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RESEARCH ARTICLE

Effect of temperature on chemosensitive locus coeruleus neurons of savannah monitor lizards, *Varanus exanthematicus*

Lucas A. Zena[1,2,3], Elisa M. Fonseca[1,2,3], Joseph M. Santin[2], Lays Porto[1,2,3], Luciane H. Gargaglioni[1,3], Kênia C. Bícego[1,3] and Lynn K. Hartzler[2,*]

ABSTRACT

Savannah monitor lizards (*Varanus exanthematicus*) are unusual among ectothermic vertebrates in maintaining arterial pH nearly constant during changes in body temperature in contrast to the typical α-stat regulating strategy of most other ectotherms. Given the importance of pH in the control of ventilation, we examined the CO_2/H^+ sensitivity of neurons from the locus coeruleus (LC) region of monitor lizard brainstems. Whole-cell patch-clamp electrophysiology was used to record membrane voltage in LC neurons in brainstem slices. Artificial cerebral spinal fluid equilibrated with 80% O_2, 0.0–10.0% CO_2, balance N_2, was superfused across brainstem slices. Changes in firing rate of LC neurons were calculated from action potential recordings to quantify the chemosensitive response to hypercapnic acidosis. Our results demonstrate that the LC brainstem region contains neurons that can be excited or inhibited by, and/or are not sensitive to CO_2 in *V. exanthematicus*. While few LC neurons were activated by hypercapnic acidosis (15%), a higher proportion of the LC neurons responded by decreasing their firing rate during exposure to high CO_2 at 20°C (37%); this chemosensitive response was no longer exhibited when the temperature was increased to 30°C. Further, the proportion of chemosensitive LC neurons changed at 35°C with a reduction in CO_2-inhibited (11%) neurons and an increase in CO_2-activated (35%) neurons. Expressing a high proportion of inhibited neurons at low temperature may provide insights into mechanisms underlying the temperature-dependent pH-stat regulatory strategy of savannah monitor lizards.

KEY WORDS: Chemosensitivity, Brainstem, CO_2 inhibition, Ventilation, Ectothermic vertebrates, pH regulation

INTRODUCTION

Regulation of intracellular and extracellular pH is a fundamental homeostatic requirement for vertebrates. Animals primarily compensate for perturbations to acid–base status of metabolic origin by adjusting the depth and rhythm of breathing through a negative feedback loop where O_2, CO_2 and pH are the main signals for ventilatory adjustments (Sundin et al., 2007). The negative feedback system that drives ventilation has been mainly elucidated in mammals that tightly regulate arterial pH. Specifically, chemosensory structures are well defined and the cellular mechanisms underlying chemosensitivity have been extensively studied in mammals (Guyenet and Bayliss, 2015; Hartzler and Putnam, 2009; Huckstepp and Dale, 2011), where the CO_2/pH-sensitive brainstem areas involved in respiratory chemosensing are located in regions surrounding the fourth ventricle (Coates et al., 1993; Huckstepp and Dale, 2011).

Chemosensitive regions contributing to the control of breathing have not been identified in reptiles. Nevertheless, at least one chemosensitive region, the locus coeruleus (LC), has been described in amphibians (toads and bullfrogs: Noronha-de-Souza et al., 2006; Santin and Hartzler, 2013) and mammals (reviewed in Hartzler and Putnam, 2009 and Gargaglioni et al., 2010), suggesting that CO_2/pH chemosensitivity of the LC may be conserved across air-breathing vertebrates. Anatomical studies have identified noradrenergic cells of the LC in a variety of reptiles (Kiehn et al., 1992; Lopez et al., 1992; Smeets and González, 2000; Wolters et al., 1984), including savannah monitor lizard brainstems (Wolters et al., 1984), showing tyrosine hydroxylase-containing cell bodies in the LC neurons as observed in anurans and rats (Biancardi et al., 2008; Noronha-de-Souza et al., 2006).

Temperature is an important variable affecting nearly all physiological processes, including neuronal activity, ventilation, cardiovascular function, metabolic rate and central chemoreception, among others, in ectothermic vertebrates (Bícego-Nahas and Branco, 1999; Branco et al., 1993; Branco and Wood, 1993; Santin et al., 2013; Stinner et al., 1998; Wood et al., 1977; Zena et al., 2015). In order to deal with environmental fluctuations of temperature, many vertebrates have developed autonomic, biochemical and behavioral strategies to handle changes in temperature. Despite these strategies, ectotherms can face large fluctuations in body temperature (T_b) that can introduce metabolic acid–base regulatory challenges for ectotherms (Stinner et al., 1998), and given the fundamental importance of pH regulation, we expect that ectothermic vertebrates would have a well-defined set of mechanisms to compensate for the effects of temperature on acid–base balance (da Silva et al., 2013). Therefore, regulation of pH requires an ability to sense pH perturbations within the brain and peripheral sites and to adjust the depth and rhythm of breathing to appropriately regulate temperature-dependent pH. For example, ventilatory requirements for acid–base balance in anuran amphibians vary proportionally with temperature (Branco et al., 1993; Gottlieb and Jackson, 1976; Mackenzie and Jacson, 1978; Santin et al., 2013; Tattersall and Boutilier, 1999a,b). Bullfrogs exhibit large ventilatory, central and cellular responses to CO_2 at high temperatures (>25°C), but no ventilatory, central or cellular chemosensitivity at low temperatures (10°C; Bícego-Nahas and Branco, 1999; Morales and Hedrick, 2002; Santin et al., 2013). Thus, the cellular basis for central chemoreception matches the ventilatory requirements of acid–base regulation of bullfrogs across its temperature range.

[1]Department of Animal Morphology and Physiology, College of Agricultural and Veterinary Sciences, São Paulo State University, Jaboticabal, São Paulo 14884-900, Brazil. [2]Department of Biological Sciences, Wright State University, Dayton, OH, USA. [3]National Institute of Science and Technology in Comparative Physiology (INCT Fisiologia Comparada), Brazil.

