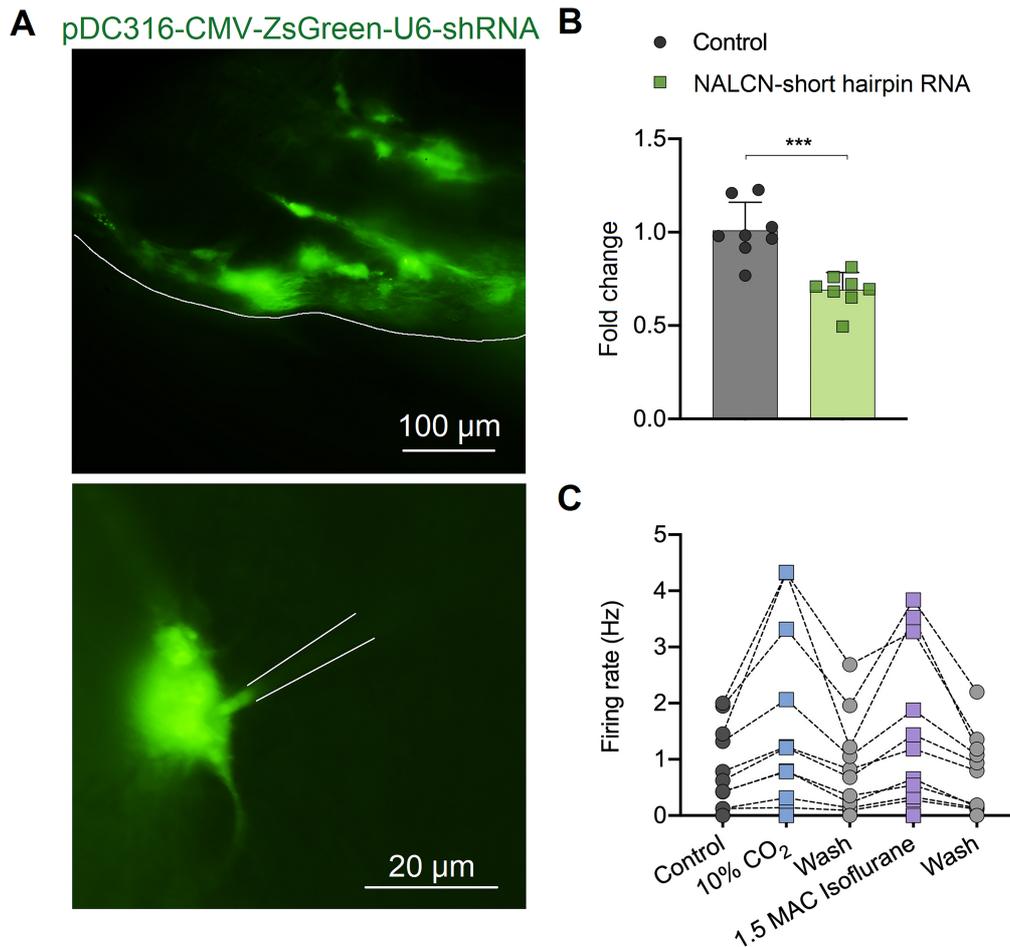


Volatile Anesthetics Activate a Leak Sodium Conductance in Retrotrapezoid

Nucleus Neurons to Maintain Breathing during Anesthesia in Mice

Supplementary materials



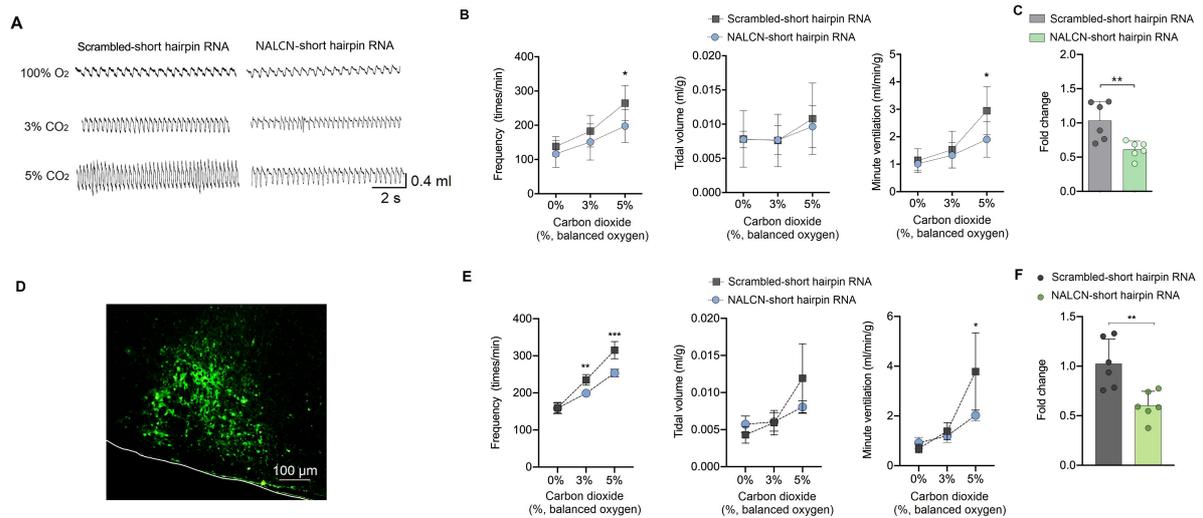
Supplementary Fig. 1. Patch-clamp recordings on NALCN genetically silenced brain slices.

