Isoflurane abolishes spontaneous firing of serotonin neurons and masks their pH/CO$_2$ chemosensitivity

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Serotonin (5-hydroxytryptamine; 5-HT) neurons from the mouse and rat rostral medulla are stimulated by increased CO$_2$ when studied in culture or brain slices. However, the response of 5-HT neurons has been variable when animals are exposed to hypercapnia \textit{in vivo}. Here we examined whether halogenated inhalational anesthetics, which activate TWIK-related acid-sensitive K$^+$ (TASK) channels, could mask an effect of CO$_2$ on 5-HT neurons. During \textit{in vivo} plethysmography in mice, isoflurane (1\%) markedly reduced the hypercapnic ventilatory response (HCVR) by 78-96\% depending upon mouse strain and ambient temperature. Using a perfused rat brainstem preparation, isoflurane (1\%) reduced or silenced spontaneous firing of medullary 5-HT neurons \textit{in situ} and abolished their responses to elevated perfusate PCO$_2$. In dissociated cell cultures, isoflurane (1\%) hyperpolarized 5-HT neurons by 6.52 ± 3.94 mV, and inhibited spontaneous firing. A subsequent decrease in pH from 7.4 to 7.2 depolarized neurons by 4.07 ± 2.10 mV, but that was insufficient to reach threshold for firing. Depolarizing current restored baseline firing and the firing frequency response to acidosis, indicating that isoflurane did not block the underlying mechanisms mediating chemosensitivity. These results demonstrate that isoflurane masks 5-HT neuron chemosensitivity \textit{in vitro} and \textit{in situ}, and markedly decreases the HCVR \textit{in vivo}. The use of this class of anesthetic has a particularly potent inhibitory effect on chemosensitivity of 5-HT neurons.
Breathing is controlled by neurons within the brainstem that generate rhythmic, patterned output to respiratory muscles. One of the most important sources of feedback comes from central respiratory chemoreceptors (CRCs) that monitor arterial PCO₂, probably indirectly via tissue pH (Feldman et al. 2003; Richerson 2004; Wang et al. 2002). There are certain properties that cells must possess to be CRCs (Richerson et al. 2005). These include intrinsic chemosensitivity to physiologically relevant changes in CO₂, and appropriate effects on other cells so that any changes induced by hypercapnia ultimately lead to stimulation of respiratory output. There are many different pH/CO₂ sensitive neurons, as well as some glia, that have been identified as candidates for CRCs (Feldman et al. 2003; Fukuda et al. 1978; Gourine et al. 2010; Mulkey et al. 2004; Nichols et al. 2008; Pineda and Aghajanian 1997; Richerson 2004; Richerson et al. 2005; Richerson et al. 1995; Trapp et al. 2008). It is possible that all or most of these various cells contribute to the whole animal response to hypercapnia in vivo. If they actually are all chemoreceptors, it remains unknown whether they play an equal, widely distributed role under all conditions, or if they each make unique contributions (e.g. depending on pathological states, developmental age, level of CO₂, etc.) (Nattie and Li 2006).

Some 5-HT neurons in the medulla are putative CRCs. 5-HT neurons are located in regions of high blood flow, similar to peripheral chemoreceptors. A subset increase their firing rate in vitro in response to hypercapnia due to intrinsic chemosensitivity, project to respiratory nuclei, and stimulate output of the respiratory control network (Bernard et al. 1996; Bradley et al. 2002; Corcoran et al. 2009; Depuy et al. 2011; Feldman et al. 2003; Hodges et al. 2004; Ptak et al. 2009; Richerson 1995; 2004; Richerson et al. 2005; Taylor et
5-HT neurons in the medulla respond to hypercapnia *in vivo* with an increase in c-fos staining (Corcoran et al. 2009; Larnicol et al. 1994; Richerson 2004; Sato et al. 1992). Recently, 5-HT neurons in the medulla of a decerebrate *in situ* perfused brainstem preparation have also been shown to increase their firing rate in response to acidosis of the perfusate (Iceman and Harris 2014). However, single unit electrophysiological recordings from an *in vivo* preparation are considered by some to be the gold standard for defining normal neuronal activity, and there have been contradictory experimental findings reported using this approach. One laboratory has reported that 5-HT neurons in the raphé obscurus (Veasey et al. 1995) and dorsal raphé (Veasey et al. 1997) of unanesthetized, behaving cats increase their firing frequency in response to as little as 3% inhaled CO$_2$. In contrast, a different laboratory has reported that 5-HT neurons in the raphé obscurus of anesthetized mice and ventrolateral medulla (VLM) of anesthetized rats *in vivo* do not increase their firing frequency in response to inhalation of 10% CO$_2$ (Depuy et al. 2011; Mulkey et al. 2004). It is important to understand why these different results have been obtained. A lack of consistent chemosensitivity *in vivo* has led some to conclude that 5-HT neurons are not central respiratory chemoreceptors (Depuy et al. 2011; Guyenet et al. 2005; Richerson et al. 2005). These contradictory data constitute the major remaining argument against the hypothesis that 5-HT neurons in the medulla are CRCs (Teran et al. 2014). One potential confounding factor is that in the two studies that failed to show 5-HT neuron chemosensitivity, animals were anesthetized with halothane or isoflurane (Depuy et al. 2011; Mulkey et al. 2004).

The halogenated anesthetics family are commonly used in humans and in laboratory animals (Eger 1981). The mechanisms of halogenated anesthetic action are not precisely
understood. Importantly, however, halogenated anesthetics activate TASK channels (Patel et al. 1999; Sirois et al. 2000). These channels are expressed widely in the central nervous system and, when activated, hyperpolarize cells that express them (Duprat et al. 1997; Talley et al. 2001). Halothane and isoflurane have been commonly used as research anesthetics, although halothane is currently less popular due to hepatotoxicity. Isoflurane is considered to be advantageous over many other inhalational anesthetics, because it has low blood solubility and does not induce cardiovascular depression (Eger 1981).

While it is widely acknowledged that halogenated anesthetics can depress breathing, there is not uniform agreement on their impact on ventilatory responses to hypercapnia. The literature is inconsistent, and differences have been reported with different anesthetic agents, concentrations, and species investigated (Groeben et al. 2003; Hirshman et al. 1977; Knill et al. 1983; Martin-Body and Sinclair 1985; Pandit 2014).

Recognizing the potential for halogenated anesthetics to alter breathing, ventilatory responsiveness, and mechanisms of chemosensitivity is critical when interpreting the results of studies conducted under anesthesia.