*Author for correspondence (lynn.hartzler@wright.edu)

L.K.H., 0000-0002-2355-0174

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List of symbols and abbreviations

aCSF	artificial cerebral spinal fluid
CI	chemosensitivity index
HA	hypercapnic acidosis
LC	locus coeruleus
Q_{10}	effect of temperature
T_b	body temperature
V_m	membrane voltage

Central chemoreceptors that induce changes in ventilation represent the principal sensory elements in the negative feedback loop that regulates pH, and, at least in bullfrogs, those chemosensitive neurons are known to be sensitive to temperature (Santin et al., 2013). Arterial pH is inversely related to temperature, so an increase in T_b decreases pH and, conversely, a decrease in T_b increases pH. For reptiles and amphibians, therefore, changes in T_b entail regulated, inversely proportional acid–base balance through adjusting ventilation; however, the temperature dependence of arterial pH regulatory strategies varies more than an order of magnitude between species of ectotherms (*Scaphiopus couchii*: $\Delta pH/\Delta T_b = -0.03$ U °C^{-1} to *Varanus exanthematicus*: $\Delta pH/\Delta T_b = -0.001$ U °C^{-1}; Withers, 1978 and Wood et al., 1977, respectively). The α-stat regulatory strategy is assumed to be the normal acid–base regulating mechanism among amphibians and reptiles, and posits that pH is adjusted with temperature to achieve a constant protonation state of critical histidine residues to maintain tertiary protein conformation, thus maintaining cell function (Reeves, 1972); therefore, savannah monitor lizards, *V. exanthematicus*, are unusual among air-breathing ectothermic vertebrates in maintaining arterial pH nearly constant during changes in T_b (from −0.001 to −0.003 U °C^{-1}; Wood et al., 1977; Wood et al., 1981). We hypothesized that LC neurons from *V. exanthematicus* exhibit depressed sensitivity to CO_2/pH changes at cooler temperatures because they retain CO_2 when cooled (Stinner et al., 1998). To test this hypothesis, we used the whole-cell patch-clamp technique to measure membrane voltage (V_m) and changes in action potential firing frequency of LC neurons in brainstem slices from *V. exanthematicus* upon exposure to hypercapnic acidosis (HA) across a physiological range of temperatures.

MATERIALS AND METHODS

Brainstem slice preparation

Juvenile savannah monitor lizards, *Varanus exanthematicus* (Bosc 1792) (N=46; 62.8±6.09 g) of both sexes were housed in tanks with access to basking areas for thermoregulation (temperature range: 25–48°C), exposed to 12 h:12 h light:dark cycles, and fed with crickets and rat pups twice weekly to satiety, with water available *ad libitum*. Experiments were approved by Wright State University's Institutional Animal Care and Use Committee. Methods were similar to those previously described (Santin et al., 2013). Lizards were anesthetized with isoflurane and, after the loss of the toe and tail withdrawal reflex, animals were rapidly decapitated. The head was submerged in 4°C artificial cerebral spinal fluid (aCSF; see 'aCSF preparation', below, for composition) equilibrated with 97.5% O_2 and 2.5% CO_2 and the brainstem was dissected. Following dissection, the brainstem was adhered to an agar block using cyanoacrylate glue then sectioned into ∼400 µm transverse slices using a Vibratome tissue slicer (Leica Microsystems Inc., Buffalo Grove, IL, USA). Slices containing the LC were identified using topographical distribution as previously described for *V. exanthematicus* (ten Donkelaar et al., 1987). Brainstem slices containing the LC were given 1 h to recover at room temperature in control aCSF equilibrated with 80% O_2, 2.5% CO_2, balance N_2 (pH ∼7.6). Following recovery, a transverse slice containing the LC was transferred to a polyethylene recording chamber with a glass coverslip base, immobilized with a nylon grid, and superfused with aCSF at a rate of ∼2.5 ml min^{-1}.

Temperature control

The temperature of the chamber was controlled with a Warner Instruments bipolar temperature controller (model CL-100, Hamden, CT, USA). The measured temperature of the bath varied ±1°C depending on the location of the thermocouple. To ensure precise temperature measurements at the exact location of the slice, the thermocouple was placed where the slice would be positioned during the experiments, and the objective was kept in touch with the bath to ensure that experimental temperatures were determined under identical conditions prior to experiments.

aCSF preparation

aCSF was composed of (in mmol l^{-1}): 134.0 NaCl, 5.0 KCl, 1.3 $MgSO_4$, 10.0 D-glucose, 26.0 $NaHCO_3$, 2.4 $CaCl_2$ and 1.24 KH_2PO_4 (Liu et al., 2006). Gases were mixed using an MFC-4 mass flow controller and gas mixer (Sable Systems International, Las Vegas, NV, USA) and continuously diffused in aCSF. Control aCSF at 20°C (pH 7.61), 30°C (pH 7.66) and 35°C (pH 7.68) was equilibrated with 80% O_2, 2.5% CO_2, balance N_2. The solubility of gases is inversely related to temperature, so the expected increase in pH of aCSF with cooling was countered by a decrease in pH due to an increase in dissolved CO_2. Hypercapnic aCSF was identical to control aCSF except that CO_2 was elevated to 7% or 10%, displacing N_2, depending on the experimental protocol. The pH changes during HA with 7% CO_2 were similar at all temperatures (ΔpH from control aCSF at 20, 30 and 35°C was 0.29, 0.29 and 0.28 pH units, respectively). The ΔpH from control aCSF solution at 20°C during HA with 10% CO_2 and during hypocapnia (0% CO_2) was 0.44 and 1.02 pH units, respectively.

Electrophysiological recordings

Whole-cell patch-clamp recordings were obtained as previously described (Santin et al., 2013). Briefly, micropipettes with resistances of 4–7 MΩ were back-filled with mock-intracellular solution (composition in mmol l^{-1}: 110 potassium gluconate, 2 $MgCl_2$, 10 Hepes, 1 Na_2-ATP, 0.1 Na_2-GTP, 2.5 EGTA; pH 7.2) and placed over a $AgCl_2$-coated Ag wire connected to an Axon Instruments CV 203BU headstage (Molecular Devices, Sunnyvale, CA, USA). The slice was visualized at 4× magnification with a Nikon FN1 fixed stage microscope (Nikon, Elgin, IL, USA) using NIS-Elements imaging software (Nikon). The LC was identified by its bilateral location adjacent to the fourth ventricle (for anatomy, see references in 'Brainstem slice preparation', below). Individual neurons of interest were visualized at 60× magnification prior to recording. A 10 ml syringe was connected to the headstage and was used to apply positive pressure through the pipette tip in order to keep the tip free of debris. A micromanipulator (Burleigh PCS 5000, Thorlabs, Newton, NJ, USA) was used to position the pipette adjacent to the neuron of interest and the pipette offset was zeroed prior to entering the on-cell configuration to ensure accurate V_m measurements. Positive pressure was removed and suction was applied until a ≥1 GΩ seal was obtained between the pipette tip and cell membrane in the on-cell configuration. Further gentle negative pressure was applied to rupture the seal and obtain whole-cell electrochemical access to the neuron. Changes in V_m were recorded

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in current-clamp mode (Axopatch 200 B integrating patch-clamp amplifier, Molecular Devices) and collected using Molecular Devices P10 Clampex software. Current-clamp recordings were analyzed off-line using pCLAMP software (Molecular Devices).

