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Isoflurane causes concentration-dependent inhibition of medullary raphé 5-HT neurons *in situ*

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1. Introduction

The cellular mechanisms and neuron types involved in producing the state of anesthesia, and associated decreases in autonomic activity, are largely undefined (Kotrly et al., 1984; Seagard et al., 1984; Pagel et al., 2007; Paisansathan et al., 2007). Serotonin-synthesizing (5-HT) neurons, primarily localized in the midline medulla, have widely distributed projections that influence homeostatic processes across the autonomic, motor, somatosensory, and limbic systems (Jacobs and Azmitia, 1992; Lovick 1997; Hornung 2003; Madden and Morrison, 2006). Serotonergic ascending projections to the forebrain mediate arousal, cognition, anxiety, and cerebral blood flow (Jacobs and Azmitia, 1992). Descending projections to the brainstem and spinal cord meditate respiratory, cardiovascular and motor control, thermoregulation, and nociception (Pérgola and Alper, 1992; Richerson 2004; Madden and Morrison, 2006; Watts et al., 2012). Investigation has shown anesthetic, analgesic, hypnotic, and immobilizing actions of volatile anesthetics such as isoflurane on post-synaptic receptor types that are targets of serotonergic projections (Franks and Lieb, 1994; Minami et al., 1997; Bardin et al., 2000; Campagna et al., 2003; Zhang et al., 2003; Mukaida et al., 2007; Nagatani et al., 2011). Although volatile anesthetics also influence presynaptic mechanisms that alter serotonergic neuron activity (Bayliss and Barrett, 2008; Orestes and Todorovic, 2010; Herold and Hemmings, 2012), such action is poorly understood and uncharacterized in intact nervous systems. One study demonstrated sensitivity of serotonergic neurons *in vitro* to various anesthetics, but omitted volatile anesthetics such as isoflurane (McCardle and Gartside, 2012). The influence of volatile

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anesthetics on 5-HT neurons is poorly understood and yet may contribute greatly to states of anesthesia.

Recently it has been demonstrated that there are distinct and specialized subsets of serotonergic neurons which may represent cells with intrinsic functional differences (Brust et al., 2014). One genetically defined subset of serotonergic neurons are intrinsically sensitive to CO_2/pH. We have recently shown that these CO_2/pH sensitive 5HT neurons are severely hyperpolarized by isoflurane, suggesting a mechanism for ventilatory and chemosensory depression during anesthesia (Massey et al., 2015). In the present study, we specifically exclude this subset of neurons to investigate a potentially more general characteristic of isoflurane sensitivity. We previously characterized the firing patterns of conclusively identified 5-HT neurons in intact unanesthetized brainstem preparations using extracellular recordings, juxtacellular labeling, and immunohistochemistry (Johansen et al., 2012; Iceman et al., 2013; Iceman and Harris, 2014; Massey et al., 2015). Using these methods, we now test the hypothesis that 5-HT neurons are inhibited by isoflurane, and we speculate that such influence could contribute to the widespread actions of anesthesia.

2. Methods

2.1 Experimental animals and preparations

All experiments were done in accordance with the National Institutes of Health “Guide for the Care and Use of Laboratory Animals” guidelines and were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee. Juvenile male rats (50 – 150 g; Sprague-Dawley strain; Simonson Laboratories, Gilroy, CA, USA) were used to generate the perfused *in situ* brainstem preparation as previously described (Paton 1996; Johansen et al., 2012; Corcoran et al., 2013; Iceman et al., 2013; Iceman and Harris, 2014; Massey et al., 2015). Briefly, animals were administered an intraperitoneal heparin sodium injection (0.5 mL of 1000 i.u./mL; Baxter, Deerfield, IL, USA) and deeply anaesthetized with isoflurane. Preparations were bisected sub-diaphragmatically, decerebrated rostral to the superior colliculi, and immersed in chilled artificial cerebral spinal fluid for the remainder of the dissection. Subsequent procedures were conducted without anesthesia.