Here we used experimental preparations at increasing levels of complexity ranging from cultured neurons to whole animals to test the hypothesis that anesthetics might prevent detection of an effect of hypercapnia on 5-HT neurons. As previously reported, 5-HT neurons were chemosensitive to acidosis in culture (Wang et al. 2002; Wang et al. 1998; Wang and Richerson 1999; Wang et al. 2001), and this property was retained in a perfused brain preparation (Iceman et al. 2013). In culture and in the perfused brainstem, 1% isoflurane abolished the firing of 5-HT neurons under control conditions, and prevented
the increase in firing frequency normally induced by hypercapnic acidosis. These results
demonstrate that the use of halogenated anesthetics masks 5-HT neuron chemosensitivity
and suggest why 5-HT neurons were unresponsive *in vivo* to inhalation of CO$_2$ in previous
studies (Depuy et al. 2011; Mulkey et al. 2004). Isoflurane (1%) also caused a severe
reduction in the HCVR *in vivo*. Inhibition of the HCVR *in vivo* could be due to inhibition of
any combination of the putative CRCs (Corcoran et al. 2009; Mulkey et al. 2004; Nichols et
al. 2008; Pineda and Aghajanian 1997; Richerson 2004), including peripheral
chemoreceptors (Lahiri and DeLaney 1975; O'Regan and Majcherczyk 1982). However,
abolition of the CO$_2$ response of 5-HT neurons would be predicted to contribute to blunting
of the HCVR by isoflurane.

**MATERIALS AND METHODS**

*Ethical approval.* All animal procedures and experiments involving mice were carried out
under the approval of The University of Iowa Institutional Animal Care and Use Committee.
All animal procedures and experiments involving rats were carried out under the approval
of The University of Alaska Fairbanks Institutional Animal Care and Use Committee. All
animal procedures were carried out in accordance with the recommendations of the
American Veterinary Medical Association Guidelines for the Euthanasia of Animals: 2013
edition. The minimal number of animals was used and care was taken to reduce the
possibility of any discomfort.

*Plethysmography.* $\dot{V}_E$ was measured using standard open-flow (700 ml/min), whole-body
plethysmography as previously used in our laboratory (Hodges et al. 2008). The chamber was a commercially available model (Buxco, Wilmington, North Carolina), but the remainder of the plethysmography equipment was custom-designed and built. The protocol consisted of >20 minutes of baseline recording in 0% CO$_2$, 50% O$_2$, balance N$_2$ followed by approximately 7 minute exposures to 3%, 5%, or 7% CO$_2$, 50% O$_2$, balance N$_2$. The same sequence of increased CO$_2$ from 0% to 7% was then performed with 1% isoflurane. Lmx1b$^{f/f}$ mice (n=16) and ePet-EYFP mice (n=10), both of which have a wildtype phenotype, were used for the whole animal experiments (Hodges et al. 2008; Scott et al. 2005; Zhao et al. 2006). Mice were exposed to 1% isoflurane mixed with 50% O$_2$ for at least 15 minutes prior to CO$_2$ exposure. Anesthesia was fully induced with 1% isoflurane, which was the only concentration used. The plethysmograph chamber was maintained at 30° C using a heat lamp and feedback controller (TCAT-2AC; Physitemp Instr., Clifton, New Jersey). All data were acquired using custom-written MATLAB software. Body temperature measurements were recorded by telemetry probes (IPTT-300; BMDS, Inc., Seaford, Delaware) inserted into the abdominal cavity at least 5 days before recordings. We did not collect O$_2$ consumption data because engineers from AEI Technologies (Pittsburgh, Pennsylvania) advised us that isoflurane would damage the O$_2$ analyzer. Isoflurane levels were maintained at 1% using a precision vaporizer (Summit Anesthesia Solutions, Bend, Oregon).

Since body temperature regulation may be compromised by isoflurane, and 30° C is below the thermoneutral range of mice, an additional set of plethysmography studies were performed while maintaining body temperature constant at the normal level. In these experiments, the protocol was altered to expose wild-type mice (n=4) to two different gas
mixtures (0% CO$_2$, 50% O$_2$, balance N$_2$; and 7% CO$_2$, 50% O$_2$, balance N$_2$) with and without isoflurane, while body temperature was maintained at 36 °C with a heat lamp to control for confounding influences of an isoflurane-induced reduction in body temperature.

Cell culture. ePet-EYFP mice were used to prepare cultures to allow identification of 5-HT neurons prior to patch clamp recordings (Scott et al. 2005). In these mice, the enhancer region of the Pet-1 ETS gene drives expression of enhanced YFP. Neonatal ePet-EYFP pups (n=20) were sacrificed on postnatal day 0-2 (P0-P2) and a wedge of tissue from the ventromedial portion of the rostral half of the medulla (including the raphé pallidus, r. magnus, and r. obscurus) was removed. The tissue was digested, triturated, and plated on poly-L-ornithine- and laminin-coated coverslips. Cultures were fed and maintained as previously described (Wang et al. 1998). Recordings were performed on YFP-positive cells after P21 (19-21 days after culturing) to allow maturation of chemosensitivity (Wang and Richerson 1999).

Patch-clamp recordings. The gramicidin perforated-patch technique was used for recordings. Electrodes (6-14 MΩ; borosilicate glass) were pulled on a micropipette puller (Model #P-97; Sutter Instrument Co., Novato, California) and filled with intracellular solution containing (in mM): 135 KOH, 135 methanesulfonic acid, 10 KCl, 5 HEPES, and 1 EGTA (pH 7.2; osmolarity 275 ± 5 mOsm). Coverslips were transferred to a recording chamber on the stage of an Axiovert 200 inverted microscope (Carl Zeiss USA, Thornwood, NY). Recordings were performed with a Multiclamp 700B microelectrode amplifier (Molecular Devices, Sunnyvale, California) and data were collected using PClamp software and a Digidata 1440A acquisition system (Molecular Devices, Sunnyvale, California).
Bath solutions. aCSF (pH 7.4) contained (in mM) 124 NaCl, 3 KCl, 2 MgCl₂, 2 CaCl₂, 1.3 NaH₂PO₄, 26 NaHCO₃, and 10 dextrose. Acidic aCSF (pH 7.15) contained (in mM) 136 NaCl, 3 KCl, 2 MgCl₂, 2 CaCl₂, 1.3 NaH₂PO₄, 13 NaHCO₃, 10 dextrose. Both solutions had an osmolarity of 305 ± 5 mOsm and were maintained isocapnic by equilibration with 5% CO₂-95% O₂. Fast glutamatergic, glycinergic and GABAergic ionotropic synaptic transmission was blocked by 100 μM picrotoxin (PTX) (Sigma-Aldrich, St. Louis, Missouri), 50 μM (±)-2-amino-5-phosphonopentanoic acid (AP-5) (Tocris, Ellisville, Missouri), and 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Tocris, Ellisville, Missouri). Solutions were equilibrated with isoflurane (1%) using a precision vaporizer (Summit Anesthesia Solutions, Bend, Oregon).

In situ brainstem recordings. Juvenile (n=22; 60-120 g) male Sprague-Dawley rats (Simonsen Laboratories, Gilroy, California) were used for a perfused brainstem preparation, whose advantages have been described previously (Harris and St -John 2003; Richerson and Getting 1990; St-John and Paton 2000; Toppin et al. 2007). Rats were heparinized (0.3 ml of 1,000 I.U./ml; i.p.) and briefly anesthetized with isoflurane until spontaneous respirations ceased. The portion of the body caudal to the diaphragm was removed, and the temperature of the rostral portion reduced by placing it in chilled perfusate containing: (in mM) NaCl 125, KCl 3.75, CaCl₂ 2.5, MgSO₄ 1.25, KH₂PO₄ 1.25, NaHCO₃ 25, glucose 10, ficoll-70 0.18. Preparations were decerebrated at a precollicular level and the forebrain removed by aspiration. A catheter with a double lumen was inserted retrogradely into the descending aorta, and perfusate was pumped into the aorta. Perfusion pressure was increased gradually to 50-75 mmHg and then held constant. Gallamine triethiodide (60 mg/l) was added to the perfusate. Perfusate passed through a heat exchanger, filter (25
μm), and “bubble trap.” The perfusate was maintained at 30-31°C. Venous outflow of perfusate was collected and recirculated.