Experimental procedures

Determination of LC chemosensitivity

As a general procedure, LC neurons were exposed to hypercapnic aCSF to determine CO_2/pH sensitivity. Upon entering the whole-cell current-clamp mode, V_m was recorded for 2–5 min under control conditions, where aCSF was equilibrated with control gas mixture (80% O_2, 2.5% CO_2, balance N_2) to establish baseline firing rate. Neurons were then exposed to 7% CO_2 or 10% CO_2 for 5 min to elicit changes in firing rate in response to HA and then returned to control aCSF solution. Only neurons returning to near-control firing rates following HA were included in the analysis. aCSF equilibrated with control gas mixture was used to determine the baseline firing rate and the CO_2 composition of the hypercapnic gas mixture was increased to 7.0% and/or 10.0%, and/or decreased to 0%. Neurons unresponsive after 5 min of HA exposure in all described experiments were considered non-chemosensitive. Neurons were considered activated or inhibited by HA if their chemosensitivity index (CI) was ≥120% or ≤80% than control firing rate, respectively (Wang et al., 1998).

Effect of temperature on firing rate and chemosensitivity of LC neurons

The preferred T_b of *V. exanthematicus* is about 35°C based on their behavioral preference in a thermal gradient (Hallman et al., 1990), so to investigate the effect of temperature (35°C) on lizard LC neurons, the chamber was heated to 35°C and maintained at this temperature for the duration of the experiment. Using a whole-cell current-clamp configuration, baseline firing rate was recorded for 2–5 min from LC neurons (N=26; N=12 chemosensitive, N=14 non-chemosensitive) in aCSF equilibrated with control gas mixture (80% O_2, 2.5% CO_2, balance N_2), at 35°C. Once control firing rates and V_m were established, the LC neurons were exposed to 7% CO_2 for 3–5 min. Only neurons that recovered firing rate and V_m after the return to control aCSF were included in this dataset.

To determine the effect of both warming and HA on firing rate and V_m of LC, we varied the chamber solution from 20 to 30°C (N=14). Well-sealed patches last longer at cooler temperatures, so for these longer experiments we decreased the temperature at which we held our slices. In whole-cell current-clamp mode, baseline firing rate was recorded for 2–5 min from LC neurons in aCSF equilibrated with control gas mixture (80% O_2, 2.5% CO_2, balance N_2) at a lower temperature, 20°C. Once control firing rate and V_m were established, LC neurons were exposed to 7% CO_2 for 3–5 min. Neurons that recovered firing rate and V_m after the return to control aCSF were used in subsequent experiments and included in this dataset.

The influence of warming on both firing rate and chemosensitivity was investigated in a subset of LC neurons (N=10 chemosensitive, N=4 non-chemosensitive). Once stable firing rates of LC neurons at 30°C were recorded, temperature was returned to 20°C and firing rate was allowed to return to near-initial values at 20°C for at least 3 min; after this, a slow warm ramp to 30°C was again applied. Once the chamber temperature reached 30°C (in ∼2 min), a new baseline firing rate at the new temperature was established for ∼2 min, and the neuron was exposed to 7% CO_2 for 3–5 min at the higher temperature (N=7). Only neurons that recovered firing rate and V_m after the return to control aCSF at 20°C were included in this dataset.

Data analysis

All values are reported as means±s.e.m. Nine LC slices at 35°C yielded nine neurons excited by HA, three inhibited by HA and 14 non-chemosensitive neurons. Fourteen additional slices recorded at 20°C had four excited, 20 inhibited and 13 non-chemosensitive neurons. The mean firing rate in control, 7% or 10% CO_2, and the return to control (washout) are reported for 20°C; mean firing rate during control, 7% CO_2 and washout are also reported for 30 and 35°C. A repeated measures analysis of variance (RM-ANOVA) was used to determine differences among control, 7% or 10% CO_2, and washout neuronal firing rates at 20, 30 and 35°C. Percentage changes in chemosensitivity (excited and inhibited LC neurons) between 20 and 35°C were accessed by a Fisher's exact test. The magnitude of the response to HA was quantified as the CI, which expresses the percentage change in firing rate due to HA normalized for a pH change of 0.2 pH units. The effect of temperature on firing rate during normocapnia was assessed by comparing the average firing rate 1 min before warming with the first minute of average firing rate once the new temperature was achieved. One-way ANOVA followed by Tukey's *post hoc* test were used to determine firing rate differences among 0% (hypocapnia), 2.5% (control), 7.0% and 10.0% CO_2 (hypercapnia) values. A two-way RM-ANOVA followed by Tukey's *post hoc* test was used for evaluating temperature effects on firing rate in chemosensitive and non-chemosensitive LC neurons. A Student's *t*-test was applied to compare the effect of temperature (Q_{10} effect) on basal firing rate between chemosensitive and non-chemosensitive LC neurons and between recovery time from HA for excited and inhibited LC neurons at 20 and 35°C. All graphics were made using GraphPad Prism (GraphPad Software, San Diego, CA, USA) and statistical analysis was carried out using SigmaPlot version 11. Significance was accepted with P≤0.05. Data were tested for unequal variance and normality, and, when necessary, log transformation was performed.

RESULTS

Chemosensitivity in savannah monitor lizard LC neurons

LC neurons in savannah monitor lizards exhibit chemosensitivity to changes in CO_2/H^+ (Fig. 1). Fig. 1A depicts the CI that was used to categorize LC neurons as excited, inhibited or non-chemosensitive in the savannah monitor lizards, while Fig. 1B shows the percentage of each response type at 35°C. On exposure to HA (7% CO_2) at 35°C, 35% (9/26) of the LC neurons increased their firing rate (2.22±0.57 versus 4.06±0.92 Hz; RM-ANOVA, P<0.01; Fig. 1C), 11% of the LC neurons (3/26) showed an inhibitory response (1.24±0.05 versus 0.09±0.09 Hz; RM-ANOVA, P<0.05; Fig. 1D) and 54% (14/26) were non-chemosensitive when exposed to HA (2.53±0.27 versus 2.49±0.25 Hz; RM-ANOVA, P=0.38; Fig. 1E). HA caused a significant depolarization in the cell membrane of excited LC neurons at 35°C (−46.6±1.7 versus −41.4±2.0 mV; paired *t*-test, P<0.05), while CO_2-inhibited LC neurons had no significant change in V_m (−47.1±4.1 versus −51.7±7.4 mV; paired *t*-test, P=0.3). While there was a significant depolarization in the cell membrane of non-chemosensitive neurons (−46.4±1.3 versus −45.1±1.4 mV; paired *t*-test, P<0.01), the absolute change in V_m was less in comparison to CO_2-excited cells.