Preparations were placed prone in the recording chamber. The descending aorta was cannulated retrogradely with a double-lumen catheter, and preparations were perfused with solution at a temperature of 31 °C. The perfusate solution contained the following (in mM): 1.0 $MgSO_4$, 125 NaH_2PO_4, 4.0 KCl, 24 $NaHCO_3$, 115 NaCl, 10 D-Glucose, 2.0 $CaCl_2$, and 0.18 Ficoll. Under baseline conditions, the perfusate was equilibrated with 95% O_2 – 5% CO_2 (PCO_2 33 mmHg; pH 7.4). The neuro-muscular blocker gallamine triethidodide (60 mg/L) was added to the perfusate to eliminate movement. The pressure of aortic perfusion was gradually increased to 50–75 mmHg and held constant.

2.2 Extracellular recording and neuron selection

Extracellular recordings were conducted using pulled-glass capillary electrodes (15–30 MΩ) filled with biotinamide hydrobromide (5%; Life Technologies, Carlsbad, CA, USA) in 0.5 M sodium acetate, as previously described (Iceman et al., 2013; Iceman and Harris, 2014).

We targeted medullary raphé (r. magnus, r. obscurus, r. pallidus) neurons along the midline (≤ 0.1 mm lateral) between 0 and 3 mm rostral of obex. Extracellular recordings were made with an intracellular amplifier (Axon Instruments Multiclamp 700B) in current clamp mode, with a high pass filter at 300 Hz and low pass filter at 1 kHz Bessel, using a high impedance headstage (Axon CV7B, Molecular Devices). Single unit action potentials were digitized at 25 kHz using Spike 2 (Cambridge Electronic Design Power 1401) and LabChart (AD Instruments) software and stored as computer data files.

Cells were initially screened for being serotonergic by real-time assessment of firing frequency and regularity. Cells with firing patterns characteristic of serotonergic neurons (tonic firing without sustained pauses or phasic bursts at frequencies below 3 Hz) were considered potential serotonergic neurons during experimental recording. Recordings of cells not matching these general criteria were not maintained. Neuronal recordings were initiated under baseline conditions (perfusate equilibrated with 95% O_2 – 5% CO_2; PCO_2 33 mmHg; pH 7.4). These conditions approximate normocapnic plasma *in vivo*. Due to the lack of haemoglobin, solution hyperoxia (PO_2 ~ 600 mmHg) was necessary to maintain O_2 content sufficient to meet tissue metabolic demands, and this unavoidable hyperoxia was constant under all conditions. We have previously considered the impact of isoflurane on the CO_2-sensitive subset of 5-HT neurons (Johansen et al., 2012; Massey et al., 2015) and designed the current investigation to determine such impact on CO_2-insensitive 5-HT cells. To exclude CO_2-sensitive cells, once stable recordings were obtained, preparations were briefly exposed to perfusate equilibrated with 91% O_2 – 9% CO_2 (PCO_2 60 mmHg; pH 7.2; 5 min). Cells that changed mean firing frequency by greater than 20% were excluded from the current analysis (Johansen et al., 2012; Iceman et al., 2013; Massey et al., 2015). Cells were returned to baseline conditions for at least 5 min prior to experimental protocols.

2.3 Isoflurane treatments

Isoflurane treatments were administered as previously described (Johansen et al., 2012). Isoflurane was bubbled into the perfusate for 10 min (1, 1.5, or 2% isoflurane in 95% O_2 – 5% CO_2). 1% isoflurane approximates 1.15%, which is 1 MAC, the minimum alveolar concentration of isoflurane necessary for surgical anesthesia (Eger 1981). Upon return to baseline conditions (0% isoflurane; 95% O_2 – 5% CO_2), the neuron was allowed to recover toward its baseline firing frequency.

2.4 Juxtacellular labeling

Subsets of recorded neurons were individually filled with biotinamide using the juxtacellular labeling method, as previously described (Pinault 1996; Iceman et al., 2013; Iceman and Harris, 2014). Extracellular recordings were conducted in current clamp mode (Axon Multiclamp 700B) to allow current to be injected through the electrode while action potentials were monitored. Positive current pulses (400-ms duration) were applied and gradually increased from 0-15 mV (0.5 mV steps) until cell discharge entrained to the stimulus for at least 30 s, resulting in uptake of the ejected biotinamide by the recorded cell. After termination of entrainment, the biotinamide was allowed to disperse for 30 min. Rats were perfused through the descending aorta with 4% paraformaldehyde (in 0.1 M PBS).

Brainstems were removed and stored in fixative overnight. Coronal sections (60 μm) were cut through the medulla (Vibratome 1000Plus), and sections were processed free-floating.

