilation in some anesthetized rats injected with Syn-AlstR-EGFP or Sst-AlstR-EGFP AAV2. **(a)** Three Syn-AlstR-EGFP rats (1-3), and **(b)** two Sst-AlstR-EGFP rats (4-5) show significant increase of amplitude and frequency transiently within 20 seconds upon AL administration. Gray bars: Averages of normalized (to control) inspiratory amplitude and instantaneous frequency for all breaths 5-20 seconds after AL administration; White bars: Control averages of normalized inspiratory amplitude and frequency for all breaths 15 seconds prior to AL administration. 3 of 5 Syn-AlstR-EGFP (a) and 2 of 4 Sst-AlstR-EGFP (b) injected rats show the transient and significant increase of both amplitude ((a) and (b) upper panels) and frequency ((a) and (b) lower panels) after AL administration (* $p < 0.05$, ** $p < 0.0001$, independent t -test). Values are mean \pm s.d.



Supplementary Figure 6 Administration of AL *icc* does not affect the breathing patterns in control (uninfected) rats. **(a)** Plethysmographic record of breathing pattern after AL *icc* ($0.42 \mu\text{mole kg}^{-1}$). **(b)** Expanded sections from (a). Signal represents tidal volume.

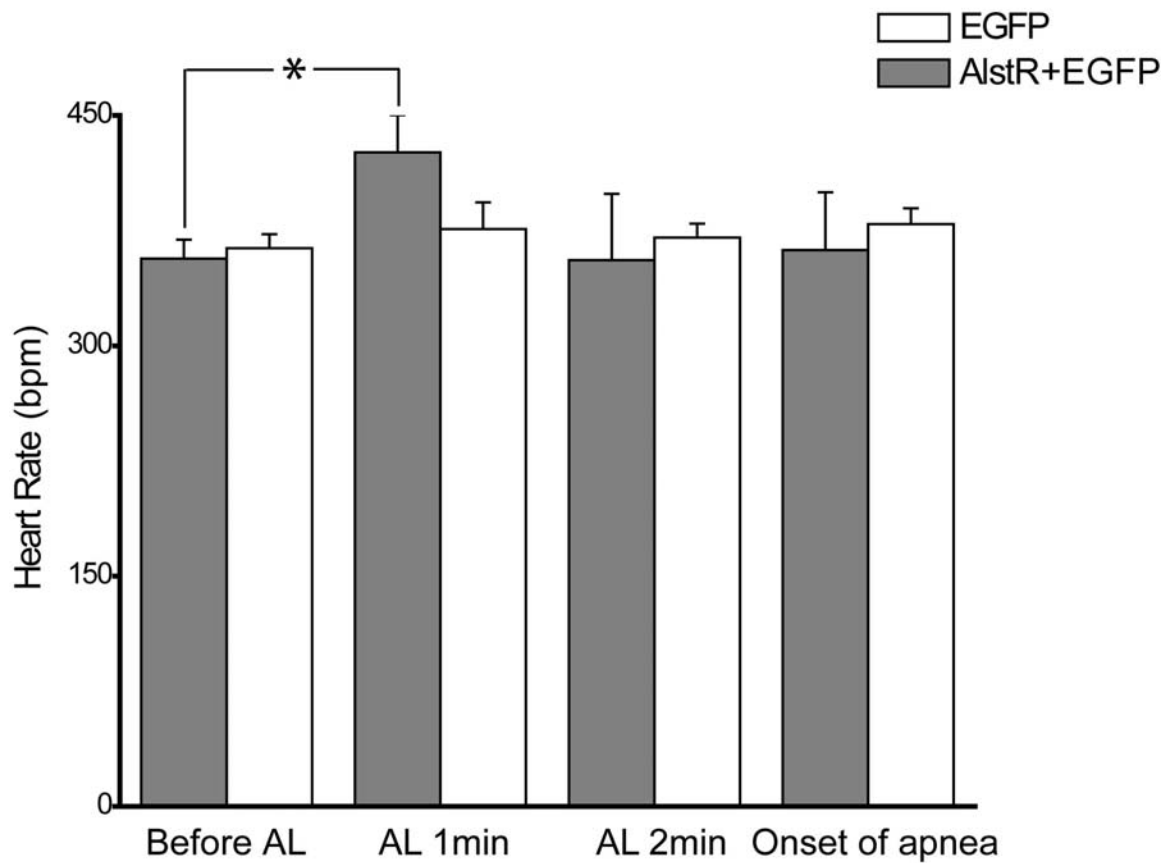


Supplementary Figure 7 Rapid silencing of preBötC AlstR-expressing neurons induces apnea in awake behaving rats. AL administered *icc* ($0.42 \mu\text{mole kg}^{-1}$) 4-6 weeks after bilateral preBötC microinjection of Syn-AlstR-EGFP. **(a)** Plethysmographic record of breathing pattern, signal represents tidal volume. **(b)** Expanded sections from (a). **(c)**, **(d)** Changes of normalized frequency (c) and amplitude (d) upon AL application. Values are mean \pm s.d. (n=6).



Supplementary Figure 8 Dose-response of AL. AL administered *icc* 4-6 weeks after bilateral preBötC microinjection of Syn-AlstR-EGFP or Syn-EGFP AAV2.

(a) Normalized frequency and (b) amplitude changes at 4.5 min after AL application in awake behaving rats. Values are means \pm s.d. of 6 rats * $p < 0.05$ (t -test).



