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Isolated adult turtle brainstems exhibit central hypoxic chemosensitivity

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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 $PO_2 = 32.6 \pm 1.2$ mm Hg) 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-HT₃ 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-HT₃ receptor activation. This is the first example of isolated reptile brainstems exhibiting central hypoxic chemosensitivity similar to other vertebrate species.

Keywords

α_1 adrenergic receptors; Breathing; Chelonian; Episodicity; Hypoxia; Reptile; Respiratory motor output; Serotonin 5-HT₃ receptors; Turtle

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

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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; Pena 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 (Cote 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 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 more than 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-HT₃ 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-HT₃ 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 O₂) 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-ethane-sulfonic acid]) buffer as follows (in mM): 100 NaCl, 23 NaHCO₃, 10 Glucose, 5 HEPES (sodium salt), 5 HEPES (free acid), 2.5 CaCl₂, 2.5 MgCl₂, 1.0 K₂PO₄, and 1.0 KCl. Standard solution was aerated with 95% O₂-5% CO₂; pH = ~7.35. To record respiratory bursts, glass suction electrodes were attached to hypoglossal nerve rootlets (Fig. 1A). Signals were amplified (10,000x) 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% N₂-5% CO₂, and parafilm© (Beemis NA, Neenah, WI) was secured over the chamber opening while 95% N₂-5% CO₂ was blown underneath the barrier. To determine bath PO₂ 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-HT₃ 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. Data were reported as the mean $\pm$ 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 < 0.05 were considered significant.

3.0 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 5–240 min following the switch to hypoxic solution. To determine the upper and lower limits of the bath PO₂ levels, solution samples were taken from flasks aerated with 95% O₂-5% CO₂ and 95% N₂-5% CO₂. The upper and lower limits were PO₂ = 569 ± 4 mm Hg and 27 ± 0.4 mm Hg, respectively (horizontal dotted lines in Fig 1C). Bath PO₂ was at 493 ± 7 mm Hg while

aerated with 95% O₂-5% CO₂, and decreased to below 100 mm Hg after 15 min of hypoxic solution application before reaching a minimum of 32 ± 63 mm Hg at 210 min (Fig. 1C).

3.2 Hypoxia-induced changes in respiratory motor output

To examine hypoxia-induced changes, isolated turtle brainstems were exposed to either control solution (n=19) or hypoxic solution (n=20; Fig. 2) for 240 min after recording baseline activity. Brainstems exposed to control solution exhibited no time-dependent changes in burst frequency for 240 min (Figs. 3A, 3B) or bursts/respiratory event for the subset of brainstems (n=6/19) that produced episodic bursts (Fig. 3D). Burst amplitude decreased from baseline by only 10-13% at the 180 and 240 min time points ($P=0.012$; Fig. 3C) similar to previous control experiments using isolated turtle brainstems (Bartman et al., 2010; Bartman and Johnson, 2012). There were no time-dependent changes observed in burst duration or percent time-to-peak during the 240 min in control brainstems (Table 1).

In contrast, exposure to hypoxic solution resulted in a biphasic frequency response. Burst frequency increased from 0.56 ± 0.05 to 0.78 ± 0.10 bursts/min after 15 min of exposure ($P=0.018$), and then decreased to 0.35 ± 0.04 bursts/min after 55 min (Fig 3A). From 120–240 min, burst frequency was decreased from baseline ($P<0.009$). When graphed as the change in burst frequency, the biphasic response was more apparent with an initial frequency increase followed by a sustained frequency decrease (Fig. 3B). When analyzing baseline and the first 15 min of hypoxia, there was a significant condition effect ($P=0.025$; hypoxia vs. controls) with the frequencies at 10 and 15 min increased compared to controls ($P<0.034$; Fig. 3B inset). The sustained frequency decrease was observed from 35–240 min of hypoxia (Fig. 3B). Hypoxic solution had no effect on burst amplitude for the first 40 min, but then a decrease from baseline was observed from 50–240 min ($P<0.017$; Fig. 3C). In brainstems that produced episodic bursts (9/20), bursts/respiratory event decreased from 2.15 ± 0.14 to 1.44 ± 0.15 after 30 min of hypoxia ($P=0.002$) where it remained relatively unchanged to the 240-min time point ($P=0.035$ for condition effect; Fig. 3D). Exposure to hypoxic solution for 240 min did not alter burst duration or percent time-to-peak (Table 1).