2.5 Immunohistochemistry

Tissue sections were first incubated in blocking buffer for 1 hr at 20 °C (5% normal goat serum, 0.3% Triton X-100 in 0.1 M PBS), then a streptavidin-Alexa 546 conjugate for 2 hr at 20 °C (Life Technologies #S-11225; 1:500 dilution in PBS with 5% NGS) to reveal biotinamide introduced into single neurons by juxtacellular labeling. To identify serotonergic cells, sections were incubated overnight at 4 °C with mouse monoclonal anti-tryptophan hydroxylase (TPH) antibody (Sigma-Aldrich #T0678; 1:1000 dilution in blocking buffer), then incubated for 2 hr at 20 °C in secondary goat anti-mouse Alexa 488 antibody (Life Technologies #A-11029; 1:500 dilution in 0.1 M PBS with 5% NGS). Fluorophores were individually excited and emission spectra collected separately to minimize interference using a Zeiss LSM510 confocal microscope: biotinamide-labeled neuron, Alexa 546, 543 nm laser, filter BP 560-615; anti-TPH, Alexa 488, 488 nm laser, filter BP 505-530. Images are presented as 20× projections of z-stacks.

2.6 Gas chromatography – mass spectrometry

All gas chromatography – mass spectrometry (GCMS) analyses were performed on an Agilent Technologies 7890A GC System and 5975C mass selective detector. Helium carrier gas was used at a constant flow rate of 20 mL min^{-1}. Samples were injected into a DB-1 ms capillary column (45 m × 0.25 mm, film thickness 0.25 μm) at a split ratio of 50:1. The system oven temperature was initially maintained at 30 °C for 6 min, and then increased at a rate of 10 °C min^{-1} from 30 to 120 °C. MS acquisition was performed in full scan mode from 40 to 200 amu. Mass conditions were as follows: ionization voltage, 70.0 eV; ion source temperature, 230 °C; detector voltage, 1.6 kV.

A saturated solution of isoflurane (15 mM) was prepared by adding aliquots of isoflurane to perfusate solution and stirring in an airtight container for 3 hr (Scheller et al., 1997). Different concentrations were prepared by diluting the saturated solution in perfusate (1:2, 1:4, 1:8, 1:16, 1:32, and 1:64). Samples (250 μL) of headspace gas from each dilution were analysed by GCMS for isoflurane intensity to generate a standard curve for isoflurane. The standard curve was fit to a linear regression line (SigmaPlot). Samples of perfusate were taken from the aortic cannula, to best approximate perfusate received by experimental preparations, at each minute during a 10-min 1% isoflurane exposure. The GCMS headspace analysis of these samples yielded a value for mean peak area, corresponding to the number of isoflurane molecules in solution, and the concentration of isoflurane in solution at each minute of the drug exposure was calculated from the standard curve equation. In all cases the standard curve and perfusate samples were run in triplicates and average isoflurane peak area was used to determine concentration.

2.7 Data analysis

Stable 1-2 min periods of individual unit firing were analysed (Spike 2; Spike Histogram, AD Instruments) before and during isoflurane treatment to provide a mean value for unit firing frequency (spikes/s) and interspike interval (ms). Paired *t*-tests or one-way ANOVA

and post-hoc Holm-Sidak pairwise comparison tests were used to compare means (SigmaPlot). Values are expressed as mean ± standard error of the mean. The criteria for statistical significance was $P < 0.05$.

2.8 Neuron classification

Putative serotonergic cells were identified based on an assessment of frequency and regularity of firing. In this assessment the mean interspike interval ($\bar{X}$) and standard deviation of the intervals (sd) are considered independent variables. The algorithm $Y(\bar{X},\bar{sd}) = 146 - \bar{X} + 0.98sd$ was used to characterize or exclude spontaneously firing neurons as serotonergic (Mason 1997). If the value of this function was less than zero [$Y(\bar{X},\bar{sd}) < 0$], the cell was predicted to be serotonergic, while function values greater than zero [$Y(\bar{X},\bar{sd}) > 0$] suggested the cell was non-serotonergic. All neurons selected for recording by real-time assessment of firing pattern and regularity were subsequently classified as putative serotonergic cells by the algorithm.