**In situ protocol.** The levels of O₂ and CO₂ in the perfusate were maintained by equilibrating a perfusate reservoir with gas mixtures produced with a precision GSM-2 gas mixer (CWE, Inc., Ardmore, Pennsylvania) and verified with a CD-3A CO₂ analyzer (AEI Technologies, Pittsburgh, Pennsylvania). Control conditions approximated normocapnic plasma *in vivo:* perfusate equilibrated with 95% O₂ - 5% CO₂ entered the aorta with a Pco₂ and pH of 33 mmHg and 7.4, respectively. Neuronal recordings were always initiated under control gas conditions, and followed by a hypercapnic challenge (91% O₂ - 9% CO₂; Pco₂ 60 mmHg; pH 7.2) for 5 minutes. Preparations were then perfused with isoflurane (1%) added to the perfusate using an anesthetic vaporizer (Draeger Medical, Inc., Telford, Pennsylvania).

Extracellular recordings were made using pulled glass electrodes fabricated to produce a tip resistance of 15-20 MΩ, and filled with biotinamide hydrobromide (Life Technologies, Grand Island, New York) dissolved at 5% in 0.5 M sodium acetate. Using a dorsal approach, electrodes were placed in the rostral medullary raphé (r. magnus and r. pallidus) under stereotactic guidance using surface landmarks. Electrodes were placed using a stepping motor (Burleigh Inchworm) held in a stereotaxic 5-axis micropositioner integrated with a Benchmark Angle Two digital brain atlas (MyNeuroLab, St. Louis, Missouri). Recordings were made with a Multiclamp 700B intracellular amplifier (Molecular Devices, Sunnyvale, California) with high pass filter at 300 Hz and low pass filter at 1 kHz Bessel via an Axon CV7B high impedance headstage (Molecular Devices, Sunnyvale, California). Signals were digitized using Spike 2 (CED, Cambridge, England), sampled (>10 kHz) and stored as computer data files.
Spike sorting and analysis. Stable 1- to 3-min periods of single-unit firing frequency were analyzed using Spike 2 software (CED, Cambridge, England). Mean single-unit firing frequencies were analyzed before (baseline), during the last minute of the 5-minute hypercapnic challenge and following a 5-minute return to control conditions. Control recordings and hypercapnic challenges were repeated after at least a 10-minute exposure to 1% isoflurane. If a neuron responded to hypercapnic perfusate with an increase in firing frequency greater than 20% relative to baseline the neuron was considered chemosensitive.

Identification of 5-HT neurons. Putative serotonergic neurons were identified using electrophysiological criteria similar to those used by Veasey et al. (Veasey et al. 1995; 1997). Mason (1997) and Mulkey et al. (2004) used intracellular or juxtacellular labeling of recorded neurons, and demonstrated that electrophysiological criteria identify 5-HT neurons with approximately 90% accuracy.

5-HT raphe neurons typically display a stereotypical slow tonic firing pattern (0.5-2.5 Hz; Mason 1997). Stable 1- to 3-min periods of single-unit firing were analyzed to provide a mean value for unit firing frequency, mean interspike interval, and standard deviation and standard error of mean interspike interval. Cell firing frequency and regularity were used to classify neurons as putative 5-HT neurons (Mason 1997). Briefly, the mean interspike interval ($\bar{x}$, in ms) and the SD of the interspike interval ($s$, in ms) were derived as independent variables and used to solve the discriminant function:

$$y(\bar{x}, s) = 146 \text{ ms} - \bar{x} + 0.98s$$
A value resulting from this function is $<0$ indicates that the cell is likely to be serotonergic (putative 5-HT cell), while a value $>0$ indicates the cell is not likely to be serotonergic. We and others have characterized recordings as putative 5-HT neurons by firing pattern analysis, and confirmed that it is accurate at least 90% of the time when compared to juxtacellular labeling and immunohistochemical neurotransmitter phenotyping (Iceman et al. 2013; Mulkey et al. 2004).

Recorded neurons were filled with biotinamide by applying positive-current pulses (400-ms duration, 50% duty cycle) of gradually increasing intensity (0 - 10 nAmp max in 0.2 nAmp steps) until entrainment of cell discharge to the current pulse was achieved (Iceman et al. 2013). Cell entrainment was maintained for at least 30 s. Current pulses trigger the iontophoretic ejection of biotinamide and entrainment facilitates uptake of this marker by the entrained cell. Entrainment was never initiated when multiple units were visible, and double neuron or ectopic labeling was not observed. Spike height, width and shape were monitored before, during and after juxtacellular entrainment to ensure that only one cell was recorded and labeled.

**Immunohistochemistry.** Preparations were perfused with 4% paraformaldehyde in PBS. Brainstems were removed and submerged in fixative overnight prior to sectioning (Vibratome; 60-µm coronal sections). Biotinamide was revealed with a streptavidin-Alexa 546 conjugate (2 µg/ml; Life Technologies, Grand Island, New York). Sections were then blocked with 1% bovine serum albumin, 3% normal goat serum, and 0.3% Triton X-100, then incubated at 4°C overnight in a mouse anti-TpOH monoclonal antibody diluted 1:1000 in blocking buffer (#T0678; Sigma-Aldrich, St. Louis, Missouri) followed by a goat anti-mouse secondary antibody conjugated to Alexa 488 (1:500; Life Technologies, Grand Island, New York).
Island, New York). Local biotinamide label and TpOH immunoreactivity were visualized using a Zeiss LSM510 confocal microscope.

**Statistical analysis.** All statistical differences were calculated using a two-way repeated measures ANOVA and Holm-Sidak Pairwise multiple comparison procedures with an overall significance level set to p<0.05, unless otherwise indicated (GraphPad Prism V6.01 & SigmaPlot V12). F statistic values reported are for the interaction between isoflurane and pH/CO$_2$. When data is presented as X ± Y, X is the group mean and Y is the standard deviation; all error bars represent the standard error of the mean. For whole-animal experiments with mice, each animal was exposed to increased CO$_2$ levels both in the absence and presence of isoflurane to act as in-subject controls.