Although there was no apparent effect of a lower temperature (20°C) on the magnitude of the chemosensitive response (two-way ANOVA, P=0.3; Fig. 2A), LC neurons had a different distribution of

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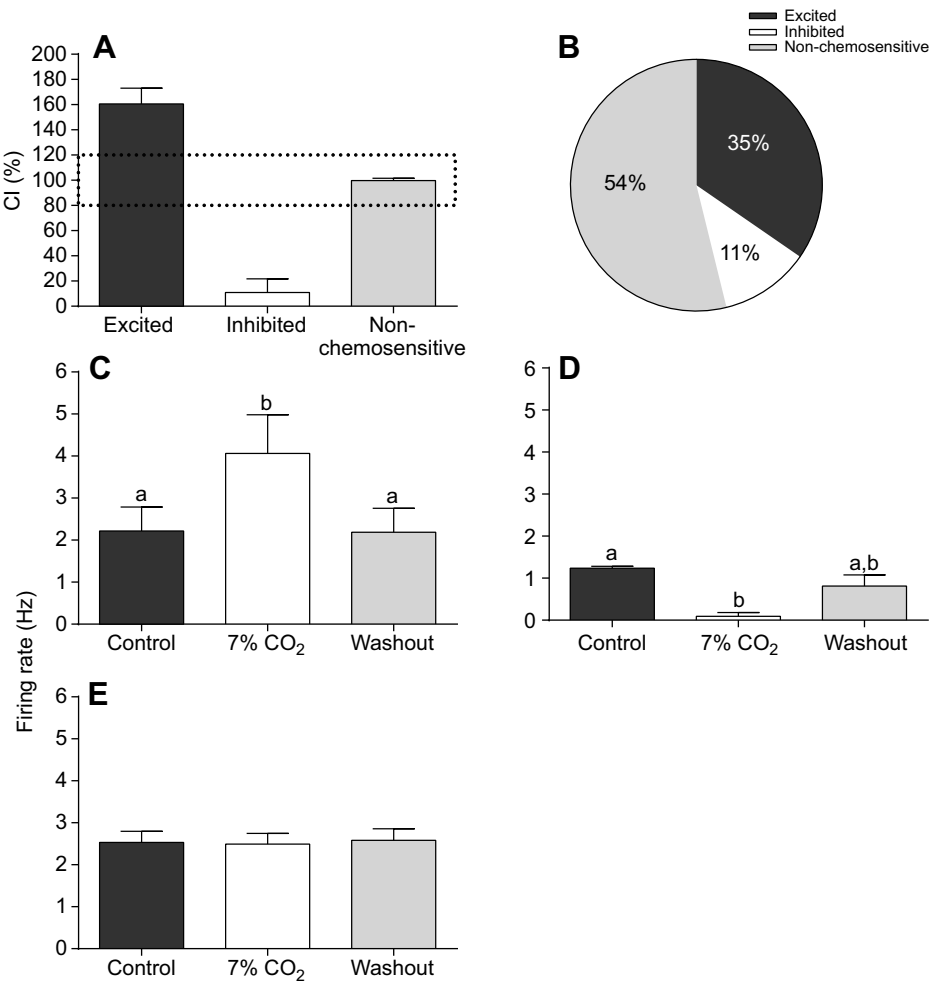

Fig. 1. *Varanus exanthematicus* locus coeruleus (LC) neurons show three types of firing rate response to hypercapnic acidosis (HA) at 35°C. (A) Neurons were considered activated (or excited), inhibited and non-chemosensitive based on their chemosensitivity index (CI). Dotted lines delineate the CI above and below which neurons are classified as excited (CI ≥120% of baseline firing rate) or inhibited (CI ≤80% of baseline firing rate) by HA, respectively. (B) The three types of chemosensitive responses to HA are shown as a percentage of chemosensitive and non-chemosensitive LC neurons. (C) Firing rate significantly increases upon exposure to 7% CO_2 (N=9) in excited neurons (RM-ANOVA, P<0.05). (D) In inhibited LC neurons, firing rate significantly decreases upon exposure to 7% CO_2 (N=3; RM-ANOVA, P<0.05). (E) Non-chemosensitive LC neurons do not change their firing rate upon 7% CO_2 exposure (N=14; P=0.38). Different lowercase letters indicate significant differences between treatments. Data are means+s.e.m.

responses to CO_2 (Fig. 2B). Most of the chemosensitive LC neurons were inhibited by HA (10/27, 37% of total population; Fisher's exact test; P=0.047). Only 15% (4/27) of LC neurons were excited by HA, but because baseline firing rate was so variable, a statistically significant change was not observed during HA (RM-ANOVA, P=0.13; Fig. 2C). If we normalize frequency, these excited neurons expressed a significant increase of 116.8±15.5% in firing frequency relative to baseline values (P=0.005, paired t-test; data not shown), while inhibited neurons at 20°C had a control firing rate of 1.35±0.38 Hz and HA decreased the firing rate to 0.33±0.20 Hz (RM-ANOVA, P=0.013; Fig. 2D), returning (on washout) to a firing rate near control values (1.09±0.41 Hz). Even when LC neurons to a greater HA, induced with 10% CO_2 (N=7), firing rate still decreased from 0.93±0.31 to 0.13±0.09 Hz, with washout firing rate increasing to 0.51±0.11 Hz (RM-ANOVA, P=0.02; Fig. 2E). Most (48%, 13/27) of the LC neurons in the savannah monitor lizard at 20°C were unresponsive to HA (RM-ANOVA, P=0.08; Fig. 2F). Additionally, the effect of hypocapnic exposure (0% CO_2; N=3) was not significantly different from that of HA with 2.5% CO_2 (control CO_2; N=10); however, firing rate during both hypocapnia and control CO_2 was significantly different from that during hypercapnia (7% and 10% CO_2; one-way ANOVA, P<0.001; Fig. 3). Furthermore, HA caused no change in V_m of excited LC neurons at 20°C (−51.6±9.1 versus −45.8± 10.2 mV; paired t-test, P=0.08) and a significant hyperpolarization in the V_m of inhibited LC neurons (−53.0±2.8 versus −58.6±3.6 mV; paired t-test, P=0.007), while non-chemosensitive neurons did not change V_m during exposure to 7% CO_2 (−47.1±2.3 versus −46.6± 2.7 mV; paired t-test, P=0.5).