Supplementary Figure 9 Heart rate in awake rats was elevated during the early onset transient hyperventilation period but returned to control levels prior to the onset of apnea. Rats were injected with Syn-AlstR-EGFP. Heart rates (beat per minute, bpm) were determined by the EMG signal using Chart 5 software (AD Instruments, Colorado Springs, CO). Four time points were plotted: prior to AL administration: 357 ± 12 ; AL 1 min: 426 ± 24 ; 2 min: 356 ± 43 ; and 4.5 min (onset of apnea) post AL application (*icc*): 362 ± 38 ; * $p < 0.05$ (*t*-test). Values are means \pm s.d. of 3 rats.

Table 1. Absence of EGFP cell bodies in brainstem and spinal cord respiratory-related regions (except for the preBötC) after local viral injection.

| | Syn promoter [*] | Sst promoter ^{**} |
|---|---------------------------|----------------------------|
| Periaqueductual grey | 0 | 0 |
| Parabrachial complex/ Kölliker Fuse | 0 | 0 |
| Parafacial respiratory group/Retrotrapezoid nucleus | 0 | 0 |
| Bötzinger complex | 0~5 ^{&} | 0~5 ^{&} |
| Hypoglossal nucleus/parahypoglossal region | 0 | 0 |
| Nucleus tractus solitarius | 0 | 0 |
| preBötzinger Complex | 590±62 | 465±26 |
| Ventral respiratory group caudal to preBötC | 0 | 0 |
| Ambiguus nucleus | 0 | 0 |
| Cervical motor neurons (C1-C4) | 0 | 0 |

2 months post-viral injection, the cerebellum, brainstem, spinal cord and part of the midbrain was removed, sectioned, and EGFP was detected by immunohistochemistry. Every slice from -7.2mm from Bregma²¹ to thoracic level of spinal cord was examined. We did not find any EGFP positive cell bodies beyond the preBötC injection sites. This data is consistent with numerous reports that AAV2 is not retrogradely transported¹⁶⁻¹⁸.

[&]~0-5 EGFP positive cell bodies were found in the most caudal part of Bötzinger Complex, no EGFP positive cell bodies were found in rostral part of Bötzinger complex.

^{*} Syn promoter AAV2: Syn-EGFP (n=3 rats), Syn-AlstR-EGFP (n=3 rats);

^{**} Sst promoter AAV2: Sst-EGFP (n=6 rats), Sst-AlstR-EGFP (n=4 rats).