**RESULTS**

**Isoflurane severely impaired the hypercapnic ventilatory response in vivo**

We measured the HCVR in awake (unanesthetized) *Lmx1b*/*f*/*f* mice (Fig. 1a), which are on a mixed background of C57BL/6 and 129 strains (Zhao et al. 2006), and are functionally wildtype, and again following at least 15 minutes of exposure to 1% isoflurane (Fig. 1b), the approximate mean alviolar concentration required for surgical anesthesia (1 MAC) (Eger 1981). Increases in inspired CO$_2$ from 0% to 3%, 5% and 7% were performed with O$_2$ maintained at 50% (increased from the normal 21%; balance N$_2$) to reduce the contribution from peripheral chemoreceptors as shown by others previously (Lahiri and DeLaney 1975). In response to 7% CO$_2$, minute ventilation ($\dot{V}_E$) increased by 338% in
unanesthetized Lmx1b/f/f mice (from $2.52 \pm 0.86 \mu l \, g^{-1} \, min^{-1}$ to $11.03 \pm 2.95 \mu l \, g^{-1} \, min^{-1}$; p<0.0001; n=16; Fig. 1c), compared to only 83% in 1% isoflurane (from $2.23 \pm 0.61 \mu l \, g^{-1} \, min^{-1}$ to $4.08 \pm 1.31 \mu l \, g^{-1} \, min^{-1}$; p=0.0004; n=16; Fig. 1c), a reduction in the HCVR to 22% of control ($F_{3,90}=50.03$, p<0.0001, n=16). In contrast, isoflurane had no effect on baseline breathing (from $2.52 \pm 0.86 \mu l \, g^{-1} \, min^{-1}$ in awake Lmx1b/f/f mice to $2.23 \pm 0.61 \mu l \, g^{-1} \, min^{-1}$ in isoflurane; p=0.4880; n=16). $\dot{V}_E$ in 7% CO$_2$ with isoflurane ($4.08 \pm 1.31 \mu l \, g^{-1} \, min^{-1}$) was significantly reduced compared to $\dot{V}_E$ in 7% CO$_2$ in the absence of isoflurane ($11.03 \pm 2.95 \mu l \, g^{-1} \, min^{-1}$; p<0.0001). The slope of the HCVR curve between 3% and 7% CO$_2$ was decreased in anesthetized Lmx1b/f/f mice to 16% of that in unanesthetized mice (Fig. 1c).

We also tested a separate strain of mice, ePet-EYFP, because we used these mice for in vitro experimentation (see below). ePet-EYFP mice are on a mixed background of C57Bl/6 and 129 strains (Scott et al. 2005), and are functionally wildtype. The effects of isoflurane on $\dot{V}_E$ were more robust in ePet-EYFP mice. $\dot{V}_E$ increased by 227% in response to 7% CO$_2$ in unanesthetized ePet-EYFP mice (from $3.41 \pm 1.15 \mu l \, g^{-1} \, min^{-1}$ to $11.14 \pm 4.18 \mu l \, g^{-1} \, min^{-1}$; p<0.0001; n=10; Fig. 1c). In isoflurane, ePet-EYFP mice no longer had a HCVR, as there was no difference in $\dot{V}_E$ at 0% CO$_2$ compared to 7% CO$_2$ ($2.16 \pm 0.53$ vs. $2.45 \pm 0.38 \mu l \, g^{-1} \, min^{-1}$, which was 4% of control; p=0.9947; n=10). In contrast to Lmx1b/f/f mice, isoflurane did decrease baseline breathing (from $3.41 \pm 1.15 \mu l \, g^{-1} \, min^{-1}$ in awake ePet-EYFP mice to $2.16 \pm 0.53 \mu l \, g^{-1} \, min^{-1}$ in isoflurane; p=0.0257; n=10). It is not known why isoflurane had greater effects in ePet-EYFP mice than in Lmx1b/f/f mice. In ePet-EYFP mice, $\dot{V}_E$ in 7% CO$_2$ with isoflurane was significantly reduced compared to $\dot{V}_E$ in 7% CO$_2$ in the absence of isoflurane ($2.45 \pm 0.38$ vs. $11.14 \pm 4.18 \mu l \, g^{-1} \, min^{-1}$; p<0.0001; n=10).
Isoflurane had a particularly profound effect on the breathing frequency ($F_R$) component of the HCVR in both $Lmx1b^{+/+}$ and ePet-EYFP mice (Fig. 1d). In unanesthetized $Lmx1b^{+/+}$ mice, when CO$_2$ was increased to 7%, $F_R$ increased by 85% (from $150.85 \pm 22.67$ breaths min$^{-1}$ to $279.71 \pm 38.95$ breaths min$^{-1}$; $p<0.0001$; $n=16$). In 1% isoflurane, there was no longer any change in $F_R$ with 7% CO$_2$ in $Lmx1b^{+/+}$ mice (from $150.69 \pm 21.86$ breaths min$^{-1}$ to $146.94 \pm 13.57$ breaths min$^{-1}$; $p=0.9413$; $n=16$). In unanesthetized ePet-EYFP mice, when CO$_2$ was increased to 7%, $F_R$ increased by 62% (from $170.94 \pm 15.93$ breaths min$^{-1}$ to $277.29 \pm 22.30$ breaths min$^{-1}$; $p<0.0001$; $n=10$). However, in the presence of 1% isoflurane, there was no increase with 7% CO$_2$ in ePet-EYFP mice (from $164.92 \pm 18.39$ breaths min$^{-1}$ to $148.03 \pm 44.50$ breaths min$^{-1}$; $p=0.3506$; $n=10$).

Isoflurane also blunted the effect of CO$_2$ on tidal volume ($V_T$), albeit less than on $F_R$ (Fig. 1e). In unanesthetized $Lmx1b^{+/+}$ mice, $V_T$ increased by 139% (from $16.44 \pm 3.80$ μl g$^{-1}$ to $39.32 \pm 7.67$ μl g$^{-1}$; $n=16$); while in 1% isoflurane, $V_T$ only increased 89% (from $14.76 \pm 3.18$ μl g$^{-1}$ to $27.85 \pm 8.40$ μl g$^{-1}$; $n=16$). The increase in $V_T$ induced by 7% CO$_2$ in $Lmx1b^{+/+}$ mice was reduced by 43% in the presence of isoflurane ($p<0.0001$). In contrast, $V_T$ in ePet-EYFP mice was more severely reduced. In unanesthetized ePet-EYFP mice, $V_T$ increased by 103% (from $19.81 \pm 6.36$ μl g$^{-1}$ to $40.13 \pm 15.02$ μl g$^{-1}$; $p<0.0001$; $n=10$); however, in isoflurane, $V_T$ only increased 41% (from $13.01 \pm 2.60$ μl g$^{-1}$ to $18.29 \pm 2.81$ μl g$^{-1}$; $p=0.0148$; $n=10$). The increase in $V_T$ induced by 7% CO$_2$ in ePet-EYFP mice was reduced by 74% in the presence of isoflurane ($p<0.0001$).