3. Results

3.1 Characteristics of medullary raphé 5-HT neurons

Cells were initially selected for firing characteristics expected of 5-HT neurons, including large spike width, long after-hyperpolarization, and regular firing with tonic frequencies below 3 Hz without sustained pauses or phasic bursts. Putative 5-HT neurons (n=38) had baseline firing frequencies between 0.1 to 2.01 Hz and firing patterns consistent with serotonergic neuron characteristics. All neurons selected for recording were classified as putative 5-HT neurons by subsequent regularity assessment (Mason 1997). In our previous work, 5-HT neurons that exhibited the above firing characteristics were conclusively identified through juxtacellular labelling followed by immunohistochemical localization of the serotonin-synthesizing enzyme tryptophan hydroxylase (TPH) (Johansen et al., 2012; Iceman et al., 2013; Iceman and Harris, 2014).

3.2 Isoflurane inhibits firing of medullary raphé 5-HT neurons

Single unit extracellular recordings performed on putative 5-HT neurons before, during, and after isoflurane treatment indicate that isoflurane inhibited the action potential discharge of these neurons. A subset of these neurons (n=3) were juxtacellularly filled with biotinamide and confirmed as serotonergic by colocalization of TPH in the biotinamide-labeled neuron. A representative cell is shown in Figure 1. All recorded neurons were classified as putative 5-HT neurons by the algorithm that distinguishes between serotonergic and non-serotonergic cells (Mason 1997).

3.3 Isoflurane inhibits firing of raphé 5-HT neurons at different concentrations

All preparations (n=38, one cell recorded per animal) received isoflurane at either 1% (n=18); 1.5% (n=10); or 2% (n=10). 36 of the 38 recorded cells (95%) were inhibited or silenced by isoflurane. For 2/38 cells, baseline firing either did not significantly change (7.89% increase) or significantly increased (48.0% increase) in response to 1% isoflurane (data not shown). Mean baseline firing frequencies during 0% isoflurane (1% isoflurane: 0.964 ± 0.11 Hz; 1.5% isoflurane: 1.00 ± 0.15 Hz; and 2% isoflurane: 0.95 ± 0.16 Hz) for

each treatment group were not significantly different ($P = 0.971$). The mean firing frequencies in the last minute of isoflurane exposure for each isoflurane dose (1% isoflurane: 0.349 ± 0.11 Hz; 1.5% isoflurane: 0.113 ± 0.04 Hz; or 2% isoflurane: 0.021 ± 0.01 Hz) were each different from those under baseline conditions prior to isoflurane exposure ($P < 0.001$, paired *t*-test), indicating that isoflurane inhibits medullary raphé 5-HT neurons throughout the range of concentrations approximating MAC.

3.4 Isoflurane-induced inhibition of raphé 5-HT neurons is concentration-dependent

The mean percent decrease of firing rate from baseline during isoflurane exposure was calculated for each isoflurane treatment group (1% isoflurane: −68.96 ± 9.57%; 1.5% isoflurane: −89.78 ± 3.52%; 2% isoflurane: −97.76 ± 1.24%). A comparison of these mean percent changes using one-way ANOVA indicated a significant difference between neurons receiving 1% and 2% isoflurane ($P < 0.05$), demonstrating that isoflurane-induced inhibition of putative 5-HT neurons was concentration-dependent (Fig. 2). The proportional changes induced by 1 versus 1.5% isoflurane were not significantly different ($P = 0.147$) and neither were the changes induced by 2% versus 1.5% isoflurane ($P = 0.541$).

3.5 Presence of isoflurane in the perfusate confirmed by gas chromatography – mass spectrometry analysis

Aliquots of the perfusate sampled during administration of isoflurane were analysed by GCMS to confirm that the experimental preparation received the intended concentration of isoflurane via the perfusate. A chromatogram of a sample of 1% isoflurane in perfusion solution indicates two distinct peaks: CO_2 and isoflurane (Fig. 3a). The peak at retention time 3.20 min was identified as isoflurane based on the mass spectra ($C_3H_2ClF_5O$; molecular weight = 184.49 g/mol), which showed characteristic molecular and fragment ions: m/z 149 ($C_3H_2F_5O$ – Cl) and 116.9 (C_2HClF_3 – $OCHF_2$) (Fig. 3b).