Table 2. Average number or percentage of preBötC neurons (within 250 μm from injection site) with phenotypic immunomarker (NeuN, Sst or NK1R) and coexpressing EGFP when expression was driven by synapsin vs. somatostatin promoter.

| | Per side in preBötC | | |
|--------------------------------------|---------------------|--------------------|-------------------|
| | NeuN | Sst | NK1R |
| Average # neurons | 2965 | 464 | 298 |
| <i>SYNAPSIN</i> | | | |
| EGFP Neurons | 590 \pm 62 (n=7) | 91 \pm 24 (n=3) | 61 \pm 12 (n=9) |
| % Double positive of total phenotype | 20 \pm 2% (n=7) | 20 \pm 4% (n=3) | 21 \pm 4% (n=9) |
| % Double positive of total EGFP | 99 \pm 1% (n=7) | 15 \pm 4% (n=3) | 10 \pm 2% (n=9) |
| <i>SOMATOSTATIN</i> | | | |
| EGFP neurons | 465 \pm 26 (n=6) | 376 \pm 28 (n=4) | 49 \pm 14(n=5) |
| % Double positive of total phenotype | 16% (n=6) | 81 \pm 8% (n=4) | 16 \pm 5% (n=5) |
| % Double positive of total EGFP | 100% (n=6) | 80 \pm 6% (n=4) | 11 \pm 3% (n=5) |

Material and Methods

Adeno-associated viral vector construction and AAV2 preparation. AAV with an expression cassette of the synapsin promoter driving AlstR/EGFP, flanked by the AAV ITRs, was described previously¹. The construct was named pA–Syn–AlstR–IRES–EGFP. The rat Sst promoter –750bp from transcriptional site is involved in cell specific expression, and contains the essential elements for basal and regulatory transcriptional activities^{2,3}. We searched the homolog of this portion corresponding to the mouse Sst gene. Generally, longer distal promoter region renders more specific activity in Sst–expressing neurons, thus we cloned around 2.0kb of mouse Sst promoter region which contains the characterized region (–750bp) and 1.2kb more distal region of mouse Sst promoter. The mouse Sst promoter (2.0kb) was amplified by the primers (5' TTC GAA AGC CTA GAG GCA GAG CAA GCG CTG 3' and 5' TTC GAA GCT ATG GAG CTC TCC ACG GTC TCC 3' or 5' ACA TGT C GCT ATG GAG CTC TCC ACG GTC TCC 3'; the underlines indicate the BstBI and PciI sites respectively) from BAC RP23–274H19 and cloned into TOPO–T vector (Invitrogen), respectively. The Sst promoter fragments were cleaved by BstBI or BstBI plus PciI and inserted into BstBI site or BstBI plus NcoI sites of pA–Syn–AlstR–IRES–EGFP respectively to construct the pA–Sst–AlstR–IRES–EGFP or pA–Sst–EGFP. All the constructs were verified by sequencing.

AAV2 was prepared by using AAV Helper–Free System (Stratagene) according to the instructions of the manufacturer. Briefly, AAV2 was produced by transfection of AAV–293 cells in 150 mm dishes with 16 µg each of pAAV–RC, pHelper and a cloning viral vector generated above by using lipofectamine (Invitrogen). The cells were harvested at 72 hours post–transfection, lysed in 15 ml of gradient buffer (10 mM Tris (pH7.6), 150 mM NaCl, 10 mM MgCl₂) by four freeze/thaw cycles in dry ice–ethanol and 37°C bath, with addition of passing through a syringe with a 23G needle ten times. The lysate was treated by 50 U/ml of Benzonase (Sigma) for 30 min at 37°C, and clarified by centrifuge at 3,000 g for 15 min. The virus was purified by using iodixanol density gradient ultracentrifuge at 350,000 g for 1 hour at 18°C as described elsewhere⁴. The titer of the AAV2 was determined by quantitative PCR.