Isoflurane treatment induced mild hypothermia (from $35.9 \pm 1.6$ °C to $34.7 \pm 1.0$ °C in $0%$ CO$_2$; $F_{(3,45)}=5.190$; $p=0.0036$; $n=16$). As such it was important to determine whether
changes in body temperature had a confounding effect on the results, perhaps indirectly through changes in metabolism. In a separate set of experiments body temperature was held constant at 36 °C during isoflurane administration using a heat lamp controlled by a feedback loop. In the absence of isoflurane, $\dot{V}_E$ increased by 430% (from 2.44 ± 0.47 μl g$^{-1}$ to 12.92 ± 2.18 μl g$^{-1}$; p=0.0016; n=4; Fig. 1f) in response to 7% CO$_2$. In isoflurane, $\dot{V}_E$ did not increase in response to 7% CO$_2$ (from 1.78 ± 0.32 μl g$^{-1}$ min$^{-1}$ to 3.09 ± 0.82 μl g$^{-1}$ min$^{-1}$, which was 13% of control; p=0.1806; n=4; Fig. 1f), $\dot{V}_E$ in 7% CO$_2$ (3.09 ± 0.82 μl g$^{-1}$ min$^{-1}$) was significantly reduced in the presence of isoflurane as compared to $\dot{V}_E$ in 7% CO$_2$ in the absence of isoflurane (12.92 ± 1.09 μl g$^{-1}$ min$^{-1}$; p=0.002). Therefore, the effect of isoflurane on the HCVR was independent of body temperature.

Isoflurane abolished the response of 5-HT neurons to CO$_2$ in the perfused brainstem

The severe depression of the HCVR by isoflurane without a significant effect on baseline ventilation in Lmx1b$^{+/}$ mice was similar to previous observations that genetic deletion (Hodges et al. 2008) or selective inhibition (Ray et al. 2011) of 5-HT neurons also led to a decrease in the HCVR without changing baseline ventilation in adult mice. We hypothesized that isoflurane induced its effects on breathing in part by inhibition of 5-HT neurons, a possibility made more likely because 5-HT neurons express TASK channels at a high level (Talley et al. 2001). To test this possibility, we conducted experiments in an unanesthetized perfused in situ rat brainstem preparation. This preparation maintains an intact and functional respiratory control network and cardiorespiratory reflexes similar to those in vivo (Harris and St -John 2003; Richerson and Getting 1990; St-John and Paton)
2000; Toppin et al. 2007). We first examined whether isoflurane affected baseline firing frequency of 5-HT neurons. Adding isoflurane (1%) to control perfusate (pH 7.4) reduced (Fig. 2a) or completely eliminated (Fig. 2b-c) spontaneous firing in 64% (n=14/22) and 36% (n=8/22) of putative 5-HT neurons, respectively. This effect was reversible (Fig. 2a and 2d). A subset of these neurons was verified to be serotonergic using juxtacellular labeling followed by TpOH immunohistochemistry (Fig. 2e-f).

Extracellular recordings from CO$_2$-stimulated neurons (n=9) meeting the electrophysiological criteria for 5-HT neurons (see Methods) in the rostral portion of the medullary raphé (r. magnus and r. pallidus) revealed that these cells increased their firing frequency when in hypercapnic perfusate (Fig. 3a-c), confirming that 5-HT neurons are chemosensitive in an intact brainstem (Iceman et al. 2013). The size of the response (71% ± 56% increase from control) was consistent with the sensitivity of 5-HT neurons documented in cats in vivo (Veasey et al. 1995; 1997) and the response of the respiratory system as a whole in this perfused brain preparation (St-John and Paton 2000; Toppin et al. 2007). These results verify that 5-HT neurons are chemosensitive even when the glial microenvironment is intact, in agreement with Veasey et al. (Veasey et al. 1995; 1997) who demonstrated that a subset of 5-HT neurons increases their firing rate in response to inspired CO$_2$ in unanesthetized cats in vivo. These data also confirm our recent results using this perfused preparation (Iceman et al. 2013), and add to what has been considered to be the key, missing evidence needed to support the 5-HT neuron chemoreceptor hypothesis; chemosensitivity, in an intact brainstem, of neurons verified to be serotonergic by anatomical methods (Depuy et al. 2011).
Isoflurane (1%) eliminated the acidosis-induced increased firing frequency of 5-HT neurons in the \textit{in situ} perfused brainstem preparation. As was found with the larger set of putative 5-HT neurons noted above, adding 1\% isoflurane to the perfusate eliminated (n=3/9) or markedly decreased (n=6/9) firing in the subset of CO$_2$-stimulated neurons. Subsequent hypercapnia in this isoflurane perfusate resulted in no increase in firing frequency in response to hypercapnia (an increase of 15 ± 72\%; n=9), thus isoflurane completely eliminated the response to hypercapnia (Fig. 3c-e). This effect of isoflurane was reversible (n=8/9; Fig. 3c). A subset of these neurons was verified to be serotonergic using juxtacellular labeling followed by TpOH immunohistochemistry (Fig. 3f).

**Isoflurane abolished firing of 5-HT neurons in culture and prevented increased firing in response to acidosis**

5-HT neurons can be studied in cell culture, where they maintain properties that closely resemble those exhibited in more intact preparations (Wang et al. 2001). We prepared medullary raphé cultures from ePet-EYFP mice (Scott et al. 2005) (Fig. 4a) to understand cellular mechanisms of the effects of isoflurane on respiratory chemoreception. For the 5-HT neuron shown in Figure 4b, acidic aCSF (pH=7.15) induced increased firing that quickly reversed on return to aCSF (pH=7.4). Acidosis consistently increased firing frequency in all 5-HT neurons tested (from 0.43 ± 0.25 Hz in aCSF to 1.90 ± 1.30 Hz in acidic aCSF, an increase of 342\%; p<0.0001; n=15; Fig. 4c), consistent with the degree of chemosensitivity previously reported for 5-HT neurons in culture (Wang et al. 2002; Wang et al. 1998; Wang and Richerson 1999; Wang et al. 2001).
In aCSF, isoflurane (1%) completely eliminated 5-HT neuron firing, and firing did not return in acidic aCSF (Fig. 4b). Firing was restored in aCSF upon washout of isoflurane, as was the acidosis-induced increase in firing frequency. This effect of isoflurane was highly consistent across all recorded neurons (n=15; Fig. 4c). When neurons were exposed to 1% isoflurane in aCSF, the firing frequency decreased from 0.43 ± 0.25 Hz to 0.00 ± 0.00 Hz (n=15). Furthermore, in isoflurane there was no change in firing frequency in response to acidosis (from 0.00 ± 0.00 Hz to 0.12 ± 0.22 Hz; p=0.6635; n=15) (Fig. 4c). The inhibition of 5-HT neurons was reversible upon washout of isoflurane (n=12), as was the acidosis-induced increase in firing frequency (n=12).

Isoflurane did not prevent underlying mechanisms of chemosensitivity in 5-HT neurons

The protocol used above (Fig. 4b) did not distinguish between whether isoflurane blocked the underlying chemosensory mechanisms, or alternatively, simply hyperpolarized 5-HT neurons and prevented them from reaching threshold for action potential generation during acidosis. To differentiate between these possibilities, we examined changes in membrane potential (E_m) in response to acidosis both in the absence and presence of isoflurane. In aCSF without isoflurane, acidosis induced a 3.10 ± 1.05 mV depolarization in E_m (n=13; Fig. 5a). In aCSF, isoflurane (1%) hyperpolarized 5-HT neurons by -6.53 ± 4.26 mV relative to aCSF without isoflurane (n=13; Fig. 5a). When acidic aCSF with isoflurane was superfused into the chamber, the E_m in these neurons depolarized by 3.68 ± 1.95 mV compared to aCSF with isoflurane (n=13; Fig. 5a). However, this was not enough to reverse
the hyperpolarization of $E_m$ caused by isoflurane. There was no difference in the
depolarization induced by acidosis in control conditions compared to acidosis in isoflurane
(p=0.3922, Wilcoxon matched-pairs signed rank test; n=13; Fig. 5a). Thus, 5-HT neurons
retained chemosensitivity, but isoflurane caused sufficient hyperpolarization so that
acidosis could not depolarize them enough to reach action potential threshold.