3.6 Standard curve for isoflurane based on GCMS analysis of perfusate

A standard curve for isoflurane was generated from a saturated solution of isoflurane (15 mM) (Scheller et al., 1997). Dilutions of the saturated solution (1:2, 1:4, 1:8, 1:16, 1:32, and 1:64) were analysed by GCMS. The mean peak area for each dilution, corresponding to the number of isoflurane molecules in solution, was used to generate the standard curve for isoflurane (Fig. 3c), which was fit to a linear regression line described by the following equation: $y = 6.2*10^6 \times + 6.0*10^5$ ($R^2 = 0.994$).

3.7 Isoflurane concentration in perfusate is equivalent to 1 volume percent

Isoflurane is poorly soluble in water, but readily soluble in plastic and rubber tubing (Eger 1981); thus, we confirmed the concentration of isoflurane received by the preparation through the perfusate using GCMS analysis. At 10 min, the time point when all neuronal responses to isoflurane were recorded, the mean equilibrium isoflurane concentration was 0.38 mM, which was equivalent to 1 volume percent (Fig. 3d; 0.38 mM = mean concentration from 6 to 10 min; 0.37 mM = 1 volume percent; Eilers et al., 1999). The 6 to 10 min range was chosen to calculate the mean equilibrium concentration because the concentration steadily increased from 3 to 6 min and remained relatively constant from 6 to

10 min. Additional samples taken at 11 and 15 min demonstrate stability throughout the recording period.

4. Discussion

In conjunction with our earlier characterization of isoflurane-sensitivity of specifically CO_2-stimulated 5-HT neurons, we present the first evidence that isoflurane inhibits firing of caudal raphé CO_2-insensitive 5-HT neurons. Isoflurane routinely inhibited 5-HT neuron discharge, or caused firing to cease completely (Fig. 1). The degree of inhibition increased as the concentration of isoflurane in the perfusion solution increased, and the difference between neuronal responses to 1 and 2% isoflurane indicated concentration-dependent responsiveness of putative 5-HT neurons to isoflurane within a clinically relevant range (Fig. 2). The observed trend toward significant inhibition in the 1 to 1.5% comparison group with little evidence for further inhibition in the 1.5 to 2% comparison may be due to neuronal activity reaching maximal inhibition at 1.5% isoflurane. 1% isoflurane approximated 1 MAC (confirmed by GCMS analysis; Fig. 3), the minimum alveolar concentration of isoflurane necessary for surgical anesthesia (Eger 1981), which allowed us to confirm that our preparations received isoflurane at clinically relevant concentrations.

We have previously reported that isoflurane hyperpolarizes and inhibits/abolishes the tonic firing of a distinct subset of CO_2-sensitive 5-HT neurons (Massey et al. 2015), and here illustrate similar isoflurane-mediated inhibition of CO_2-insensitive neurons. Both of these cell types fire in a manner expected of 5HT neurons (Mason 1997). Importantly, there are populations of 5-HT neurons that do not fire with this expected discharge pattern. These, as well as non-5-HT neurons, have not been assessed for sensitivity to isoflurane. As such, we cannot conclude that isoflurane sensitivity is either a universal or exclusive feature of 5HT neurons. Isoflurane binds to two-pore domain potassium (TASK) channels (Bayliss and Barrett, 2008), which mediate instantaneous open-rectifier background K+ currents that are potentiated by volatile anesthetics, resulting in hyperpolarization. (Sirois et al., 1998; Patel et al., 1999; Sirois et al., 2000; Talley et al., 2000). Two subtypes of TASK channels, TASK-1 and TASK-3, are highly expressed in the raphé nuclei (70-90% of 5HT neurons) compared to other brain regions (Talley et al., 2001; Washburn et al., 2002). We postulate that potentiation of the TASK channel-mediated background K+ current by isoflurane may have hyperpolarized our target population of raphé 5-HT neurons. Isoflurane sensitivity may be absent in cells lacking TASK channels, and would be expected in any cell type that expresses these channels.

Many studies on the influence of anesthetics on 5-HT neurons have focused on molecular action at postsynaptic 5-HT receptors (Minami et al., 1997; Bardin et al., 2000; Mukaida et al., 2007; Nagatani et al., 2011). Our demonstration that isoflurane inhibits action potential generation in 5-HT neurons suggests that isoflurane acts on presynaptic sites, a hypothesis that, until recently (Griffiths and Norman, 1993; Pocock and Richards, 1993), has been overshadowed by consideration of postsynaptic anesthetic effects. Presynaptic binding sites for volatile anesthetics have been reported (Orestes and Todorovic, 2010; Herold and Hemmings, 2012; Hemmings 2009). Blockade of Na^+ channels (Wu 2004; Ouyang and Hemmings, 2005) and inhibition of Ca^{2+} currents (Study 1994; Nikonorov et al., 1998;

Kamatchi et al., 1999) by anesthetics can inhibit action potential conductance and depress neurotransmitter release. The influence of isoflurane on these channels could also contribute to the inhibition of action potential discharge of 5-HT neurons by isoflurane.