3.3 Reversibility of hypoxia-induced changes in respiratory motor output

To determine if the long-term changes in respiratory burst variables were reversible following reoxygenation, some of the isolated brainstems (n=14/20) that were exposed to hypoxia for 240 min were then exposed to oxygenated standard solution for 2 h. Burst frequency decreased from 0.53 ± 0.05 to 0.30 ± 0.05 bursts/min after 240 min of hypoxia ($P=0.011$ compared to baseline), and partially recovered to 0.40 ± 0.06 bursts/min after reoxygenation ($P=0.095$ compared to baseline; Fig. 4A). Burst amplitude decreased to 0.66 ± 0.04 after 240 min of hypoxia ($P<0.05$ compared to baseline) and was still decreased at 0.73 ± 0.05 ($P<0.05$ compared to baseline) during reoxygenation (Fig. 4B). Bursts/respiratory event were reduced from 2.22 ± 0.17 (baseline) to 1.08 ± 0.06 bursts/respiratory event following 240 min of hypoxia ($P<0.05$ compared to baseline) and was still decreased at 1.59 ± 0.21 bursts/respiratory event following 2 h of reoxygenation ($P<0.05$; Fig. 4C).

3.4 Hypoxia-induced changes in respiratory output: role of α_1 -adrenergic receptor activation

To determine which features of the acute hypoxic response required α_1 -adrenergic receptor activation, 0.5 μ M prazosin (n=12 brainstems; α_1 -adrenergic receptor antagonist) was bath-applied 30-min prior to and during the 60-min hypoxic exposure. Based on a previous study, 0.5 μ M prazosin has no effect on burst frequency and a minimal effect on burst amplitude during a 2-h bath application (Bartman et al., 2012). Prazosin application prevented the initial increase in burst frequency at 15 min ($P=0.564$ compared to baseline), as well as the hypoxia-induced decrease in burst frequency during the 30–60 min of hypoxia ($P=0.044$ for drug effect; Fig. 5A). When graphed as the change in frequency, the initial hypoxia-induced increase in burst frequency within the first 15 min ($P=0.012$ for hypoxic vs. control data) was abolished with prazosin application ($P=0.928$ for prazosin/hypoxia vs. control data; Fig. 5B, inset). There was also little decrease in burst frequency during 20–60 min of hypoxia with prazosin application (Fig. 5B). There were no differences in burst amplitude ($P=0.53$ for drug effect; Fig. 5C) or for bursts/respiratory event (6/12 brainstems exhibited episodic discharge; $P=0.412$ for drug effect; Fig. 5D) with prazosin treatment during bath hypoxia.

3.5 Hypoxia-induced changes in respiratory output: role of 5-HT₃ receptor activation

To determine which features of the hypoxic response required serotonin 5-HT₃ receptor activation, 50 μ M MDL7222 (n=13 brainstems; 5-HT₃ receptor antagonist) was bath-applied 30 min prior to and during the 60-min hypoxic exposure. This concentration of MDL7222 has minimal effects on burst frequency and bursts/respiratory event during a 2.5 h application (Bartman et al., 2010). MDL7222 pretreatment prevented the initial hypoxia-induced increase in burst frequency as well as the sustained hypoxia-induced frequency decrease (Figs. 6A, 6B). When graphed as the change in frequency, the initial hypoxia-induced increase in burst frequency within the first 15 min ($P=0.015$ for condition effect [hypoxia vs. controls]) was abolished with MDL7222 application ($P=0.981$ for MDL7222/hypoxia vs. controls; Fig. 6B, inset). Instead of a sustained hypoxia-induced decrease in burst frequency, there was a sustained burst frequency increase above baseline during the 35–60 min of hypoxia ($P<0.001$ for drug effect; Fig. 6B). In the presence of MDL7222, hypoxia decreased burst amplitude during the 35–60 min period ($P<0.005$ compared to baseline) and to 0.70 ± 0.04 of baseline after 60 min ($P=0.006$ for drug effect; Fig. 6C). In brainstems with episodic motor activity (n=6/13), the hypoxia-induced decrease in bursts/respiratory event was blocked by MDL7222 pretreatment because bursts/respiratory event at 30–60 min were not decreased compared to baseline ($P=0.09$ for drug effect; Fig. 6D).

