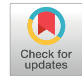
Comparative Biochemistry and Physiology, Part A 225 (2018) 65–73

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Isolated adult turtle brainstems exhibit central hypoxic chemosensitivity



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ARTICLE INFO

Keywords:
α_1 adrenergic receptors
Breathing
Chelonian
Episodicity
Hypoxia
Reptile
Respiratory motor output
Serotonin 5-HT_3 receptors
Turtle

ABSTRACT

During hypoxia, red-eared slider turtles increase ventilation and decrease episodic breathing, but whether these responses are due to central mechanisms is not known. To test this question, isolated adult turtle brainstems were exposed to 240 min of hypoxic solution (bath $PO_2 = 32.6 \pm 1.2$ mmHg) and spontaneous respiratory-related motor bursts (respiratory event) were recorded on hypoglossal nerve roots. During hypoxia, burst frequency increased during the first 15 min, and then decreased during the remaining 35–240 min of hypoxia. Burst amplitude was maintained for 120 min, but then decreased during the last 120 min. The number of bursts/respiratory event decreased within 30 min and remained decreased. Pretreatment with either prazosin (α_1-adrenergic antagonist) or MDL7222 (5-HT_3 antagonist) blocked the hypoxia-induced short-term increase and the longer duration decrease in burst frequency. MDL7222, but not prazosin, blocked the hypoxia-induced decrease in bursts/respiratory event. Thus, during bath hypoxia, isolated turtle brainstems continued to produce respiratory motor output, but the frequency and pattern were altered in a manner that required endogenous α_1-adrenergic and serotonin 5-HT_3 receptor activation. This is the first example of isolated reptile brainstems exhibiting central hypoxic chemosensitivity similar to other vertebrate species.

1. Introduction

The hypoxic ventilatory response in ectothermic vertebrates is poorly understood due to the variable time course of the hypoxic response (minutes to days), and differences in the hypoxic sensitivity (Porteus et al., 2011). Semi-aquatic, red-eared slider turtles (*Trachemys scripta*) provide a unique perspective on respiratory changes induced by hypoxia because these turtles tolerate hypoxic conditions for days at room temperature (Jackson, 2000). Adult turtles breathe mostly episodically under normoxic conditions, with respiratory events containing clusters of 4–6 breaths separated by apneas of various lengths (Funk and Milsom, 1987; Johnson et al., 2008). During hypoxia, semi-aquatic turtles increase breathing frequency by decreasing the length of respiratory apneas (Boyer, 1963; Glass et al., 1983; West et al., 1989), and decreasing the number of breaths that occur within each respiratory event (Frische et al., 2000). Red-eared slider turtles produce a distinctive highly regular, singlet-breath pattern during hypoxia (Frische et al., 2000; Johnson et al., 2015). The physiological mechanisms underlying these hypoxia-induced changes in breathing rhythm and pattern in turtles are not known.

The hypoxic ventilatory response is mainly due to sensory afferent input from peripheral chemoreceptors, but significant contributions are proposed to come from central chemosensitive regions in mammals (Neubauer and Sunderram, 2004; Powell et al., 2009; Angelova et al., 2015; Funk et al., 2015; Gourine and Funk, 2017), such as the pre-Bötzinger Complex (Solomon, 2000; Solomon et al., 2000; Solomon, 2004, 2005; Peña and Ramirez, 2005; Hill et al., 2011), nucleus tractus solitarius (Pascual et al., 2002), and locus coeruleus (Nieber et al., 1995). Central hypoxic chemosensitivity may be due to hypoxia-induced changes in neuronal function (e.g., Pascual et al., 2002) or in neuronal-glial interactions (e.g., Huxtable et al., 2010; Angelova et al., 2015; Funk et al., 2015; Gourine and Funk, 2017). In contrast, for non-mammalian species, little is known regarding the expression of central hypoxic chemosensitivity. For example, isolated goldfish brainstems increase respiratory motor bursts during bath hypoxia (Côté et al., 2014). For isolated premetamorphic tadpole brainstems, bath hypoxia induces a biphasic lung frequency response with a short (10 min) increase in respiratory motor burst frequency that requires α_1-adrenergic receptor activation, followed by a decrease in respiratory frequency for 3 h (Winmill et al., 2005; Fournier et al., 2007; Fournier and Kinkead, 2008; Rousseau et al., 2016). Isolated adult frog brainstems respond to hypoxia similarly with an initial increase in lung-related respiratory motor burst frequency (Rousseau et al., 2016), but then there is an α_1-adrenergic receptor-dependent sustained decrease in respiratory motor