We next used depolarizing current injection to reverse the hyperpolarization of 5-HT neurons that was induced by isoflurane, and determined whether acidosis would then alter their firing rate. 5-HT neurons were first exposed to acidic aCSF without isoflurane to quantify their chemosensitivity (Fig. 5b). Bath solutions were then switched to aCSF with 1% isoflurane, which hyperpolarized neurons and caused them to stop firing. A sufficient amount of current was then injected to return firing frequency back to the baseline level, and chemosensitivity was reassessed. Prior to isoflurane exposure, tested cells increased action potential frequency in response to acidosis from 0.52 ± 0.31 Hz to 2.02 ± 1.12 Hz, an increase of 288% (p=0.0022; n=12; Fig. 5c). Despite continued exposure to isoflurane, neurons with compensatory current injection still increased their firing frequency when exposed to acidic aCSF from 0.45 ± 0.40 Hz to 2.15 ± 2.09 Hz, an increase of 378% (p=0.0009; n=12). Thus, when hyperpolarization induced by isoflurane was reversed with current injection, there was no difference in firing frequency between control and isoflurane conditions in aCSF (p=0.9285) and acidic aCSF (p=0.7842), indicating that isoflurane did not abolish underlying chemosensory mechanisms in 5-HT neurons.
DISCUSSION

Here we studied the effects of isoflurane on pH/CO$_2$ chemosensitivity of 5-HT neurons in culture and correlated our findings with that of 5-HT neurons \textit{in situ} and with respiratory motor output \textit{in vivo}. Using this approach, we found that isoflurane hyperpolarized 5-HT neurons in culture and eliminated their firing, consistent with activation of TASK channels. This hyperpolarization prevented expression of chemosensitivity, as normally assessed by a change in firing rate in response to acidosis. However, hyperpolarized neurons still depolarized with acidosis, and the firing response to acidosis returned when hyperpolarization was reversed by current injection. Recordings from a perfused brain preparation demonstrated that similar cellular responsiveness existed when 5-HT neurons were embedded within an intact nervous system. Spontaneous firing of 5-HT neurons \textit{in situ} was reduced or abolished, and firing frequency responses of CO$_2$-stimulated neurons were absent in the presence of isoflurane.

Our collection of data from three different preparations, and from different species and strains provide confidence that the results are of general relevance. ePet-EYFP mice were used for both \textit{in vivo} and \textit{in vitro} experiments, allowing the data to be directly compared. Data from whole animal plethysmography were obtained from two different mouse strains, and in both cases there was a similar strong reduction in the HCVR. The inhibition of 5-HT neuron chemosensitivity in cultures from ePet-EYFP mice was recapitulated in the perfused rat brainstem preparation. These findings at the single cell level can explain, in part, why isoflurane (1\%) greatly attenuated the ventilatory response to hypercapnia \textit{in vivo}. These data demonstrate that isoflurane introduces a significant decrease in the ability to detect chemoreception of 5-HT neurons and other neurons that
express significant levels of TASK channels (Talley et al. 2001).

5-HT neurons and central CO₂ chemoreception

5-HT neurons are chemosensitive across many different preparations (see above). However, conflicting interpretations of data (Mulkey et al. 2004) (Depuy et al. 2011) have led to the suggestion that chemosensitivity of 5-HT neurons may be an unnatural property induced by culture conditions. Despite evidence of chemosensitivity *in vitro*, absence of 5-HT neuron response to CO₂ in anesthetized animals *in vivo* has been taken to indicate that 5-HT neurons do not express chemosensitivity within the intact nervous system and, thus, are not central chemoreceptors (Depuy et al. 2011; Mulkey et al. 2004). However, our results demonstrate that 5-HT neurons are chemosensitive in an intact nervous system. The two studies that failed to document 5-HT neuron chemosensitivity *in vivo* used either halothane (Mulkey et al. 2004) or isoflurane (Depuy et al. 2011) anesthesia. Our current results suggest that halogenated anesthetics may have prevented detection of 5-HT neuron chemosensitivity in these two previous *in vivo* studies. This possibility is further supported when the ventilatory sensitivities reported in the various studies are considered. Studies in unanesthetized mice (Hodges et al. 2008) or rats (Davis et al. 2006; Taylor et al. 2005) typically report at least 250% increases in $\dot{V}_E$ with elevation of inspired CO₂ from 0% to 7%. In contrast, during experiments in isoflurane anesthetized rats in which 5-HT neurons were reported to be insensitive to CO₂, $\dot{V}_E$ increased by only 35% when inspired CO₂ was elevated from 0% to 10% (Depuy et al. 2011). Thus, in the studies that failed to detect 5-HT neuron chemosensitivity it is likely that halogenated anesthetics hyperpolarized 5-HT
neurons, obscuring their chemosensitivity and blunting the HCVR.

Previous recordings from 5-HT neurons in the parapyramidal (ppy) region showed that these neurons are not responsive to hypercapnia/hyperoxia in urethane-anesthetized animals after carotid body denervation (Takakura and Moreira 2013). This is consistent with the likelihood that 5-HT neurons in different nuclei and regions serve different physiological roles. For example, 5-HT neurons from rhombomere five (primarily in raphe magnus) are more chemosensitive than 5-HT neurons originating from rhombomeres six and seven, and the former project to integrative respiratory nuclei (Brust et al. 2014b).

**Role of TASK channels in 5-HT neurons**

Isoflurane is well known to potentiate TASK channel currents (Sirois et al. 2000). TASK channels mediate a leak K\(^+\) current, and potentiation of this current causes hyperpolarization. TASK channels are present throughout the nervous system and 5-HT neurons express them at very high levels (Talley et al. 2001). Thus, isoflurane will hyperpolarize and inhibit many neurons, including 5-HT neurons. The effects seen when 5-HT neurons were exposed to isoflurane in cell culture and in the *in situ* perfused brain preparation were consistent with what would be expected with activation of TASK channels. Additionally, it is possible that depression of arousal is due in part to isoflurane-induced inhibition of 5-HT neurons in the midbrain, which have previously been shown to be involved with CO\(_2\)-induced arousal (Buchanan and Richerson 2010).