(A) Green fluorescence was found in retrotrapezoid nucleus 2-3 days after injection of adenovirus (upper), which is helpful to the visualized patch-clamp recordings (lower). The white pipe is part of recording electrode.

(B) The expression level of NALCN in retrotrapezoid nucleus is decreased by $31.8\% \pm 14.0\%$ ($P < 0.001$, $n = 8$) at 3 days after

injection of NALCN-shRNA adenovirus, detected by qRT-PCR. Data are mean \pm SD. **(C)**

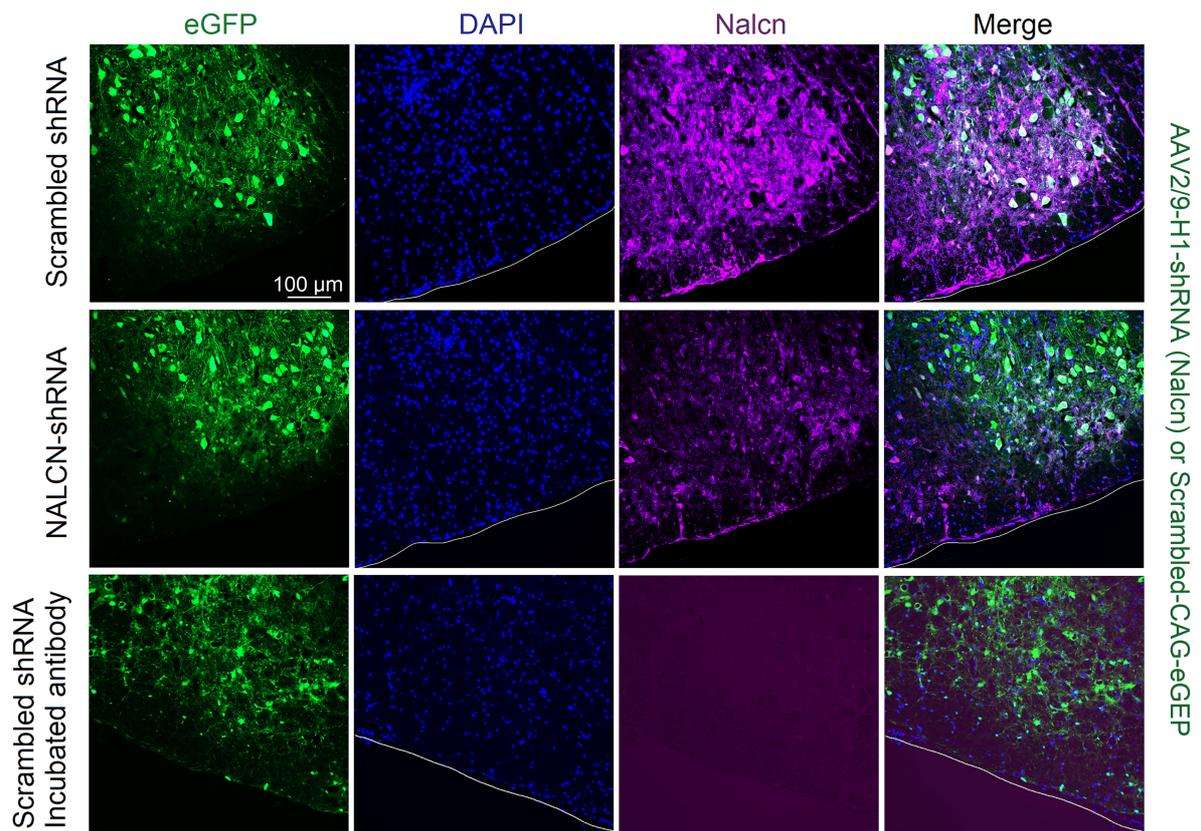
Isoflurane at concentration of 0.42-0.50 mM (~1.5 MAC) only slightly increases firing rate in retrotrapezoid nucleus neurons of NALCN genetically silenced brain slice, without statistical significance (n = 15 neurons). There are 5/15 (~33%) neurons that was found no spontaneous firing and no response to CO₂ after genetic silencing of NALCN (identified by green fluorescence). The data was analyzed by one-way repeated measured ANOVA. *** $P < 0.001$ by two-tailed independent sample t test **(B)**.