The effect of anesthetics on 5-HT neuron activity in the dorsal raphé ascending system that mediates arousal, cognition, anxiety, and cerebral blood flow has been characterized (McCardle and Gartside, 2012), but the effect of anesthetics on 5-HT neurons in the caudal raphé descending system remains a pertinent question. This caudal raphé population of 5-HT neurons that we targeted mediates autonomic output, respiratory, cardiovascular and motor control, and nociception (Richerson 2004). Inhibition of this serotonergic system by isoflurane may contribute to the immobilization, hypnosis, sedation, analgesia, and cardiorespiratory depression characteristic of isoflurane anesthesia (Minami et al., 1997; Bardin et al., 2000; Mukaida et al., 2007; Nagatani et al., 2011).

In summary, we demonstrate that exposure to isoflurane inhibits 5-HT neuron firing in a concentration-dependent manner. Our work contributes to the growing body of literature that identifies presynaptic sites as critical components in mechanisms of anesthetic action. As 5-HT neurons have broad modulatory and homeostatic effects, isoflurane influences on these neurons could contribute to widespread actions of anesthesia.

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Highlights

- Serotonin-synthesizing (5HT) neurons influence a variety of homeostatic processes
- We examine the effect of isoflurane exposure on raphé 5HT neuron firing *in situ*
- The volatile anesthetic isoflurane inhibits 5HT neurons
- Isoflurane sensitivity of 5HT neurons may contribute to actions of anesthesia

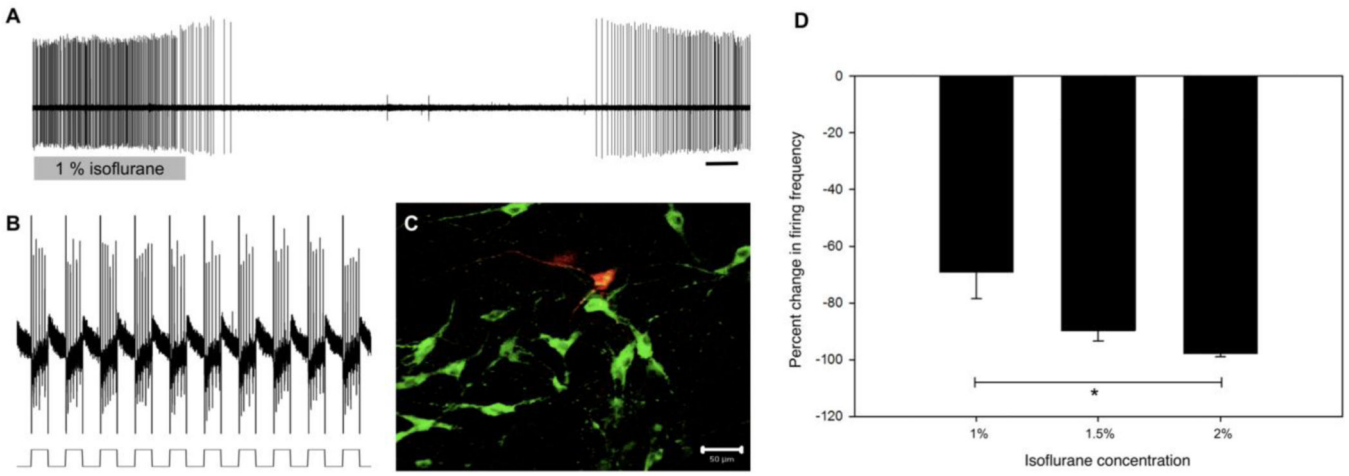

Figure 1. Medullary raphé 5-HT neurons are inhibited by isoflurane

a) Representative extracellular recording of a 5-HT neuron that ceased firing during isoflurane treatment. The neuron returned to baseline firing rate upon washout. Under baseline conditions, firing frequency (1.12 Hz) and pattern was characteristic of serotonergic cells. Scale bar = 30 s. **b)** The same neuron was entrained to fire simultaneously with an applied stimulus and filled with biotinamide. **c)** Positive staining for TPH (green) and biotinamide (red) colocalize (yellow) to indicate the recorded neuron was serotonergic. Scale bar = 50 μm.