Surgical procedures. All experimental procedures were approved by the Chancellor's Animal Research Committee at the University of California, Los Angeles. The surgical procedures were described previously^{5,6}. Briefly, male Sprague–Dawley rats (220–250g) were anaesthetized with Ketamine (100 mg kg⁻¹) and Xylazine (10 mg kg⁻¹) injected intraperitoneally (i.p.). Rats breathed 1–1.5 vol% isoflurane in O₂ throughout the surgery. The skull was positioned in a stereotaxic apparatus with Bregma 5mm below Lambda. The dorsal medullary surface was exposed. Using a micropipette (40 µm tip diameter) guided by a micromanipulator, 0.5 µl of AAV2 virus (10¹⁰ particles ml⁻¹) in phosphate buffered saline (PBS) was injected into the preBötC at coordinates: 0.9 mm rostral, ±2 mm lateral and 2.7 mm ventral to the calamus scriptorius. In all rats, injections were made bilaterally. Microinjections were made at the rate of 0.2 µl min⁻¹ using a series of pressure pulses (PicoSpritzer III, Parker Hannifin Corporation). The micropipette was left in place for 5 minutes post-injection to allow the viral solution to be absorbed and to minimize the backflow of the solution up the micropipette track. After microinjection, a catheter (0.6 mm diameter) was implanted into the cerebellomedullary cistern and fixed to the skull using bone cement. Pairs of biocompatible wire electrodes (Cooner Wire, Chatsworth, CA) were implanted for intercostal muscle electromyography and ECG measurement and tunneled subcutaneously to a socket secured to a skin button (Instech Laboratories, Inc., Plymouth Meeting, PA) implanted in between the shoulder blades. Muscles were sutured in layers using 6–0 absorbable sutures. Skin was sutured using 5–0 nylon. Sutures were removed 7 days post-surgery. After surgery, the rats were housed for 3–8 weeks to allow for expression of AlstR and EGFP.

Awake rats: Breathing was recorded using a whole body plethysmograph (Buxco Research Systems, Sharton, CT). The signal from the plethysmograph represents tidal volume. While in the plethysmograph, rats could move freely, with access to water and rat chow. Rats were studied for 4 hours in the plethysmograph for 8–12 times in between AAV2 injection and AL administration. AL was dissolved in saline to make the final concentration as 2 mM. At the end of a 4-hour recording session, AL (0.42 µmol kg⁻¹) was slowly (~30 sec.) injected via the implanted catheter (intracerebellomedullary cistern (*icc*)). The maximum dose was 0.16 µmole, even in rats that weighed >400 grams. Rats

were allowed to recover. In some rats, the procedure was repeated 3–4 weeks later, with similar results.

The initial response to AL administration in awake rats was a significant increase in ventilation. During this period rats stood “frozen” – a position associated with fear in rats, with an associated transient increase of heart rate that returned to control levels prior to the onset of apnea (Supplementary Figure 9). We speculate that this response was initiated by the mechanisms underlying the small but significant transient increase in ventilation seen in anesthetized rats, but manifested as a much larger response in the awake state. In humans complaining of dyspnea, a dissociation between metabolic needs and actual ventilation can lead to a sensation of discomfort. We suggest that this effect underlies the noticeable increase in ventilation in awake rats, but regardless of cause could not be sustained as preBötC neurons hyperpolarized.

During progressing hypoventilation and apnea, after a brief struggle, initially awake rats went passive, then limp. In initial experiments we were not able to rescue 5 awake rats whose apneas were 477, 215, 196, 170, or 162 seconds long; none of these rats gasped prior to death. One report⁷ suggests the preBötC network underlies generation of multiple breathing patterns, including eupnea, sigh, and gasp. The lack of gasping is consistent with an essential role of the preBötC in gasping. In subsequent experiments all rats were rescued after 90 seconds of apnea. They were removed from the plethysmograph and connected to a ventilator *via* a sterile lubricated silicone tube inserted in one nostril. Rats are nose–breathers and their elongated soft palate facilitates effective ventilation via the nose. This mode of ventilation is comparable to mechanical ventilation using a nasal mask in humans. Ventilation continued until rats were able to breathe on their own (typically ~40 min. later). Rats were not restrained and free to remove the tube and detached themselves from the ventilator. Only two rats attempted to remove the nose tube after 20 min of ventilation. During prolonged ventilation these rats were in their natural (prone) position and were completely covered with a small towel.

Anesthetized rats: In anesthetized rats a tube was inserted in one nostril and connected to a flow head to continuously measure airflow. After rats were anesthetized, AL ($0.42 \mu\text{mol kg}^{-1}$) was slowly (~30 sec.) added *icc*. After 90 seconds of apnea, rats

were mechanically ventilated until they were able to breathe on their own, typically ~40–60 minutes.

Airflow and ECG signals were digitized and stored and analyzed using software from AD Instruments (Grand Junction, CO).