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https://doi.org/10.1016/j.cbpa.2018.07.001
Received 31 January 2018; Received in revised form 27 June 2018; Accepted 3 July 2018
Available online 09 July 2018
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burst frequency (Fournier et al., 2007). Transections of the pons in isolated adult frog brainstems removes the locus coeruleus and abolishes the hypoxia-induced decrease in lung-related respiratory motor burst frequency, suggesting that the locus coeruleus is necessary for central hypoxic chemosensitivity (Fournier and Kinkead, 2008). For reptiles, less is known regarding central hypoxic chemosensitivity and potential underlying mechanisms. In isolated brainstems from adult red-eared slider turtles, a 2-h bout of bath hypoxia did not alter respiratory burst frequency and amplitude (Johnson et al., 1998), but the bath PO_2 levels and other variables associated with the pattern of respiratory bursts were not quantified in that study.

To address these questions, adult turtle brainstems were exposed to hypoxic bath conditions to determine the effects on respiratory motor burst pattern. Adult turtle brainstems are ideal because they produce respiratory output similar to intact turtles (i.e., expiratory and inspiratory motor activity; Johnson and Mitchell, 1998) for > 40 h under in vitro conditions (Wilkerson et al., 2003). Specifically, this study exposed isolated turtle brainstems to a 240-min bout of bath hypoxia to determine (1) the acute and long-lasting effects of hypoxia on respiratory burst frequency, amplitude, and the number of bursts/respiratory event, and (2) whether α_1-adrenergic or serotonin 5-HT_3 receptor activation is necessary for the expression of central hypoxic chemosensitivity. α_1-adrenergic receptor activation is required for the adult frog central hypoxic response (Fournier et al., 2007), and bath-applied phenylephrine (α_1-adrenergic receptor agonist) to isolated turtle brainstems increases burst frequency and decreases the number of bursts/respiratory event (Bartman and Johnson, 2012), similar to the hypoxic response intact freely-breathing turtles (Bartman and Johnson, 2012). Likewise, bath-application of specific 5-HT_3 receptor agonists to isolated turtle brainstems decreases the number of bursts/respiratory event (Bartman et al., 2010). Thus, we hypothesized that these two receptors may contribute to the response of the central turtle respiratory network to hypoxia.

2. Methods

2.1. Procedures

All procedures were approved by the Animal Care and Use Committee at the University of Wisconsin-Madison School of Veterinary Medicine. Adult turtles (*Trachemys scripta*, n = 89, weight = 749 ± 29 g) were obtained from commercial suppliers and kept in a large open tank where they had access to water for swimming, and heat lamps and dry areas for basking. Room temperature was set to 27–28 °C with light 14 h per day. Turtles were fed ReptoMin® floating food sticks (Tetra, Blacksburg, VA) 3–4 times per week. To minimize seasonal differences in metabolism and ventilation in this species (Reyes and Milsom, 2010), newly arrived turtles were allowed to acclimate in tanks for 1–4 weeks before experiments were performed, and drug experiments were distributed over multiple seasons.

Turtle brainstems were isolated as described previously (Johnson et al., 1998). Turtles were intubated and anesthetized with 5% isoflurane (balance O_2) until limb withdrawal to noxious foot pinch was eliminated. Turtles were rapidly decapitated and decerebrated. Brainstems were removed and pinned down in a small-volume recording chamber (1.5 ml volume) with the ventral surface facing upwards (Fig. 1A). Brainstems were superfused (5–6 ml/min) with standard solution containing HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]) buffer as follows (in mM): 100 NaCl, 23 $NaHCO_3$, 10 Glucose, 5 HEPES (sodium salt), 5 HEPES (free acid), 2.5 $CaCl_2$, 2.5 $MgCl_2$, 1.0 K_2PO_4, and 1.0 KCl. Standard solution was aerated with 95% O_2–5% CO_2; pH = ~7.35. To record respiratory bursts, glass suction electrodes were attached to hypoglossal nerve rootlets (Fig. 1A). Signals were amplified (10,000 ×) and band-pass filtered (1.0–500 Hz) using a differential AC amplifier (model 1700, A-M Systems, Everett, WA) before being rectified and integrated (time constant = 200 ms) using a moving averager (MA-821/RSP, CWE, Inc., Ardmore, PA; Fig. 1B). Analysis was performed using Axoscope software (Axon Instruments, Foster City, CA).