Influence of temperature on chemosensitive and non-chemosensitive lizard LC neurons

In a separate set of LC neurons, we investigated the effects of changing temperature while recording V_m. Neurons inhibited by CO_2 at 20°C were thermosensitive (N=10). Increasing the temperature from 20 to 30°C caused an increase in firing rate from 0.79±0.23 to 3.66±0.63 Hz (RM-ANOVA, P<0.001; Fig. 4), and decreasing the temperature back to 20°C slowed firing rate back down to 1.15 ±0.45 Hz. Elevation in temperature from 20 to 30°C caused a significant increase in V_m in those neurons that were chemosensitive (CO_2 inhibited) at 20°C (−47.2±1.9 versus −40.5±1.8 mV; paired t-test, P=0.001). Non-chemosensitive LC neurons (N=4) also increased their firing rate from 0.90±0.23 Hz at 20°C to 3.0± 0.76 Hz at 30°C (RM-ANOVA, P<0.05; Fig. 4); firing rate slowed down to 0.38±0.12 Hz after the return to 20°C with no significant change in V_m (−47.4±2.5 versus −43.2±3.8 mV; paired t-test, P=0.08).

Chemosensitive (inhibited) LC neurons were returned to 30°C and then exposed to HA (7% CO_2) for 3–5 min (Fig. 5). The inhibited pattern observed in chemosensitive LC neurons when exposed to HA at 20°C (1.47±0.43 versus 0.33±0.12 Hz; N=10; RM-ANOVA, P<0.01) was no longer observed at 30°C (2.88±0.59 versus 2.77± 0.67 Hz; N=7; RM-ANOVA, P=0.40) (Fig. 5A), and a diminished membrane potential exhibited at 20°C during HA (−48.9±1.5 versus

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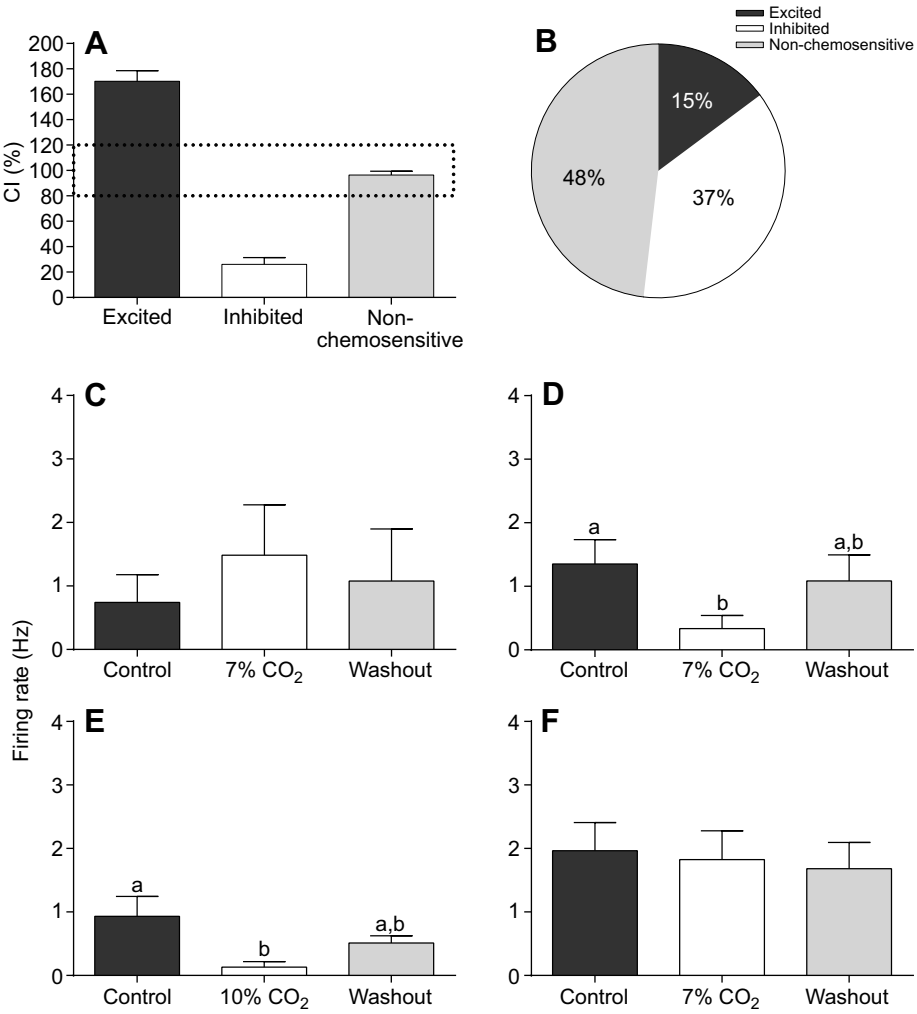

Fig. 2. *Varanus exanthematicus* LC neurons show three types of firing rate response to HA at 20°C. (A) Neurons were considered activated (or excited), inhibited and non-chemosensitive based on their CI. Dotted lines delineate the CI above and below which neurons are classified as excited (CI ≥120% of baseline firing rate) or inhibited (CI ≤80% of baseline firing rate) by HA, respectively. (B) The three types of chemosensitive responses to HA are shown as proportions of chemosensitive and non-chemosensitive LC neurons. Fewer excited neurons and more inhibited neurons were found at 20°C compared with 35°C. (C) Firing rate of excited LC neurons tends to increase upon exposure to 7% CO_2 (N=4). (D,E) Exposure to 7% CO_2 (D, N=10) and 10% CO_2 (E, N=7) induced significant decreases in firing rate in inhibited LC neurons (RM-ANOVA, P<0.05). (F) Non-chemosensitive LC neurons do not respond to 7% CO_2 exposure (N=13). Different lowercase letters indicate significant differences between treatments. Data are means+s.e.m.

−53.7±2.0 mV; paired t-test, P=0.001) was also no longer observed during exposure to HA at 30°C (−41.4±2.5 versus −40.7±2.2 mV; paired t-test, P=0.7). An example of this pattern in integrated firing rate and V_m for one representative LC neuron inhibited by HA at 20°C and unresponsive to HA when exposed to 30°C is shown in Fig. 5B,C. Reversal of the response to HA took 3.6±0.6 and 5.6±2.4 min for excited and inhibited neurons at 35°C (t-test, P=0.562), respectively, while at 20°C, excited and inhibited neurons took 2.8±0.4 and 9.4±1.9 min for recovery (t-test, P=0.004), respectively.