Rats were monitored for 1–12 weeks after AL administration. Their breathing was normal.

Immunohistochemistry. Rats were anesthetized with Nembutal (100 mg kg⁻¹, i.p.) and perfused transcardially with 4% paraformaldehyde in PBS. The brainstem was removed and put in 4% paraformaldehyde/PBS for another 2 hours, followed by cryoprotection overnight in 30% sucrose in PBS. Forty μ m thick transverse sections were cut using a freezing microtome and incubated in primary antibodies, with 10% normal donkey serum for 24 or 48 hours at 4°C. The origins and dilutions of primary antibodies used in this study were: rabbit anti-Sst-14 (Pennisula Laboratories, LLC; 1:500 or 1:5000 for TSA see below); Alexa 488-conjugated rabbit anti-GFP (Molecular Probes, CA; 1:500); Chicken anti-GFP (Aves Labs, Inc., OR; 1:500); mouse anti-Neuronal Nuclei (NeuN) (Chemicon; 1:500); guinea pig polyclonal antibody to neurokinin-1 receptor (NK1R) (Biomol; 1:5000); rabbit anti-NK1R (Chemicon; 1:1000); and mouse anti-tyrosine hydroxylase (TH) (Chemicon; 1:500). Sst expression decreases with age^{8,9}. We found significantly more Sst-ir neurons in preBötC in young compared to old rats. To facilitate detection of Sst positive neurons in older rats (>300grams) we used slightly different protocol. The animals were treated by 150 μ g colchicine in 50 μ l (*icc*) per rat for 24 hours before perfusion. The brainstems were fixed in 4% paraformaldehyde/PBS at 4°C overnight after transcardial perfusion, followed by cryoprotection overnight in 30% sucrose in PBS; Rabbit anti-Sst-14 (1:5000) was incubated for 24 hours at 4°C and detected by using TSA/Tetramethyl-rhodamine system (Perkin Elmer, MA.). In some cases, guinea pig anti-NK1R polyclonal antibodies were used and signals were amplified by TSA system as well. Others primary antibodies were detected by rhodamine-conjugated donkey anti-rabbit secondary antibody (Jackson; 1:250); rhodamine-conjugated donkey anti-mouse secondary antibody (Jackson; 1:250); Alexa 488-

conjugated donkey anti–chicken secondary antibody (Jackson; 1:250); or FITC–conjugated donkey anti–rabbit secondary antibody (Jackson; 1:250).

Brainstem sections were grouped as one–in–four of sequential series of 40 μm transverse sections, thus four groups of brainstem sections were collected from each rat and immunohistochemistry was performed using the following four groups of primary antibodies: NK1R and EGFP, Sst and EGFP, NeuN and EGFP, and TH and EGFP. Images were acquired by confocal laser scanning microscope (LSM510 META, Carl Zeiss, Germany). High resolution Z–stack confocal images were taken at 0.45 μm intervals. Confocal images were presented as Z–stack projections. Data measuring proportions of cells with EGFP colocalization with various other markers were obtained by counting the number of immunofluorescent–positive neurons within a 250 μm radius circle. This circle was positioned with the dorsal most point at the lower edge of the compact portion of the nucleus ambiguus.

Discussion

Specificity of AL: The inhibitory effects of AL on AlstR–expressing mammalian neurons *in vitro* and *in vivo* are well documented^{1,10,11}: i) effects of AL are restricted to neurons expressing AlstR, and highly specific, and; ii) neurons expressing AlstR can be completely inactivated. Patch clamp recordings *in vitro* in AlstR–EGFP–transfected neurons demonstrate that neurons expressing EGFP are quickly hyperpolarized after administration of AL, whereas neurons that do not express EGFP remain unaffected. Here, we show that: i) in control rats (in which AlstR was not expressed) there was no response to AL (Fig 2, supplementary Fig 3, 4), and; ii) transfected neurons were found around the injection site in preBötC but not anywhere else (see Supplementary Table 1 and further comments below).

We have established that activation of AlstR on preBötC Sst neurons¹², presumably silencing them, leads to persistent apnea. The determination of the spatiotemporal details of the disturbances in preBötC circuits (and other connected populations, such as within the ventral respiratory column) will require electrophysiological recording.