4.0 Discussion

This is the first study to demonstrate central hypoxic chemosensitivity in isolated adult reptile brainstems. Our data suggest that the central respiratory network contributes, in part, to the increase in breathing frequency and reduction in breath clustering observed during hypoxia *in vivo*. Isolated adult turtle brainstems responded to hypoxia within 15 min, and several changes in respiratory burst variables persisted for the duration of the 240-min hypoxic exposure. The hypoxia-induced initial increase and later sustained decrease in respiratory burst frequency required α_1 -adrenergic and 5-HT₃ receptor activation. The

hypoxia-induced reduction in bursts/respiratory event may require 5-HT₃ receptor activation although caution is warranted in interpreting these data (see below).

4.1 Central chemosensitivity in ectothermic vertebrates

For isolated ectothermic vertebrate brainstems, hypoxia-induced changes in respiratory motor output are generally similar with minor differences due to species or maturation. For example, bath hypoxia increases respiratory burst frequency in brainstems isolated from pre- and postmetamorphic tadpoles and adult bullfrogs (Winmill et al., 2005; Rousseau et al., 2016), and adult turtles in this study (Figs. 3B). With prolonged hypoxia, respiratory motor bursts are abolished in postmetamorphic tadpole and adult bullfrog brainstems, and burst frequency is significantly reduced after 3 h in premetamorphic tadpole brainstems (Winmill et al., 2005). As mentioned before, our previous study found no changes in respiratory motor output during a 2-h exposure to hypoxia (Johnson et al., 1998). We speculate that the lack of change in respiratory motor output was due to insufficient low levels of bath hypoxia. In this study with bath PO₂ reaching 32.6 mm Hg, hypoxia increased burst frequency within 15 min, followed by a sustained decrease in burst frequency from 40–240 min of hypoxia. Taken together, these data suggest that frequency response to hypoxia is conserved between isolated brainstems from adult turtles and frogs at different developmental ages (Fournier et al., 2007). The ability of brainstems from adult turtles and premetamorphic tadpoles to continue producing respiratory motor output during sustained bath hypoxia is due to their inherent hypoxia-resistance. Red-eared slider turtles are among the most hypoxia-resistant vertebrates (Bickler et al., 2002; Bickler and Buck, 2007), while premetamorphic tadpoles are more hypoxia-resistant than postmetamorphic tadpoles and adult frogs (Winmill et al., 2005).

Less is known with respect to how hypoxia alters respiratory burst episodicity. In isolated brainstem preparations from postmetamorphic tadpole and adult bullfrogs, bath hypoxia increases respiratory burst frequency by increasing bursts/respiratory event (Winmill et al., 2005). On the other hand, there is no change in lung burst episodicity during hypoxia in premetamorphic tadpole brainstems (Winmill et al., 2005). In contrast to the adult amphibian brainstems, adult turtle brainstems in this study decreased the number of bursts/respiratory event for 30–240 min of the hypoxic exposure. The different hypoxia-induced changes in respiratory burst episodicity among the species tested so far suggests that this feature of the central hypoxic ventilatory response is not well conserved.

4.2 Hypoxia-induced changes in respiratory burst shape

In intact mammals, hypoxia decreases the frequency and duration of inspiratory motor bursts, and alters the single burst pattern from a slowly incrementing/rapidly decrementing or bell-shaped burst into a large rapidly incrementing/slowly decrementing burst (St. John, 1990; St. John and Paton, 2000). Likewise, bath hypoxia decreases rise time, duration, and frequency of inspiratory motor bursts within the pre-Bötzinger complex of neonatal murine rhythmically active medullary slices (Lieske et al., 2000; Hill et al., 2011). In contrast, isolated amphibian brainstems do not undergo changes in respiratory burst shape, duration, amplitude, or rise time during sustained bath hypoxia at any developmental stage (Winmill et al., 2005). Similarly, this study showed no changes in burst duration or rise time (percent

time-to-peak) in isolated adult turtle brainstems exposed to hypoxia, suggesting that the isolated turtle respiratory rhythm generator does not switch to a gasp-like respiratory motor output. In support of this hypothesis, intact turtles breathing pure nitrogen for several hours do not switch to a gasp-like respiratory motor pattern (Johnson et al., 2015). Instead, there is an increase in inspiratory and expiratory duration with a somewhat larger increase in inspiratory drive, which may have some mechanistic similarities to the augmented inspiratory drive during mammalian gasping (Johnson et al., 2015).