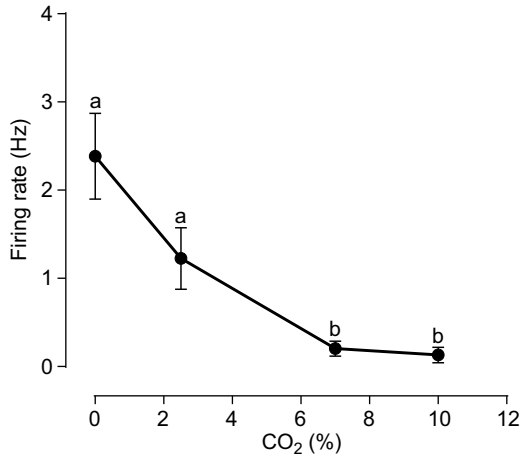

Fig. 3. LC neurons inhibited by HA have an increased integrated firing rate response to hypocapnic alkalosis (0% CO_2). Most chemosensitive LC neurons were inhibited by CO_2 at 20°C (see Fig. 2B). Control CO_2 was 2.5% (N=10), with two levels of HA (7.0%, N=10; and 10.0%, N=7), and hypocapnia at 0% CO_2 (N=3). There was no further decrease in firing rate with 10% CO_2 compared with 7% CO_2. Different lowercase letters indicate significant differences between treatments. Data are means+s.e.m.

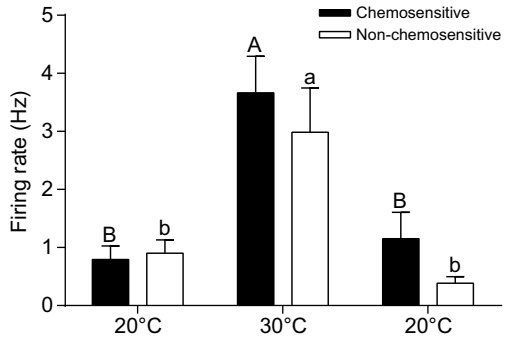

Fig. 4. Increasing the temperature increases the basal firing rate of *V. exanthematicus* LC neurons. A 10°C increase in temperature (from 20 to 30°C) induced the same magnitude rise in basal firing rate in chemosensitive (N=10; Q_{10}=12.6±6.2) and non-chemosensitive LC neurons (N=4; Q_{10}=3.4±0.5). Different lowercase letters indicate significant differences among treatments within non-chemosensitive LC neurons; and different uppercase letters indicate significant differences among treatments within chemosensitive LC neurons. Data are means+s.e.m.

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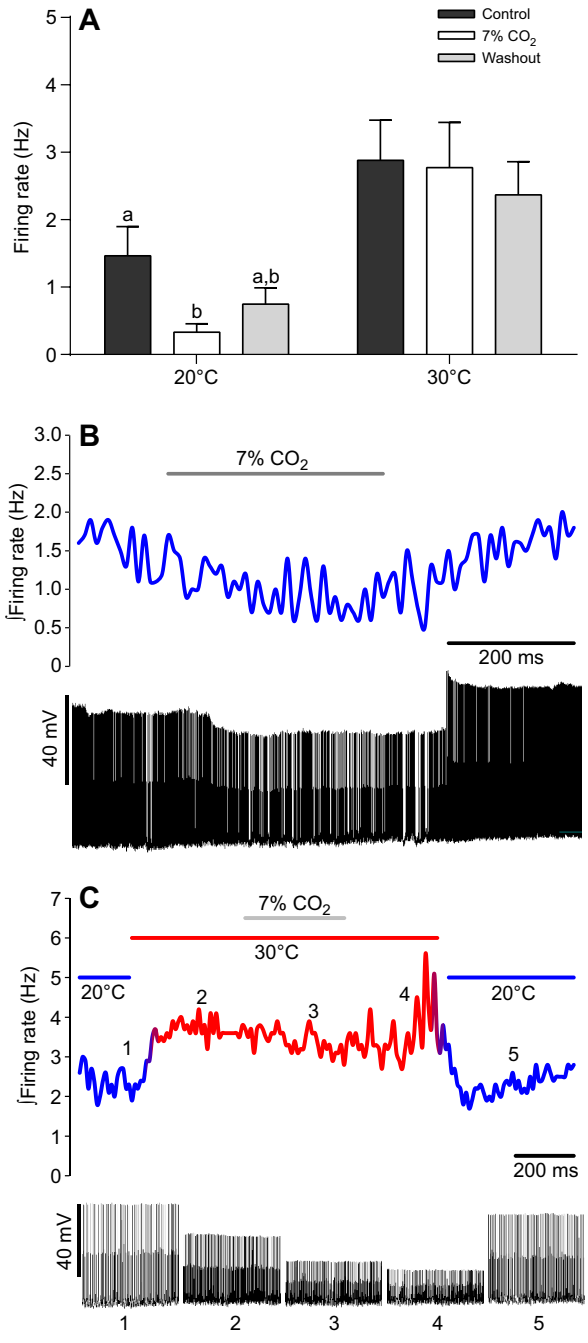

Fig. 5. The hypercapnic response of inhibited LC neurons in *V. exanthematicus* is temperature dependent within single neurons. (A) The firing rate of LC neurons significantly decreased upon exposure to 7% CO_2 (N=10) in inhibited neurons at 20°C (RM-ANOVA, P<0.01), but no longer responded to HA (became non-chemosensitive) when temperature was increased to 30°C (N=7). Different lowercase letters indicate significant differences between treatments. Data are means+s.e.m. (B) Example integrated firing rate (top) and action potential trace (bottom) from an inhibited chemosensitive LC neuron showing the decrease in firing rate during HA at 20°C. (C) When temperature was increased to 30°C, previously inhibited LC neurons no longer changed firing rate upon exposure to 7% CO_2. The corresponding action potential trace (numbers 1–5) is shown below.

DISCUSSION

In the present study, we describe chemosensitive LC neurons in the lizard *V. exanthematicus*, a pH-stat regulating ectothermic vertebrate. We quantified how temperature affects LC neuron firing rate activity under HA exposure. Relatively few lizard LC neurons were excited by HA (Figs 1 and 2), in marked contrast to a previous study from our laboratory (Santin et al., 2013) where we reported that LC neurons from bullfrogs, a classically α-stat regulating animal, have a high percentage of CO_2-activated LC neurons (>80%), and the percentage of chemosensitive neurons is not temperature dependent. In addition, in bullfrogs, increases and decreases in temperature influence the magnitude of the chemosensitive response; however, the CI in monitor lizards was not temperature dependent. Therefore, our study shows that in *V. exanthematicus*, despite fewer LC neurons being activated by HA, a higher proportion of the LC neurons responded by decreasing their firing rates during exposure to high CO_2 (7–10% CO_2) at 20°C (37%) – a response that was no longer exhibited at a higher temperature (30°C). Interestingly, further increases in temperature (35°C) again changed chemosensitive LC neurons in the monitor lizards: a reduction in the percentage of CO_2-inhibited LC neurons at 35°C (11%) and an increase in the percentage of LC neurons stimulated by HA (35%).