Apnea was complete: After rats stopped breathing they were intubated through the nose and connected to an airflow head. Our flow measurements were so sensitive that in addition to flow resulting from contraction of the respiratory pump muscles, minute flow changes related to contraction of accessory respiratory muscles and heart beat could be detected. After administration of AL only flow changes related to the heart beat were observed, indicating inactivation of *all* respiratory muscles. The most parsimonious explanation for absence of respiratory activity in muscles supplied from variety of different motoneuronal pools is the loss of respiratory rhythm at its source.

Rhythmic expiratory muscle activity persists after inspiratory muscle activity is eliminated by silencing of preBötC neurons expressing the μ -opiate receptor (μ OR) by means of systemic administration of μ OR agonist–fentanyl in neonatal and juvenile rats¹³. Here we report that after rapid hyperpolarization of preBötC Sst neurons all respiratory muscle activity, including that of the expiratory muscles, ceased. Why are these responses different? We suggest several possibilities. i) Expiratory oscillations may be inhibited by pontine neurons expressing μ OR, and fentanyl lifts this inhibition¹⁴. Thus, fentanyl could promote rhythmic expiratory activity during inspiratory apnea in the adult rat; ii) In experiments with neonatal rats, they were kept hyperoxic. In contrast, in our experiments, rats became severely hypoxic, which inhibits expiratory oscillations; iii) Expiratory activity is robust in newborn and juvenile rats, but difficult to elicit in adults¹⁵; iv) The preBötC is the dominant oscillator in the adult; perhaps any second oscillator is incapable of independent oscillations in adult rats, and/or requires excitation from the preBötC in order to do so.

Localization of viral expression: In order to determine whether the virus was expressed distantly from the preBötC injection sites, e.g., due to long–distance diffusion or retrograde transport, we systematically looked for EGFP–positive cell bodies throughout the whole brainstem. Thus, all rats (n=24) infected with AAV2 were sacrificed and perfused; we cut sections (40 μ m) from the medulla beginning at the level of the obex rostral through the caudal half of the facial nucleus. No EGFP–positive cell bodies were found outside of the region of the preBötC injection site, including in the nucleus tractus solitarius (NTS), and retrotrapezoid nucleus–parafacial respiratory group (RTN/pRFG).

In 16 of these virally injected rats, we also examined histological sections from the midbrain to the spinal cord including the cerebellum. In all cases, there was robust EGFP expression in the preBötC but we did not find *any* EGFP positive cell bodies beyond the injection sites (Supplementary Table 1). These results are consistent with numerous reports showing that AAV2 is not retrogradely transported^{16–18}. Given the design of the viral vectors, EGFP is expressed wherever AlstR is expressed. Thus we conclude from the absence of EGFP outside the preBötC that there was no AlstR expression elsewhere except in EGFP-positive preBötC neurons, and all responses to AL were mediated by them. We also have another relevant observation. We injected the AAV2–EGFP virus into the hypoglossal nerve in 3 rats and into the genioglossus muscle in 3 rats, hoping to transfect motoneurons via axonal transport. No transfected motoneurons were found within the hypoglossal motor nucleus (data not shown).

Incidental damage to preBötC: In each experiment, we carefully examined the morphology of neurons and neuronal numbers in and around the preBötC after immunostaining. We did not find scar tissue, or obvious damage, such as gliosis, nor did we observe quantitative changes in total neurons, NK1R neurons, TH neurons and Sst neurons in the preBötC (Supplementary Table 2) after months of viral expression, which suggest there was no cytotoxicity or inflammation. At a behavioral level, infected rats gained weight, breathed and moved normally, etc. Rats were still healthy five months after initial surgery (n=8). These observations are in contrast to the long-term effects of injection of Substance P-saporin into the preBötC⁵, where we readily observed proliferation of glia at the injection site. The AAV2 virus does not seem to trigger any immune reaction in the preBötC in adult rats, consistent with many reports showing that AAV2 causes very little cytotoxicity or inflammation^{1,19}.