Supplementary Fig. 2. Genetic silencing of NALCN in mice reduces respiratory outputs under control condition and exposure to CO₂.

Generally, this figure indicates the baseline changes of respiratory outputs after genetic silencing of NALCN in retrotrapezoid nucleus by shRNA adeno associated virus. One group of mice (**A-C**) ($n = 7/\text{group}$) were used for testing isoflurane in original manuscript. Another group of mice (**D-F**) ($n = 7/\text{group}$) were used for comparing effects of sevoflurane and propofol during peer review. **(A)** Representative traces of respiratory outputs from a control (scrambled-shRNA) and NALCN genetically silenced mice during exposure to 100% O₂, 3% CO₂, 5% CO₂. **(B)** For the mice in original manuscript, summary data of respiratory frequency (left), tidal volume (normalized with body weight, ml/g) (middle) and minute ventilation (normalized with body weight, ml/min/g) (right) are compared between NALCN genetically silenced mice and control mice under different concentrations of CO₂. **(C)** Level of NALCN mRNA in retrotrapezoid nucleus is decreased by $42.0\% \pm 11.8\%$ ($P = 0.007$) in the NALCN genetically silenced mice compared to scrambled-shRNA treated animals. **(D)**

Enhanced green fluorescent protein (eGFP) was detected in retrotrapezoid nucleus neurons 4 weeks after injection of virus. **(E)** For the mice used in additional experiments during peer review, summary data of respiratory frequency (left), tidal volume (normalized with body weight, ml/g) (middle) and minute ventilation (normalized with body weight, ml/min/g) (right) are compared between NALCN genetically silenced mice and control mice under different concentrations of CO₂. **(F)** Level of NALCN mRNA in retrotrapezoid nucleus is decreased by 42.2% ± 24.4% ($P = 0.004$) in the NALCN genetically silenced mice compared to scrambled-shRNA treated animals. Data are mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by two-tailed independent sample t-test **(C, F)** or two-way repeated measured ANOVA **(B, E)**.



Supplementary Fig. 3. Immunofluorescent staining of NALCN after expression of NALCN-shRNA adeno associated virus.

The expression of NALCN in retrotrapezoid nucleus from control mice (top) or NALCN genetically silenced mice (middle) was detected by immunofluorescence staining. (Bottom) After incubation of NALCN primary antibody to NALCN antigen (provided by antibody manufacturer), there is no NALCN positive neurons found in control mice, which can confirm specificity of the NALCN primary antibody. AAV: adeno-associated virus.

Supplementary Table 1: Changes of body temperature before and after general anesthesia in whole-body plethysmography

	Pre-anesthesia (°C)		Post-anesthesia (°C)	
	Control mice	Knockdown mice	Control mice	Knockdown mice
0.8% Isoflurane ¹	35.3 ± 0.3	35.9 ± 0.6	34.3 ± 0.8 *	35.3 ± 1.0
1.5% Isoflurane ¹	36.0 ± 0.6	35.7 ± 0.7	33.1 ± 1.0 *	33.8 ± 1.1 *
0.8% Isoflurane ²	35.5 ± 0.4	35.4 ± 0.8	34.5 ± 0.7 *	34.9 ± 0.5
1.5% sevoflurane ²	35.7 ± 0.8	35.2 ± 1.0	33.5 ± 0.8 *	34.2 ± 0.9
70 mg/kg propofol ²	35.1 ± 0.6	35.1 ± 0.9	32.7 ± 1.0 *	32.5 ± 1.2 *

There is no significant difference between control mice and NALCN genetically silenced mice under same condition; * $P < 0.05$, pre-anesthesia vs. post-anesthesia in the same group of mice. ¹ Original animal data, ² additional animal data in response to peer review.