CO_2 chemosensitivity in vertebrates

Adjustments of ventilation are initiated via negative feedback where O_2, CO_2 and pH are the primary stimuli to which peripheral and central chemoreceptors are capable of responding through activation of O_2- and CO_2/pH-sensitive neurons. Central chemoreceptors are widely distributed in the brains of mammals, contributing to control of breathing and maintenance of systemic pH/CO_2 homeostasis (Guyenet and Bayliss, 2015; Huckstepp and Dale, 2011; Nattie, 1999). Studies investigating central respiratory chemoreceptors have focused mainly on neurons that are excited by HA, because some of the most studied chemosensitive areas like the nucleus tractus solitarius (NTS), retrotrapezoid nucleus (RTN) and the LC have a high percentage of HA-responsive neurons that are activated by CO_2 (Conrad et al., 2009; Mulkey et al., 2004; Nichols et al., 2009; Ritucci et al., 2005); however, CO_2-inhibited neurons also possibly play a role in the ventilatory control network in mammals (Iceman et al., 2014; Wang et al., 1998; Wang and Richerson, 1999). Extracellular recordings in the rat *in situ* preparation identified CO_2-inhibited GABAergic neurons in the medullary raphe (Iceman et al., 2014). CO_2-inhibited neurons of the raphe may be involved in central CO_2/pH chemoreception in mammals, providing a tonic inhibitory drive on respiratory output that is diminished as CO_2 is increased (Wang et al., 2001). Other chemosensitive regions contributing to the control of breathing also contain CO_2-inhibited neurons. The solitary complex [NTS and dorsal motor nucleus of the vagus (DVN); Conrad et al., 2009; Nichols et al., 2009] in adult rats seems to exhibit a significant plasticity when exposed to chronic hypoxia, increasing the percentage of CO_2-inhibited neurons from about 10% up to about 30% without any change in the proportion of CO_2-activated neurons (Nichols et al., 2009).

A significant gap in our knowledge of ventilatory control in vertebrates is that we know little of the fundamental cellular mechanisms of individual chemosensitive neurons in ectothermic vertebrates and how changes in their T_b would affect central cellular chemosensitivity. In mammals, LC neurons are of particular interest for CO_2 challenges as >80% of these neurons respond to HA by increasing their firing rate (Filosa et al., 2002; Oyamada et al., 1998; Pineda and Aghajanian, 1997). A CO_2-chemosensitive LC region may be conserved across vertebrates as it has been described in bullfrogs, toads and rats (Gargaglioni et al., 2010; Hartzler et al., 2008; Nichols et al., 2008; Noronha-de-Souza et al., 2006; Santin

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and Hartzler, 2013). As in mammals, more than 80% of LC neurons in bullfrogs are CO_2 activated (Santin and Hartzler, 2013; Santin et al., 2013), reinforcing its importance as a site for central chemoreception in anurans (Noronha-de-Souza et al., 2006). Furthermore, given the similarities between LC responses in anurans and mammals in which focal acidification of the LC region stimulates ventilation and ablation reduces CO_2-induced increases in ventilation in *in vivo* experiments (Biancardi et al., 2008; Noronha-de-Souza et al., 2006), it is clear that the LC has an analogous role, and possibly a homologous role, in respiratory control in terrestrial vertebrates. The present study provides data showing that the LC in the savannah monitor lizard is also chemosensitive within a normal, physiological range of pH/P_{CO_2}, containing excited, inhibited and non-chemosensitive neurons, albeit in different proportions from those in rats and bullfrogs. In order to establish the function of the LC in respiratory control across air-breathing vertebrates, it is imperative that future *in vivo* studies show that the LC contributes to ventilatory control during CO_2 stimulation in reptiles.

The most striking finding in our study is how temperature is able to alter the proportion of LC neurons excited or inhibited by, or not sensitive to HA. The percentage of inhibited neurons was relatively high at 20°C (37%; Fig. 2B,C) compared with published data from bullfrogs and rats, none of which reported CO_2-inhibited LC neurons (Filosa et al., 2002; Santin and Hartzler, 2013; Santin et al., 2013), although we have observed some inhibited LC neurons in rats (L.K.H., J. B. Dean and R. W. Putnam, unpublished data). In addition, at 20°C, we found relatively few CO_2-activated LC neurons (15%; Fig. 2B,D), which is distinctly different from studies in bullfrogs at the same temperature (>80% activated by HA; Santin et al., 2013). The decrease in firing rate on exposure to 7% or 10% CO_2 is striking where the magnitude of the chemosensitive response is very low (CI <40%; Fig. 2A). Further, following washout (return to control CO_2), CO_2-inhibited LC neurons in aCSF solution equilibrated with 0% CO_2 (pH 8.6 at 20°C) increased their firing rate slightly higher than those neurons exposed only to control solution (2.5% CO_2; pH 7.6 at 20°C; L.A.Z., personal observation; combined data are shown in Fig. 3, but individual data are not shown). Surprisingly, inhibited neurons recorded at 20°C became unresponsive to HA at 30°C (Fig. 5). This pattern suggests that temperature has a differential modulatory effect on how CO_2 affects *in vitro* chemosensitive LC neurons in savannah monitor lizards at the cellular level. It is possible that the differences in the chemosensitive response to CO_2 could be due to whether that particular LC neuron is noradrenergic; however, given the temperature dependence of CO_2 chemosensitivity within the same neuron, it is unlikely that whether a neuron is noradrenergic is the only factor that determines whether a neuron will respond to CO_2 in savannah monitor lizards. It will be important to identify morphological characteristics and the ion channel/neurotransmitter phenotype of LC neurons in conjunction with electrophysiological recordings to address this point.

Maintaining the temperature at 35°C during the entire experiment resulted in a different scenario for chemosensitive LC neurons. The number of CO_2-inhibited neurons was lower (11%) compared with that at 20°C, but, still, the presence of inhibited LC neurons at even this higher temperature is in marked contrast to the absence of CO_2-inhibited neurons in mammals at a similar temperature (Filosa et al., 2002). Even the effect of temperature on basal firing rate (Q_{10}) was different between savannah monitor lizards (Figs 4 and 6) and bullfrogs (Santin and Hartzler, 2015; Santin et al., 2013). In lizards, warming increased basal firing rate in both chemosensitive and non-chemosensitive LC neurons. In contrast, LC chemosensitive neurons in bullfrogs were cold activated and warm inhibited, allowing a very small Q_{10} effect, while non-chemosensitive LC neurons increased firing rate with increases in temperature, exhibiting a higher Q_{10} effect (Fig. 6). At present, it is unclear how these temperature responses, per se, affect the activity of the respiratory network.