Rates of viral expression and differences in responses to AL between viruses with Syn or Sst promoters: After transfection, AAV2 DNA integrates with the neuronal genome, thus protein expression becomes constitutive, and the expression rate depends on the promoter activity. The onset of detectable EGFP signal is ~2 weeks postinjection in Syn–EGFP injected rats, and ~3 weeks postinjection in Sst–EGFP injected rats (data not shown).

Thus, the Sst promoter activity can be considered weaker than the Syn promoter in preBötC neurons. EGFP expression is weaker under IRES in Syn (or Sst) –AlstR–EGFP than under Syn (or Sst) promoter in Syn (or Sst)–EGFP. Thus, it will take more time to have equivalent levels of EGFP signal in AlstR–EGFP viruses (4 weeks for Syn promoter and 6 weeks for Sst promoter) than EGFP control viruses. Efficient AlstR expression in preBötC is necessary in order to elicit a response by AL in awake rats. Apnea could be successfully induced by application of AL at 4 weeks post-injection of Syn–AlstR–EGFP or 6 weeks post-injection of Sst–AlstR–EGFP, which is consistent with timing of EGFP expression by these viruses. Moreover, the onset of apnea following AL administration was sooner in rats transfected with viruses carrying the Syn promoter compared to the Sst promoter. There are several additional possible explanations for these differences: i) more neurons were transfected by Syn compared to Sst (Table 2); ii) Syn transfected another phenotype of preBötC neuron that when silenced had a more powerful effect on breathing compared to preBötC Sst neurons, and; iii) more AlstRs were expressed per neuron by the Syn promoter compared to the Sst promoter resulting in more rapid silencing in the former case. Further investigation will be required to distinguish among these possibilities, including identification of the neuronal phenotypes and AlstR expression rates among subpopulations transfected by the AAV2 viruses and electrophysiological recording of the activity of preBötC neurons before and after AL administration.

Differences in responses to AL between anesthetized and awake rats: Our results show that the time profiles of the perturbations of inspiratory amplitude and frequency in the anesthetized versus awake animals differ. Anesthesia does (at least) two things of relevance: i) by depressing consciousness, anesthesia depresses volitional/emotional responses to changes in ventilation such as might be associated with anxiety or panic in response to dyspnea, and; ii) depresses neuronal excitability, e.g., by potentiating GABAergic neurotransmission or inhibition of NMDA receptors. preBötC neurons are most proximal to the ventral medullary surface (preBötC neuronal somas are 150–700 μm to the ventral surface in adult rats), with dendrites extending to the ventral, but not dorsal surface, of the medulla. As AL diffuses from the CSF into the brainstem it would affect the distal, then proximal, dendrites, then somas of transfected preBötC neurons.

We suggest two possibilities: initial activation of dendritic AlstRs induced ectopic currents in preBötC neurons, paradoxically exciting them prior to their ultimate depression, and/or; AL induced synchronous opening of K^+ channels that led to extracellular accumulation of K^+ in the vicinity of transfected neurons that depolarized adjacent preBötC neurons to transiently increase respiratory rate. Respiratory-modulated oscillations of K^+ as large as 1.3–1.5 mM are seen in the vicinity of respiratory neurons in the cat²⁰. In some anesthetized rats (3 out of 5 Syn–AlstR–EGFP, and 2 out of 4 Sst–AlstR–EGFP), there was a transient, slight but significant increase of amplitude and frequency with 20 seconds upon AL administration (Supplementary Fig. 5), though group data did not reach statistical significance. These results show transient hyperventilation upon AL administration in both anesthetized and awake animals.

Differences between slow lesions and rapid silencing of preBötC neurons: When substance P –Saporin is used to lesion preBötC NK1R neurons in adult rats, the breakdown in normal breathing occurs over ~1 week^{5,6}, giving sufficient time for compensatory mechanisms to act to ultimately produce a (pathological) rhythm, i.e., no persistent apnea, during wakefulness. In contrast, in rats expressing AlstR, the silencing of preBötC neurons following AL administration occurred within minutes and resulted in a persistent apnea that would not recover before asphyxiation. Our results suggest that compensatory mechanisms cannot act rapidly enough when the depression of preBötC neuronal excitability is sudden, which may provide insight into mechanisms of catastrophic respiratory failure in some cases of sudden infant death syndrome or sudden infant unexplained death, and central sleep apnea.

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