4.3 Recovery of respiratory output during reoxygenation following hypoxia

Reversible cessation of breathing may be an adaptive, energy-saving response to sustained hypoxia, especially in hypoxia-tolerant animals (Winmill et al., 2005). Following 1.0 h of anoxia in isolated neonatal rat brainstem-spinal cord preparations, spinal respiratory motor activity recovers to baseline burst frequency levels following reoxygenation (Ballanyi et al., 1992), whereas perfused adult rat brainstems cease hypoglossal respiratory motor output after only 5–10 min under anoxic conditions (Ballanyi et al., 1992). In isolated post-metamorphic and adult frog brainstems, lung burst frequency is abolished within 25 min of bath hypoxia, but recovers to baseline levels after 2 h of apnea (Winmill et al., 2005). Isolated brainstems from pre-metamorphic tadpoles, in contrast, continue producing respiratory bursts during 3 h of sustained bath hypoxia (Winmill et al., 2005). Similar to neonatal rats and pre-metamorphic tadpoles, isolated adult turtle brainstems produce respiratory motor output for at least 240 min during hypoxic conditions. A 2-h reoxygenation period nearly restored the burst frequency, but burst amplitude and bursts/respiratory event were not restored to baseline levels. These data suggest that the isolated turtle brainstem is robust under long-lasting hypoxic conditions, but may undergo potentially irreversible tissue damage *in vitro*, or irreversible changes in respiratory rhythm generation and pattern.

4.4 Role of α_1 -adrenergic or serotonin 5-HT₃ receptors in the hypoxic ventilatory response

α_1 -adrenergic receptor activation is hypothesized to be involved in the central chemosensory response to hypoxia in mammals and ectothermic vertebrates. In neonatal sheep, hypoxia results in a biphasic phrenic burst frequency change with an immediate increase followed by a decrease (Moore et al., 1996). The frequency decrease is prevented by focal cooling of the hypoxia-sensitive locus coeruleus, the principal site for norepinephrine in the brainstem (Moore et al., 1996; Breen et al., 1997; Neubauer and Sunderram, 2004). Similarly, in isolated adult frog brainstems, the hypoxia-induced decrease in respiratory burst frequency is blocked by an α_1 -adrenergic receptor antagonist drug, and mimicked by noradrenaline application (Fournier et al. 2007). In contrast, α_1 -adrenergic receptor activation in turtles increases, rather than decreases, respiratory burst frequency and decreases bursts/respiratory event (Johnson et al., 1998; Bartman and Johnson, 2012). Accordingly, blockade of α_1 -adrenergic receptors in this study prevented a significant increase in burst frequency within 15 min of hypoxia as well as the longer term decrease in burst frequency (Fig. 5B), while the hypoxia-induced decrease in bursts/respiratory event was not altered (Fig. 5D). Thus, α_1 -adrenergic receptors appear to play a key role in the frequency response to hypoxia in turtles.

With respect to serotonin 5-HT₃ receptors, the hypoxia-induced shift to a singlet breathing pattern in intact turtles is hypothesized to be attenuated by pretreatment with a 5-HT₃ receptor antagonist (Johnson et al., 2015). In isolated turtle brainstems, 5-HT₃ receptor activation converts the respiratory motor output to singlet bursts, whereas blockade of 5-HT₃ receptors increases bursts/respiratory event (Bartman et al., 2010). This suggests that there is continuous endogenous activation of 5-HT₃ receptors in isolated turtle brainstems. In this study, pretreatment with MDL7222 prior to hypoxia prevented the hypoxia-induced decrease in burst frequency and bursts/respiratory event, but decreased burst amplitude at 40–60 min of hypoxia. These data are difficult to interpret because blockade of hypoxia-induced effects and MDL7222 effects are expected to produce similar results with a sustained increase in burst frequency and bursts/respiratory event. Thus, at best, one can only state that the hypoxia-induced decreases in burst frequency and bursts/respiratory event were not able to “break through” 5-HT₃ receptor blockade. MDL7222 could have blocked hypoxia-induced changes or alternatively counteracted hypoxia-induced effects by mechanisms independent of mechanisms underlying hypoxia-induced changes. Since a detailed understanding of the turtle respiratory rhythm generator is lacking along with an understanding as to how 5-HT₃ receptors alter respiratory neuron properties, it’s even possible that MDL7222 both blocked hypoxia-induced effects and altered other mechanisms involved in regulating the respiratory motor pattern.