After allowing the brainstems to equilibrate for 2–6 h, baseline data were obtained by recording 30 min of spontaneous respiratory activity before changing to hypoxic solution or adding drugs to the reservoir. To induce hypoxia, standard solution was aerated with 95% N_2–5% CO_2, and parafilm© (Beemis NA, Neenah, WI) was secured over the chamber opening while 95% N_2–5% CO_2 was blown underneath the barrier. To determine bath PO_2 under hyperoxic and hypoxic conditions, ~0.2 ml solution samples were obtained by pulling the solution into 1.0 ml syringes and injecting the solution into a blood-gas analyzer (ABL 500; Radiometer, Copenhagen, Denmark). Values were corrected to solution temperature (24 °C).

Prazosin hydrochloride (prazosin, α_1 adrenergic receptor antagonist) was obtained from Sigma/RBI Aldrich (St. Louis, MO), while the Tropanyl 3,5-dichlorobenzoate (MDL7222, 5-HT_3 receptor antagonist) was obtained from Tocris Bioscience (Ellisville, MO).

2.2. Data analysis

Respiratory burst variables were measured as previously described (Johnson et al., 1998; Bartman et al., 2010; Bartman and Johnson, 2012). Burst frequency was calculated as the total number of respiratory motor bursts per minute. Percent time-to-peak was calculated by dividing the time from burst onset to time of the peak by the burst duration. Respiratory events included single motor bursts (singlets) and episodes (e.g., doublets, triplets, quadruplets; Fig. 1B). Two or more bursts separated by less than twice the average duration of a single burst were considered part of the same respiratory event. A motor burst pattern was considered ‘episodic’ if the average baseline bursts/respiratory event was > 1.75. To calculate changes in bursts/respiratory event, only preparations with a bursts/respiratory event > 1.75 were considered (Figs. 3D, 5D, 6D). Burst amplitude was measured at the highest point of integrated hypoglossal nerve discharge in arbitrary units and normalized to the average amplitude during the baseline period. Baseline data were averaged into one 30-min bin, burst frequency was averaged into 5-min bins for the first 60 min (to capture rapid changes), while amplitude and number bursts/respiratory event were averaged into 10-min bins (because there were no rapid changes). Data for the 120, 180, and 240 min time points were averaged into 30-min bins. Date were reported as the mean ± S.E.M. A two-way ANOVA with repeated measures design (Sigma Stat, Jandel Scientific Software, San Rafael, CA) was used to compare data with respect to burst frequency, burst amplitude, and bursts/respiratory event (Figs. 3, 5, 6). A one-way ANOVA with repeated measures design was used to compare baseline, hypoxia, and recovery data for hypoxia-exposed brainstems (Fig. 4). If normality and equal variance assumptions were not satisfied (no data transformations were successful), data were ranked first and then two-way ANOVAs with repeated measures design were performed (Figs. 3A-C, 5A-B, 6A-B) similar to previous publications (Majewski et al., 2008; Johnson and Creighton, 2005; Johnson et al., 2008, 2010). Post-hoc comparisons were made using the Student-Newman-Keul's test. P-values < .05 were considered significant.

3. Results

3.1. Bath hypoxia following switch to hypoxic solution

To determine the bath level of hypoxia, samples (0.2 ml) of bath solution (n = 3 samples per time point) were drawn from the bath under hyperoxic conditions, and from 5 to 240 min following the switch to hypoxic solution. To determine the upper and lower limits of the bath PO_2 levels, solution samples were taken from flasks aerated with 95% O_2–5% CO_2 and 95% N_2–5% CO_2. The upper and lower limits were PO_2 = 569 ± 4 mmHg and 27 ± 0.4 mmHg, respectively (horizontal

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