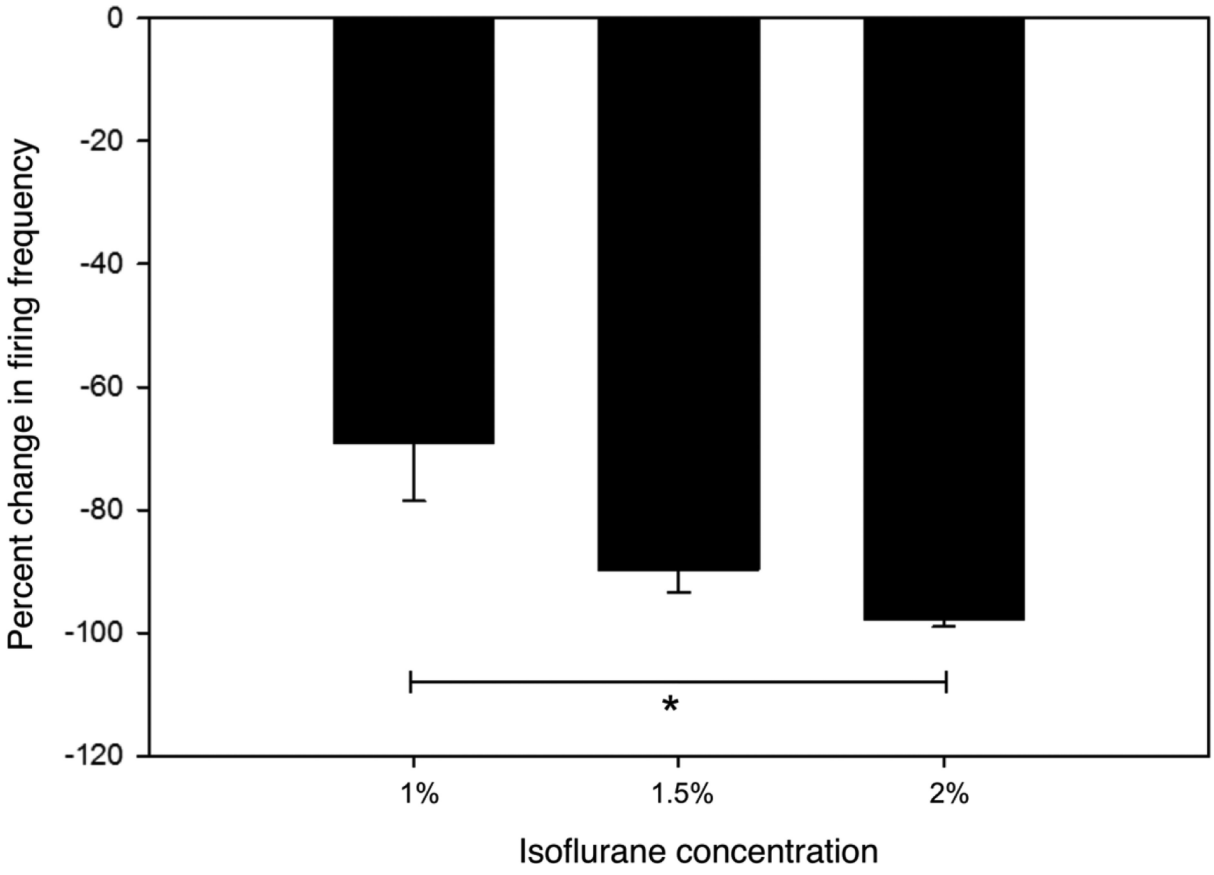

Figure 2. Isoflurane-induced inhibition of raphé 5-HT neurons is concentration- dependent

Values presented are mean percent decrease from baseline for each isoflurane concentration [1 % (n=18); 1.5 % (n=10); or 2 % (n=10)]. Between groups comparison of percent change from baseline during isoflurane exposure indicated that 2 % isoflurane produced greater inhibition than 1 % isoflurane (one-way ANOVA; **P* < 0.05).