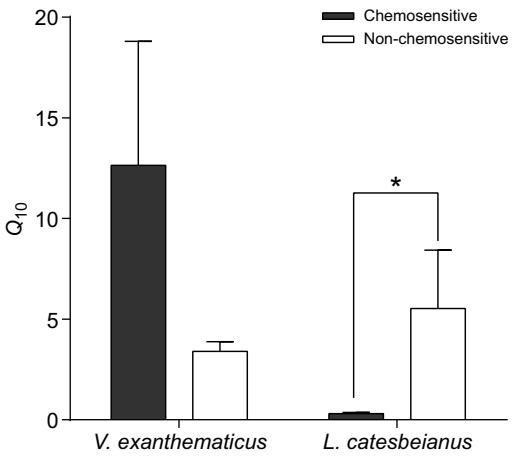

Fig. 6. The effect of temperature (Q_{10} effect) on the basal firing rate of chemosensitive LC neurons is completely different between *V. exanthematicus* and *Lithobates catesbeianus*. In lizards, warming increased the basal firing rate in both chemosensitive (N=10) and non-chemosensitive (N=4) LC neurons (shown in Fig. 4); in bullfrogs, chemosensitive LC neurons (N=33) were cold activated and warm inhibited, whereas non-chemosensitive LC neurons (N=5) increased firing rate with increases in temperature (data from Santin et al., 2013). *Significant difference between chemosensitive and non-chemosensitive LC neurons. Data are means+s.e.m.

Temperature effects on CO_2 chemosensitivity and pH regulation in savannah monitor lizards

Based on our data for LC chemosensitivity, temperature is of considerable importance as a modulatory factor of how LC neurons respond to hypercapnic stimuli in savannah monitor lizards. These animals exhibit a different modulatory pattern not previously reported in any of the putative chemosensitive areas that contribute to the respiratory network in vertebrates. Temperature influences on central chemosensitivity in ectothermic vertebrates have drawn attention to how chemosensitive areas respond to increases in environmental CO_2 across temperatures (Branco et al., 1993; Branco and Wood, 1993). For instance, in bullfrogs, a decrease or increase in the magnitude of the chemosensitive response (Santin et al., 2013) is the main effect of temperature and hypercapnic stimuli, which corroborates temperature effects in the whole-animal ventilatory response to general chemoreceptor stimulation by hypercapnia in amphibians (Bícego-Nahas and Branco, 1999; Branco et al., 1993). In contrast, there is a different chemosensitivity ratio between LC neurons activated or inhibited by CO_2 across temperatures in savannah monitor lizards; therefore, we emphasize the need to consider temperature when interpreting data of CO_2 stimuli on putative brainstem areas related to control of breathing, especially in ectothermic vertebrates. Experiments on some chemosensitive brainstem areas, such as the LC, NTS and RTN, in neonatal rats used physiological T_b for understanding how the respiratory network is modulated by CO_2 stimuli (Filosa et al., 2002; Filosa and Putnam, 2003; Nichols et al., 2008; Ritucci et al., 2005), while in others, recordings were obtained at room

temperature (Huang et al., 1997; Mulkey et al., 2004). Although no differences were found in the percentage of CO_2-activated neurons in the RTN between experiments performed at room temperature and 37°C in neonatal rats (46% and 42%, respectively; Mulkey et al., 2004; Ritucci et al., 2005), it is not clear whether those neurons were responding to a change in pH or CO_2, as both pH and the solubility of CO_2 are temperature dependent (the effects are opposite and may cancel each other out). Therefore, an appropriate temperature should be used, or at least considered, while studying CO_2 chemosensitivity within brainstem areas that may be involved in the control of breathing, especially in studies with ectothermic vertebrates that have the additional challenge of acid–base regulation during changes in T_b (Stinner et al., 1998).

The response to temperature-induced metabolic acid–base disturbances requires an ability to sense those perturbations and appropriately adjust the depth and rhythm of breathing. Mechanisms underlying the diverse acid–base regulating strategies of ectothermic vertebrates may be related to differences in the temperature dependence of central chemosensitivity at the cellular level. Uncovering differences in central respiratory chemoreception during changes in temperature in reptiles with distinct acid–base regulatory strategies has the potential to define a comprehensive, mechanistic explanation for a fundamental, but poorly understood, aspect of vertebrate homeostasis. Although we found strikingly different temperature-dependent mechanisms of chemosensitivity in the LC of monitor lizards compared with bullfrogs and rats, we cannot rule out possible contributions from other pH/CO_2-sensitive areas in the central nervous system. There are multiple CO_2/pH-sensitive areas in the brainstems of mammals (Nattie and Li, 2009), so it is likely there are also multiple CO_2/pH-sensitive areas in the brainstems of reptiles. Motor output to the breathing muscles would be modified by the networked chemosensory responses to CO_2/pH of all of the regions contributing to ventilatory control.

These are among the many remaining questions that need to be addressed, especially in closely related species that, despite phylogenetic proximity, differ from each other in pH-regulatory strategy. In addition, studies are necessary to determine the temperature dependence of hypercarbic stimuli and the contribution of the LC as a chemosensing brainstem region in the total ventilation of savannah monitor lizards in order to contextualize the neuronal CO_2 chemosensitivity found in our study within the functioning of the whole animal.

Contrary to our initial hypothesis, savannah monitor lizards do not exhibit a depressed LC chemosensitivity to pH/CO_2 changes at cooler temperatures. Instead, we found a more complex chemosensitivity pattern where LC neural sensory responses (activated/excited by HA versus inhibited by HA) in *V. exanthematicus* change with temperature, while in bullfrogs it is the magnitude of the chemosensitive response that is temperature dependent (Santin et al., 2013). Expressing a relatively high proportion of CO_2-inhibited LC neurons at low temperature may provide insight into mechanisms underlying the temperature-dependent pH-regulatory strategy of *V. exanthematicus* (Wood et al., 1977, 1981). Our findings on the chemosensory mechanisms affected by temperature are only the beginning of studies that should address whether differences in central CO_2 chemosensitivity are key mechanisms underlying differences in pH-regulatory strategies among ectothermic vertebrates. It is possible that the differences we see in chemosensitivity in lizard LC neurons are because they are pH-stat regulators while bullfrogs are α-stat regulators. Alternatively, it is possible that there are other chemosensitive sites in lizard brainstem that have a greater influence in the total chemosensitive response to HA. We do not have sufficient information at this early stage to differentiate the reason for variable chemosensitivity, and further studies are necessary to address this question.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
L.A.Z., E.M.F., J.M.S., L.P., L.H.G., K.C.B. and L.K.H. designed the research, performed data analysis and prepared the manuscript prior to submission. L.A.Z., E.M.F., J.M.S. and L.P. performed the experiments.

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