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Regulation of respiratory-related hypoglossal motor output by α_1 adrenergic and serotonin 5-HT_3 receptor activation in isolated adult turtle brainstems

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ABSTRACT

The effects of brainstem α_1 adrenergic receptor activation on respiratory control in reptiles are poorly understood. Isolated adult turtle brainstems were exposed to phenylephrine (α_1 adrenergic agonist) and respiratory motor bursts were recorded on hypoglossal nerves. Phenylephrine acutely increased burst frequency, amplitude (low concentrations only), and regularity of the time interval between the start of respiratory events (single or clustered bursts), and decreased bursts/respiratory event. Burst frequency and timing changes persisted during a 2.0 h washout. Acute increases in burst frequency and amplitude were blocked by prazosin (α_1 adrenergic antagonist). Pretreatment with prazosin and tropisetron (5-HT_3 antagonist) blocked the increase in respiratory event regularity, but did not alter the decrease in bursts/respiratory event. Intermittent phenylephrine application (4×5.0 min separated by 20 min) did not produce long-lasting changes in burst frequency and amplitude, bursts/respiratory event, or respiratory event regularity. Thus, sustained α_1 adrenergic receptor activation in turtle brainstems produces acute and long-lasting changes in respiratory burst frequency and pattern.

1. Introduction

Brainstem adrenergic receptors modulate respiratory frequency, amplitude and timing in mammals and amphibians, but the manner by which adrenergic receptor activation alters respiratory motor output in reptiles is poorly understood. In mammalian *in vitro* preparations, respiratory burst frequency is either increased or decreased depending on the type and location of the activated adrenergic receptor, the age of the preparation, and pontine inputs (Corcoran and Milsom, 2009). In general, sustained α_1 adrenergic receptor activation increases respiratory burst frequency in isolated neonatal rodent brainstem-spinal cord and medullary slice preparations (Hakuno et al., 2004; Viemari et al., 2004; Jodkowski et al., 1997). Likewise, bath application of an α_1 adrenergic receptor agonist (phenylephrine) to isolated adult turtle brainstems increases respiratory burst frequency (Johnson et al., 1998a). The effects of α_1 receptor activation on respiratory motor output in isolated in amphibians are different. The noradrenaline-dependent decrease in respiratory motor burst frequency requires α_1 receptor activation, but phenylephrine application does not change lung burst frequency (Fournier and Kinkead, 2006).

With respect to respiratory burst amplitude in neonatal rat medullary slices, sustained α_1 adrenergic receptor activation acutely (during drug application) increases hypoglossal amplitude (Selvaratnam et al., 1998; Viemari and Ramirez, 2006), whereas intermittent activation of α_1 adrenergic receptors elicits both acute and long-lasting (60 min post drug application) increases in hypoglossal amplitude (Neverova et al., 2007; Saywell et al., 2010). Further, the long-lasting amplitude increase induced by intermittent α_1 adrenergic receptor activation is blunted by blockade of serotonin 5-HT_2 receptors, suggesting an interaction between adrenergic and serotonergic pathways (Neverova et al., 2007). In isolated adult turtle brainstems, phenylephrine application decreases hypoglossal burst amplitude in a cumulative dose response (Johnson et al., 1998a), but the acute and long-lasting effects of sustained and intermittent central α_1 adrenergic receptor activation are not known. Furthermore, potential interactions between adrenergic and serotonergic systems on respiratory output in ectotherms have not been investigated, despite recent findings that serotonin 5-HT_3 receptors regulate burst regularity and episodicity in isolated adult turtle brainstems (Bartman et al., 2010).

While the primary focus of most adrenergic studies is acute changes in respiratory burst frequency and amplitude following sustained drug application, few studies investigate α_1 adrenergic modulation of respiratory motor burst timing, including regularity and episodicity (bursts/respiratory event). In Mecp2 mutant mice (Rett Syndrome animal model), reduced norepinephrine levels and tyrosine hydroxylase positive neurons in the medulla are