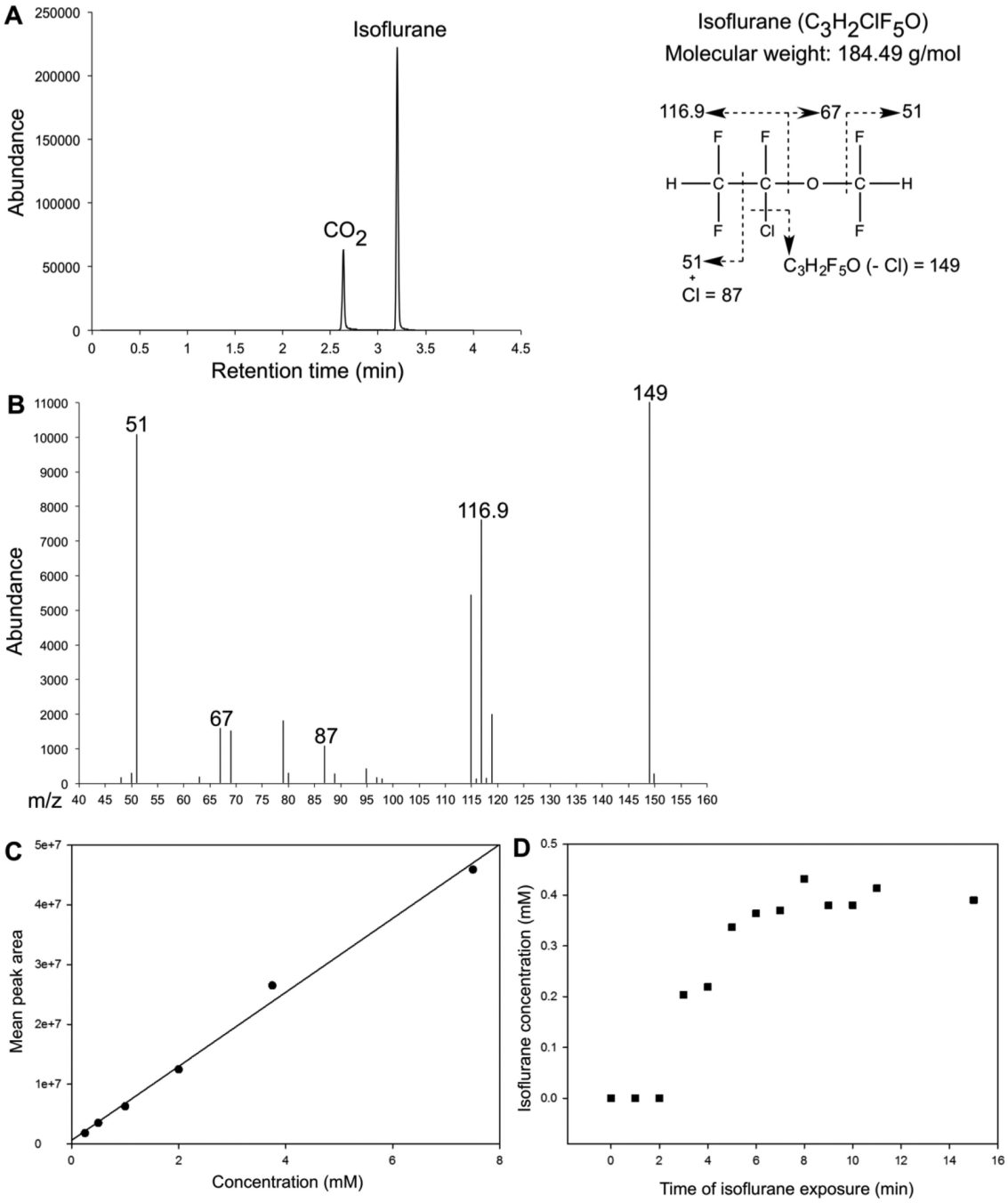

Figure 3. Isoflurane concentration in perfusate is confirmed by gas chromatography – mass spectrometry analysis

a) Chromatogram of 1 % isoflurane in perfusate solution. **b)** Mass spectra identified peak at retention time 3.20 min as isoflurane. **c)** Standard curve for isoflurane followed a linear regression line described by the equation: $y = 6.2*10^6 x + 6.0*10^5$ ($R^2 = 0.994$). **e)** GC-MS analysis of perfusate samples during 1 % isoflurane treatment confirmed that at 10 min, isoflurane concentration was equivalent to 1 volume percent.