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Noradrenergic modulation determines respiratory network activity during temperature changes in the *in vitro* brainstem of bullfrogs

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ABSTRACT

Among vertebrate ectotherms, air breathing frequency is generally constrained across warmer temperatures, but decreases during cooling. The brainstem mechanisms that give rise to this ventilatory strategy are unclear. Neuromodulation has recently been shown to stabilize motor circuit output across temperatures. Therefore, we tested the hypothesis that an important neuromodulatory system in respiratory control network, norepinephrine, produces this pattern of respiratory motor activity across temperatures. To this end, we used *in vitro* brainstem-spinal cord preparations from adult bullfrogs, *Lithobates catesbeianus*, to assess the role of noradrenergic signaling in shaping the frequency response of the respiratory network during temperature changes. We identified that noradrenergic signaling through the α_1 adrenergic receptor constrains motor output from the respiratory network across warm temperatures. In contrast, the α_2 adrenergic receptor actively inhibits respiratory motor output during cooling. These results indicate that noradrenergic tuning, rather than passive thermal responses, produces temperature responses of the respiratory circuits.

1. Introduction

Although temperature has potential to influence all physiological systems, the ability of the central nervous system to control regulatory processes plays an important role in determining thermal relationships between animals and their environments (Robertson and Money, 2012). Neural networks that control vital functions must therefore be tuned to operate appropriately over their temperature range to produce proper behaviors, especially in poikilothermic animals that do not maintain body temperature during ambient temperature changes. In general, temperature increases the rate of physical and chemical processes to varying extents. This is commonly described by a temperature coefficient, Q_{10}; change in rate resulting from a 10 °C increase. A predictable relationship generally exists between temperature and biophysical and biochemical processes at smaller scales of organization (*e.g.,* enzyme activity, ion channel conductance, *etc.*) (Behrisch and Hochachka, 1969; Hochachka, 1974). However, recent work indicates that the effects of temperature on neuronal firing and circuit output may be challenging to predict because multiple interacting processes with different Q_{10}s produce their responses (Marder et al., 2015). Although some neurophysiological properties (*e.g.,* axonal conduction and action potential duration) may follow straightforward Q_{10} effects originating from their molecular underpinnings (Hodgkin and Katz, 1949) neural systems may be tuned to maintain some degree of consistency across a range of temperatures (Tang et al., 2010; Roemschied et al., 2014; Städele et al., 2015). Thus neural systems appear to be tuned to respond to temperature variations in ways that can defy simple Q_{10} effects, which may reflect the behaviors they control.

In this context, the respiratory central pattern generating circuit from anuran amphibians represents an interesting example from the standpoint of temperature-dependent tuning. In the intact awake animal, breathing frequency is relatively stable across warm temperatures, but decreases at lower temperatures (Bícego-Nahas and Branco, 1999; Kruhoeffer et al., 1987). Lung ventilation and accompanying motor patterns then cease near ~5–10 °C (Mackenzie and Jackson, 1978; Feder and Burggren, 1985; Tattersal, 2006; Santin and Hartzler, 2017). These general trends are conserved in respiratory motor output of the isolated brainstem (Morales and Hedrick, 2002). The mechanisms that shape the patterns of respiratory output from the brainstem during temperature changes remain undetermined.

The central respiratory neural network is a complex circuit composed of rhythm generating, sensory integrating, and neuromodulatory nuclei (Smith et al., 2013; Richter and Smith, 2014; Baghdadwala et al., 2015) any of which has the potential to influence temperature-dependent tuning. Among these modules involved in respiratory control, neurons in the noradrenergic locus coeruleus (LC) of bullfrogs have

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been shown to exhibit firing responses to temperature changes (Santin et al., 2013; Santin and Hartzler, 2015) that could contribute to the interesting frequency response of the respiratory network during temperature changes. Specifically, ~85% of putative noradrenergic neurons found in the LC have firing responses that vary inversely with temperature changes and ~15% of LC neurons have firing rates that vary proportionally with temperature (Santin and Hartzler, 2013). Although the downstream neuronal targets of this response remain unknown, specific receptors, termed α_1 and α_2 adrenergic receptors (AR), bind norepinephrine to elicit respiratory modulation in bullfrogs (Fournier and Kinkead, 2006; Fournier et al., 2007). Given that neurons within the LC have firing responses to temperature that may either be excitatory or inhibitory (Santin et al., 2013; Santin and Hartzler, 2015) and that norepinephrine modulates the respiratory circuit through AR, we assessed the extent to which norepinephrine signaling mediates the stereotyped temperature sensitivity of the bullfrog respiratory network. Here we tested the hypothesis that maintenance of respiratory output across higher temperatures and its diminution during cooling occurs as a result of noradrenergic signaling in the respiratory network rather than a passive response to temperature changes predictable by Q_{10} effects. To this end, we used the *in vitro* brainstem-spinal cord preparation from adult bullfrogs to determine whether α_1 and α_2 AR are involved in producing these characteristic responses of the bullfrog respiratory network to temperature changes.

2. Materials and methods

2.1. Ethical approval

The use of animals for these protocols was approved by Wright State University's Institutional Animal Care and Use Committee.

2.2. Experimental animals

Experiments were performed in adult female bullfrogs *Lithobates catesbeianus* weighing 112 ± 12 g. Animals were purchased and shipped from *Rana* Ranch (Twin Falls, ID, USA) and kept for at least 1 week in the Laboratory Animal Resources (LAR) facilities prior to experimental procedures.