TASK channels are inhibited by acidosis (Duprat et al. 1997), and have been
proposed to mediate the response of neonatal mouse 5-HT neurons in brain slices to changes in pH from 7.5 to 6.9 (Mulkey et al. 2007). As rat and mouse 5-HT neurons mature they develop a much larger response to acidosis, and are responsive over a narrower pH range (such as between pH 7.4 and 7.2 (Brust et al. 2014a; Wang and Richerson 1999)). Preliminary evidence indicates that a calcium-activated nonselective cation current is the major contributor to chemosensitivity in adult rats and mice (Wu et al. 2009). It is possible that TASK channels also contribute to chemosensitivity of mature 5-HT neurons, but this has not yet been examined. If so, it is unlikely that TASK channels are the sole mediator, as we show here that chemosensitivity of 5-HT neurons is seemingly unchanged by TASK channel potentiation from isoflurane, when the hyperpolarization expected by the influence of isoflurane on TASK channels is compensated by experimental injection of depolarizing current. Whole-animal plethysmography of two different transgenic mouse lines demonstrated that TASK channels were not required for the HCVR in vivo (Mulkey et al. 2007), but were necessary for peripheral chemoreception (Trapp et al. 2008). Previous work from our laboratory has shown that inhibition or deletion of 5-HT neurons decreases central respiratory chemoreception in vivo (Brust et al. 2014a; Hodges et al. 2008; Ray et al. 2011). Therefore, if TASK channels mediate chemosensitivity of 5-HT neurons, then there should also be a decrease in the HCVR after genetic deletion of TASK channels, but that is not the case (Mulkey et al. 2007).

In 5-HT neurons, the change in conductance of TASK channels is small over the pH range studied here (7.4 to 7.15) (Teran et al. 2014; Washburn et al. 2002), so it would not be expected to cause the large change in firing frequency seen in 5-HT neurons over this pH range. It is also unlikely that such a small shift in TASK conductance caused by acidosis
would be enough to cause the firing frequency of these neurons to increase to 300% of baseline, when motor neurons express higher levels of TASK channels but are not strongly depolarized by acidosis (Talley et al. 2001; Washburn et al. 2002). In fact, the effect of pH on motor neurons is actually the opposite, with the common, reproducible clinical observation that respiratory alkalosis causes tetany (Brown 1953). The firing frequency responses of 5-HT neurons to acidosis were neither exaggerated nor blunted in isoflurane, when the hyperpolarizing influences were compensated. Based on these and other reasons, it is unlikely that TASK channels are the mediators of 5-HT neuron chemosensitivity (Corcoran et al. 2009; Teran et al. 2014).

Effect of isoflurane on respiratory chemoreception

Here we have shown that cell culture can be used to study 5-HT neuron chemosensitivity, and how it is influenced by the anesthetic isoflurane. We then recorded from 5-HT neurons in a perfused brainstem preparation and determined how isoflurane affected those neurons and their chemosensitivity within an intact respiratory network and a normal glial-vascular microenvironment. Finally, we studied the effect of isoflurane on the response of the whole animal to inhalation of CO₂ and illustrated that the influence of isoflurane on chemoreception in vivo is consistent with its influence on chemosensitive 5-HT neurons in vitro and in situ. Our ability to correlate the effect of a perturbation on a single neuron, with the effect of that perturbation on the motor behavior of the intact animal allowed us to define a cellular mechanism that may contribute to the effect of isoflurane on respiratory chemoreception in vivo.
Isoflurane, halothane, and other halogenated anesthetics have been frequently used in both research and clinical settings. These agents can alter the outcome of experiments on chemoreception and control of breathing more than is appreciated by some investigators. In the current experiments, isoflurane markedly depressed the HCVR, but had no effect on baseline breathing in Lmx1b/f/f mice. This is reminiscent of what is seen when 5-HT neurons are genetically deleted in adult Lmx1b/f/f/p mice (Hodges et al. 2008) or selectively silenced in adult mice expressing DREADD receptors on 5-HT neurons (Brust et al. 2014a; Ray et al. 2011). The reason for dissociation of the effects on baseline breathing and the HCVR in Lmx1b/f/f is unknown. TASK channels are widely expressed in the brainstem, including on 5-HT neurons, motor neurons and in respiratory nuclei, and when activated would cause inhibition at multiple sites in the respiratory network. Therefore, isoflurane should cause a blunted HCVR and depressed breathing at baseline, as seen in ePet-EYFP mice. However, neurons in the RTN are one of the exceptions, because they express TWIK-related halothane-inhibited K⁺ (THIK-1) channels, which are K⁺ channels that are inhibited, rather than activated, by halogenated anesthetics (Lazarenko et al. 2010; Rajan et al. 2001). As a result RTN neurons would be activated by isoflurane and halothane, and should stimulate the respiratory network, counteracting inhibition at other sites, potentially explaining why ventilation is maintained constant at baseline in isoflurane in Lmx1b/f/f mice.

TASK channels are also expressed in peripheral chemoreceptors. Our use of 50% O₂ during plethysmography recordings was designed to minimize contributions of peripheral chemoreceptors as previous work has demonstrated (Hodges et al. 2008; Lahiri and DeLaney 1975). The effect of halogenated anesthetics on the HCVR in our experiments could potentially be explained largely via inhibition of central chemoreceptors, including 5-
HT neurons. Our data do not, however, exclude an effect on other isoflurane-sensitive chemoreceptors, including peripheral chemoreceptors. They also do not exclude an effect on other elements of the respiratory network.

The gold standard for defining normal neuronal activity is widely considered to be extracellular recording from neurons in vivo. However, this approach typically requires the use of anesthesia, and two of the most common and convenient agents used are halothane and isoflurane. These halogenated anesthetics alter respiratory physiology so severely that in vivo preparations using these agents should not be assumed to reflect normal physiology in studies of breathing. In particular, the use of these anesthetics may greatly underestimate the relative contribution of chemoreceptors that express TASK channels, such as 5-HT neurons.
Competing interests: The authors declare no competing financial interests.

Author contributions: C.A.M. performed all plethysmography and patch-clamp recording experiments. K.E.I. and S.L.J. performed all perfused brainstem recordings and analyzed the data. Y.W. prepared, optimized and maintained medullary raphé cultures. C.A.M., M.B.H., and G.B.R. designed the experiments, analyzed the data, and wrote the first draft of the manuscript. All authors edited the manuscript.

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FIGURE LEGENDS

**Figure 1. Isoflurane severely impaired the HCVR in vivo.**

a) Whole animal plethysmography recordings from an Lmx1b\(^{-/-}\) mouse in 0% CO\(_2\) (above) and 7% CO\(_2\) (below), both in 50% O\(_2\). b) Identical recordings from the same mouse as in (a) except for the addition of 1% isoflurane to the inspired gas. c) Isoflurane caused a large reduction in the slope of \(\dot{V}_E\) vs. inspired CO\(_2\) in both Lmx1b\(^{-/-}\) (black lines; \(F_{3,45}=50.03, p<0.001, n=16\)) and ePet-EYFP mice (green lines; \(F_{3,27}=39.37, p<0.001, n=10\)). d) Isoflurane ablated the increase in FR as inspired CO\(_2\) increased in both Lmx1b\(^{-/-}\) (\(F_{3,45}=69.08, p<0.001, n=16\)) and ePet-EYFP mice (\(F_{3,27}=35.64, p<0.001, n=10\)). e) Increases in VT were reduced in isoflurane as inspired CO\(_2\) increased to 5% and above in both Lmx1b\(^{-/-}\) (\(F_{3,45}=14.42, p<0.001, n=16\)) and ePet-EYFP mice (\(F_{3,27}=20.85, p<0.001, n=10\)). f) Isoflurane greatly reduced the HCVR in response to 7% CO\(_2\) when body temperature during anesthesia was held at 36 °C (\(F_{1,3}=73.04, p=0.0034, n=4\)). * - p<0.05, *** - p<0.001 for Lmx1b\(^{-/-}\) mice; + - p<0.05, +++ - p<0.001 for ePet-EYFP; † - p<0.0001 for both Lmx1b\(^{-/-}\) and ePet-EYFP mice.