4.5 Central non-respiratory adaptations to hypoxia in ectothermic vertebrates

To survive in a hypoxic environment, hypoxia-tolerant ectothermic vertebrates must reduce their metabolic rate and protect their brain from hypoxic excitotoxicity. Accordingly, many hypoxia-tolerant species have central non-respiratory adaptations that allow them to survive during hypoxia. Pre-metamorphic tadpole brainstems rely on anaerobic respiration during prolonged hypoxia because application of a glycolysis inhibitor silences respiratory rhythm following 40 min of hypoxia (Winmill et al., 2005). Central adaptive neuronal mechanisms may contribute to respiratory changes in isolated turtle brainstems during prolonged hypoxia. Turtles increase anaerobic metabolism during prolonged hypoxia (Bickler and Buck, 2007) and reduce metabolic demand in the brain by down-regulating excitatory ion channel conductances (Buck and Hochachka, 1993; Bickler et al., 2002). Also, hypoxic turtles augment inhibitory GABAergic synaptic transmission (Buck et al., 2012) to hyperpolarize neurons and prevent hypoxic excitotoxicity (reviewed in Jonz et al., 2016). Brain electrical activity in turtles is reduced by about tenfold after a 60 min exposure to anoxia (Fernandes et al., 1997). Thus, it is possible that some of the hypoxia-induced changes in respiratory motor output recorded from isolated turtle brainstems involved these non-respiratory adaptive neuronal mechanisms.

A legitimate question to address is whether the hypoxia-induced changes in respiratory motor output in isolated turtle brainstems was due to hypoxia or to potential acidosis due to increased glycolytic activity during hypoxia. A pH decrease with hypoxia could activate central pH chemoreceptors and thereby alter respiratory motor output. When cerebrocortical brain slices from the same turtle species are subjected to anoxic conditions (in air-tight sealed chambers), the pH decreases by -0.11 pH units from a baseline of ~7.0 pH units with a 25-min anoxic exposure (Bickler, 1992) or by -0.25 to -0.30 pH units from a baseline of

~7.25 pH units with a 200-min anoxic exposure (Buck et al., 1998). In isolated turtle brainstems, decreasing the pH by ~0.4 pH units from 7.60 to 7.22 pH units had no effect on burst frequency or bursts/respiratory event (Johnson et al., 1998). Since the bath conditions in this study were hypoxic (and not anoxic), the potential change in brainstem tissue pH was probably not as severe as with the turtle slices exposed to anoxia. In addition, the pH decrease within this range from 7.60 to 7.20 does not produce similar effects on burst frequency or bursts/respiratory event that hypoxia caused in this study. Thus, it is unlikely that hypoxia-induced decrease in pH contributed significantly to the hypoxia-induced changes in respiratory motor output.

4.6 Conclusions

This study quantified the response of isolated adult turtle brainstems to sustained bath hypoxia. In contrast to a previous study suggesting that the central respiratory network of turtles was unresponsive to hypoxia, this study suggests that respiratory burst frequency, amplitude, and episodicity are all impacted by hypoxia. Measurements of bath PO₂ provide a possible explanation for the discrepancy in results. Our bath PO₂ reached minimum values of 32.6 ± 1.2 mm Hg while bath PO₂ was not quantified in our previous study. Further, our results suggest α_1 -adrenergic receptor activation is required for both the initial hypoxia-induced increase in burst frequency as well as the prolonged decrease in burst frequency. The role of serotonin 5-HT₃ receptor activation is not clear, but hypoxia was unable to decrease burst frequency and bursts/respiratory event during 5-HT₃ receptor blockade.

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Fig. 1.

Respiratory motor output produced by isolated turtle brainstems and quantification of PO₂ during hypoxic solution application. (A) Drawing of turtle brainstem with labeled cranial nerves. A suction electrode attached to the hypoglossal (XII) nerve roots records spontaneous respiratory motor output. (B) Integrated and rectified traces of hypoglossal motor output show respiratory events. Doublets = two bursts/respiratory event; singlet = one burst/respiratory event. (C) Samples taken from solution aerated with 95% O₂-5% CO₂ resulted in an upper limit of PO₂ = 569 ± 4 mm Hg (small dashed line). Solution aerated with 95% N₂-5% CO₂ resulted in a lower limit of PO₂ = 27 ± 0.4 mm Hg (large dashed line). Values for bath PO₂ are shown for solution samples drawn from the bath over a 4-h period.

Fig. 2.