2.3. Brainstem-spinal cord preparations

Brainstem-spinal cord dissections were performed as previously described (Santin and Hartzler, 2013). Briefly, animals were killed by rapid decapitation and brainstem-spinal cord were dissected in ice-cold artificial cerebral spinal fluid (aCSF; composition in mM: 104NaCl, 4 KCl, 1.4 $MgCl_2$, 7.5 glucose, 40 $NaHCO_3$, 2.5 $CaCl_2$ and 1 NaH_2PO_4) (Kinkead et al., 1994) and transected caudal to spinal nerve II. The preparation was then placed in a ~7 mL chamber made from a petri dish and Sylgard (DowCorning, Midland, MI, USA) and pinned with the ventral side up allowing access to the cranial nerve roots. The preparation was superfused with aCSF at a rate of 6 mL min^{-1}. Temperature of aCSF was controlled with a Warner Instruments bipolar temperature controller (model CL-100; Hamden, CT, USA) and recorded next to the brainstem-spinal cord in the petri dish using a temperature probe (ADInstruments Inc., Colorado Springs, CO, USA). All preparations were given at least one hour to recover in the experimental chamber at 20 °C while the solution was equilibrated with 90% O_2, 1.3% CO_2 and balance N_2 and superfused using a peristaltic pump (Rainin Instrument Co., Oakland, CA, USA).

2.4. Whole nerve recording

To record respiratory-related motor nerve activity from cranial nerves V and IX-X, trigeminal and joined rootlet of the glossopharyngeal and vagus nerves respectively, we used borosilicate glass bipolar suction electrodes that were pulled using a two-stage micropipette puller (PC-10; Narishige, East Meadow, NY, USA). The glass electrode tips were broken down to an appropriate size to fit snugly around the nerve, fire polished, and then attached to the nerve by suction. Nerve activity was amplified ×1000 using differential amplifiers (DP-311; Warner Instruments, Hamden, CT, USA), filtered (100–1000 Hz), full-wave rectified, integrated (time constant, 60 ms) and recorded using the Powerlab 8/35 data acquisition system (ADInstruments Inc., Colorado Springs, CO, USA).

2.5. Experimental procedures

To identify the effect of temperature on the respiratory network we changed temperature from 20 °C to 25° then to 15 °C. To determine the role of norepinephrine in producing this response we performed this same temperature protocol in the presence of AR antagonists. To block the α_1 AR we superfused the preparation with a standard antagonist; prazosin; 1 μ mol L^{-1} (Ovadia et al., 1996; Hilaire et al., 2004; Fournier and Kinkead, 2006; Fournier et al., 2007; Oliveira et al., 2016) and for α_2 AR we used the standard antagonist, RX821002 (RX) 25 μ mol L^{-1}. (Vauquelin et al., 1990; Ovadia et al., 1996; Fournier and Kinkead, 2006; Fournier et al., 2007; Romero et al., 2013). The following is a detailed description of the temperature and drug protocols. After a recovery period in the experimental chamber, we attached the suction electrodes to the nerves and recorded motor neuron activity. We kept the superfusate of aCSF at 20 °C and recorded for 45–60 minutes until prolonged stable activity was observed. Once stable firing was achieved, we changed from regular aCSF to a solution containing aCSF and either prazosin or RX. We maintained flow of aCSF + antagonist at 20 °C for 30–40 minutes until stable bursting was observed at which point we applied the following temperature adjustments. From 20 °C we increased the temperature of the bath to obtain 25 °C in the recording chamber. After 10 min stable neural output was achieved, so each temperature interval was 15 min. After 15 min of activity at 25 °C, we decreased the temperature to 15 °C in the bath and recorded activity for 15 min. Control animals were exposed to the same protocol but without drug application. We used 24 frogs (control n = 11, α_1 AR antagonist n = 6 and α_2 AR antagonist n = 7).

Based on our results from the first temperature protocol, we designed a second protocol to validate the effect of each drug at specific temperatures. In these experiments we adjusted temperature first, waited for a stable recording, and then applied the drug. We initially set the superfusate of aCSF at 20 °C and recorded for 45–60 minutes until prolonged stable activity was observed. Then, we adjusted the temperature to be either 15 °C or 25 °C in the bath. After 15 min of activity we changed the superfusate from regular aCSF to a solution containing aCSF and either prazosin or RX. We maintained flow of aCSF + antagonist for 30-40 min until stable bursting was observed and then recorded 15 more minutes and analyzed this 15 min period. We used 5 frogs for the prazosin application at 25 °C and 4 frogs for the RX application at 15 °C. In total for both protocols we used 33 frogs (control n = 11, α_1 AR antagonist n = 11 and α_2 AR antagonist n = 11).

2.6. Data analysis and statistics

In this study we only analyzed fictive lung burst frequency. Neural activity was defined as a fictive lung burst when large amplitude activity occurred near-synchronously on both CN IX-X and V rootlets. For the temperature protocols we analyzed the last 5 min of respiratory-related nerve discharge for each of the three temperatures assessed here to determine the fictive lung burst frequency (25 °C, 20 °C and 15 °C). We used fictive lung burst frequency at 20 °C as baseline and calculated the percent change at 15° and 25 °C. We compared burst frequency for each treatment at all temperatures with a repeated measurements one-way analysis of variance (RM-ANOVA) followed by a Tukey's multiple comparison *post hoc* test. Additionally, we presented and compared