**Figure 2. Isoflurane inhibited baseline firing of 5-HT neurons in the perfused brainstem preparation.**

a) A putative 5-HT neuron in the perfused brainstem preparation decreased its firing frequency in response to 1% isoflurane, with recovery upon washout. b) Spontaneous firing of a putative 5-HT neuron (different than in (a)) exposed to control perfusate. c) Isoflurane (1%) abolished firing of neuron in (b). d) Spontaneous firing of neuron in (b) returned after washout of isoflurane. e-f) Photomicrographs of the two juxtcacellularly labeled cells in a (e) & b-d (f), confirmed they were 5-HT neurons.
Figure 3. Isoflurane abolished the change in firing rate of a 5-HT neuron \textit{in situ} in response to acidosis. \textbf{a)} Spontaneous firing of a putative 5-HT neuron exposed to control perfusate (pH 7.4) \textit{in situ}. \textbf{b)} Firing frequency increased in response to hypercapnic perfusate (pH 7.2). \textbf{c)} Summary of recordings testing 5-HT neuron chemosensitivity \textit{in situ} (n=9). 5-HT neurons were chemosensitive under control conditions (p=0.03). Isoflurane significantly reduced firing frequency (p=0.02) and caused loss of the response to hypercapnic perfusate. Firing frequency returned to baseline levels after isoflurane was washed out. \textbf{d)} Isoflurane (1%) caused a decrease in firing frequency of the neuron in (a). \textbf{e)} In isoflurane, the neuron no longer responded to hypercapnic perfusate with an increase in firing frequency. \textbf{f)} Juxtacellular labeling confirmed this was a 5-HT neuron. Biotinamide (red); TpOH immunostaining (green); Co-localization (yellow). Scale bar - 50 µm. * - p<0.05, *** - p<0.001.

Figure 4. Isoflurane inhibited firing of 5-HT neurons \textit{in vitro} and abolished their response to CO$_2$. \textbf{a)} Confocal microscopy image of a cultured medullary ePet-EYFP neuron. EYFP – green. DAPI nuclear stain – blue. Scale bar - 25 µm. \textbf{b)} Firing frequency and pH of a recording from a cultured medullary 5-HT neuron. Firing increased during acidosis. 1% isoflurane eliminated firing in aCSF and firing did not return in acidosis. \textbf{c)} Summary of current clamp recordings. 5-HT neurons had a robust increase in firing frequency in response to acidosis (Ctrl, n=15). Isoflurane eliminated firing and prevented any change in
response to acidosis (1% Iso, n=15). These effects were reversible (Wash, n=12). A two way repeated measures ANOVA revealed a significant effect of pH ($F_{1,39}=41.77; p<0.0001$) and isoflurane ($F_{2,39}=12.25; p<0.0001$). Furthermore there was an interaction between pH and isoflurane ($F_{2,39}=8.568; p=0.0008$). * - $p<0.05$, *** - $p<0.001$.

**Figure 5. Isoflurane did not abolish underlying chemosensitive mechanisms in 5-HT neurons in culture.**

**a)** Summary of changes in $E_m$ of cultured 5-HT neurons induced by acidic aCSF (Acid., black bar), isoflurane (1% Iso, gray bar), and acidic aCSF in isoflurane (Acid. + 1% Iso, white bar) ($F=56.12; 1p<0.001; n=13$). **B)** Recording from a 5-HT neuron in culture whose firing was abolished by isoflurane. Current injection was increased from 77.7 pA to 159.8 pA to reverse the hyperpolarization induced by isoflurane. The response to acidosis was then as large as control despite the continued presence of isoflurane. **C)** Summary of the effect of acidosis while giving current injection to reverse the hyperpolarization induced by isoflurane. Shown are firing frequencies in aCSF (pH 7.4) and acidic aCSF (pH 7.15) under control conditions (Ctrl, n=12), in isoflurane while giving extra depolarizing current (1% Iso + depol., n=12), and during washout of isoflurane (Wash, n=7). Chemosensitivity remained intact in isoflurane ($F_{1,28}=40.01; p<0.0001$) and there was no effect of isoflurane after an increase in current injection ($F_{2,28}=0.4537; p=0.6399$) or an interaction between pH and isoflurane ($F_{2,28}=0.1227; p=0.8850$). * - $p<0.05$, *** - $p<0.001$. 

844

845

846

847
Figure 1

(a) Control

(b) 1% Isoflurane

(c) Emax vs. Inspired CO2

(d) Frequency vs. Inspired CO2

(e) Ymax vs. Inspired CO2

(f) Vel vs. Inspired CO2
Figure 2

(a) 1% Isoflurane

(b) Control

(c) 1% Isoflurane

(d) Washout

(e) Immunochemistry of a rat brain slice stained for a specific protein.

(f) Enlargements of images showing the distribution of the stained protein.
**Figure 3**

(a) Control

(b) 5% CO₂ and 9% CO₂

(c) Firing Rate (Hz)

(d) 1% Isoflurane

(e) 5% CO₂ and 9% CO₂

(f) Immunofluorescence images
Figure 4

(a) Image showing a neuroanatomical structure with labeled cells. Scale bar indicates the scale of the image.

(b) Graph showing firing rate (Hz) vs pH. The x-axis represents pH values ranging from 7.4 to 7.2, and the y-axis represents firing rate in Hz. A line graph indicates a peak firing rate at pH 7.3, with a 1% Iso label at the top.

(c) Bar chart showing firing rate (Hz) for pH 7.4 and pH 7.15. The x-axis represents different conditions: Ctrl, 1% Iso, and Wash. The y-axis represents firing rate in Hz. The chart includes statistical significance labels: *** for pH 7.4 and *** for pH 7.15, with a p-value of 0.0622 for the 1% Iso condition.
Figure 5

(a) Graph showing the change in membrane potential ($\Delta E_{m}$) with acid, 1% iso, and acid + 1% iso conditions. The p-value is 0.3922.

(b) Graph showing firing rate (Hz) with pH 7.4 and 1% Iso, and pH 7.2 and 1% Iso conditions. The firing rates are 77.7 pA and 159.8 pA, respectively.

(c) Bar graph showing firing rate (Hz) with pH 7.4, pH 7.15, 1% Iso + depol., and wash conditions. The bars are labeled as follows: Ctrl, 1% Iso, Wash, and depol. with significance levels indicated by ***.