Hypoxia-induced changes in respiratory motor output pattern. Respiratory bursts from one isolated turtle brainstem are shown during baseline conditions (control solution) (A) and following 1-h (B) and 2-h (C) exposures to hypoxic solution. There is a decrease in burst frequency and episodicity with reduced number of bursts/respiratory event.

Fig. 3.

Hypoxia-induced changes in respiratory motor output. Data are shown for control brainstems exposed to standard solution only (circles), and brainstems exposed to hypoxic solution (squares). There were no time-dependent changes in respiratory burst variables for the control brainstems for up to 240 min. (A) During hypoxia, burst frequency increased within the first 15 min, and then decreased. (B) When plotted as the change in burst frequency, the increase during the first 15 min due to hypoxia was significant with respect to drug effect compared to control solution (see inset). Hypoxia decreased burst frequency relative to baseline from 35–240 min. (C) Burst amplitude was relatively unchanged in both solutions for the first 40 min, but then hypoxic solution decreased burst amplitude at 60, 180, and 240 min. (D) Bursts/respiratory event decreased after 30 min of hypoxic solution and remained decreased up to the 240 min time point. BL = baseline conditions (30 min prior to solution change). Filled symbols represent $P < 0.05$ compared to baseline for that data set. Asterisks indicate $P < 0.05$ for hypoxic data vs. control data at that time point. Daggers indicate $P < 0.05$ for a condition effect.

Fig. 4.

Reoxygenation partially reverses hypoxia-induced changes in respiratory motor output. (A) Burst frequency was reduced and partially reversed after 2 h of reoxygenation. (B) Burst amplitude and (C) bursts/respiratory event were reduced by hypoxia, but only partially recovered with reoxygenation. Asterisks indicate $P < 0.05$ compared to baseline values.

Fig. 5.

Role of α_1 -adrenergic receptor activation during hypoxic response. Data are shown for hypoxia (squares), and prazosin (0.5 μ M) bath-applied prior to and during hypoxia (triangles). (A,B) Prazosin pretreatment prevented the hypoxia-induced initial increase in burst frequency at 15-min as well as the sustained frequency decrease from 35–60 min. The inset in panel (B) shows the change in burst frequency during the first 15-min with control data added (circles). The 15-min hypoxia data point was greater than baseline with a significant condition effect. Prazosin did not significantly alter the hypoxia-induced decrease in burst amplitude (C) or bursts/respiratory event (D). Filled symbols represent $P < 0.05$ compared to baseline (0-min time point) for that data set. Asterisks indicate $P < 0.05$ for prazosin data vs. hypoxia data at that time point. Daggers indicate $P < 0.05$ for a condition effect.

Fig. 6.

Role of 5-HT₃ receptor activation during hypoxic response. Data are shown for hypoxia (squares), and MDL7222 (50 μ M) bath-applied prior to and during hypoxia (triangles). (A, B) MDL7222 pretreatment abolished the initial hypoxia-induced burst frequency increase and prevented the subsequent hypoxia-induced decrease from 30–60 min of the hypoxic exposure. The inset in panel (B) shows the change in burst frequency during the first 15-min with control data added (circles). The 15-min hypoxia data point was greater than baseline with a significant condition effect. (C) MDL7222 pretreatment and hypoxia decreased burst amplitude after 40–60 min of hypoxia. (D) The hypoxia-induced decrease in respiratory events at 30–60 min was blocked by MDL7222, but see Discussion for interpretation. Filled symbols represent $P < 0.05$ compared to baseline (0-min time point) for that data set. Asterisks indicate $P < 0.05$ for MDL7222/hypoxia data compared to hypoxia data. Dagger indicates $P < 0.05$ for a condition effect.

Table 1

Hypoxia did not alter burst duration and percent time-to-peak.

	Standard solution	Hypoxic solution
Burst Duration (s)		
Baseline	14.6 ± 1.0	14.7 ± 1.1
30–60 min	14.7 ± 1.0	16.1 ± 1.0
150–180 min	14.5 ± 0.9	16.0 ± 1.2
210–240 min	14.7 ± 1.0	13.1 ± 1.0
Percent Time-to-Peak		
Baseline	40.3 ± 2.2	44.0 ± 2.1
30–60 min	40.2 ± 2.5	43.5 ± 1.8
150–180 min	38.8 ± 2.7	43.4 ± 1.4
210–240 min	38.9 ± 2.7	44.6 ± 1.7