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correlated with high respiratory cycle variation (Viemari et al., 2005). Bath application of norepinephrine to medullary slices from mutant Mecp2 mice increases the regularity of hypoglossal motor bursts (Viemari et al., 2005). Administration of norepinephrine reuptake inhibitors to intact mutant Mecp2 mice reduces apnea frequency and prolongs their life span (Roux et al., 2007). These data suggest that in mammals, adrenergic receptor activation modulates respiratory regularity, but the adrenergic receptor subtypes involved are not known. Likewise, relatively little is known regarding adrenergic modulation of respiratory burst clustering in vertebrates. In intact turtles, baseline respiratory events typically contain 4–6 breaths (Johnson et al., 2008). In contrast, in isolated turtle brainstems, respiratory bursts occur in clusters of 1–7 bursts/respiratory event, with an average of 1.4 ± 0.4 bursts/respiratory event (Johnson and Creighton, 2005). In a previous study where phenylephrine was applied in sequentially increasing concentrations to turtle brainstems (Johnson et al., 1998a), bursts/respiratory event decreased from 1.4 to 1.0 bursts/respiratory event, but the data were not shown, nor were statistical tests applied. Acute and long-lasting phenylephrine-dependent changes in respiratory burst episodicity in turtle brainstems were not carefully examined.

This study investigates acute (during drug application) and long-lasting (>90 min post-drug application) changes in respiratory motor output due to sustained and intermittent α_1 adrenergic receptor activation in isolated adult turtle brainstems. Our experiments were designed to (1) determine the acute and long-lasting effects of α_1 adrenergic receptor activation on frequency, amplitude and timing of hypoglossal respiratory bursts, (2) investigate interactions between α_1 adrenergic and 5-HT_3 receptor activation and (3) determine if the effects of α_1 adrenergic receptor activation are pattern-sensitive. Integrated respiratory motor output on hypoglossal nerve rootlets was recorded from isolated adult turtle brainstems prior to, during, and after drug exposure. Adult turtle brainstems are a unique model system for respiratory studies because the isolated brainstems from fully mature adult animals are able to produce respiratory-related motor output for more than 40 h (Wilkerson et al., 2003), despite PO_2 and pH gradients within the tissue (*e.g.*, see Torgerson et al., 1997). In addition, *in vitro* turtle brainstems are studied at physiologically relevant temperature, pH and K^+ levels in the bath (Johnson et al., 1998b).

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 red-eared slider turtles (*Trachemys scripta*, $n = 151$, weight = 693 ± 11 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/day. Turtles were fed ReptoMin® floating food sticks (Tetra, Blacksburg, VA) 3–4 times/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., 1998b). 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 recording chamber (13 ml volume) with the ventral surface facing upwards (Fig. 1A). Brainstems were superfused (4–6 ml min^{-1}) with standard solution containing HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethane-sulfonic 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 bubbled with 5% CO_2–95% O_2; pH ~ 7.35. To record respiratory motor 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 3–6 h, baseline data were obtained by recording 30 min of spontaneous respiratory motor activity before adding drugs to the reservoir. All drugs used in this study were obtained from Sigma/RBI Aldrich (St. Louis, MO) and include:

(R)-(−)-Phenylephrine hydrochloride (phenylephrine, α_1 adrenergic agonist), prazosin hydrochloride (prazosin, α_1 adrenergic antagonist), 3-tropanyl-indole-3-carboxylate hydrochloride (tropisetron, 5-HT_3 antagonist), and 1-(3-Chloro-phenyl)biguanide hydrochloride (mCPBG, 5-HT_3 agonist).

2.2. Data analysis

Respiratory burst variables were measured as previously described (Johnson et al., 1998b; Bartman et al., 2010), and reported as baseline values (before drug application; 30-min time point), acute (during 1.0 h drug application; *i.e.*, 60- and 90-min time points) or long-lasting effects (minimum of 90 min following end of drug application; *i.e.*, 180–210-min time points). Burst frequency was calculated as the total number of 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 of clustered motor bursts (*i.e.*, doublets, triplets, *etc.*) (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. To quantify the degree of respiratory event regularity, the time interval between the start of respiratory events in seconds was measured and the standard deviation calculated within a 30-min bin. Respiratory event interval coefficient of variation was calculated by dividing the mean interval by the standard deviation within the bins. With respect to "episodicity" of respiratory events, a motor burst pattern was considered 'episodic' if the average baseline bursts/respiratory event was >1.75, whereas 'singlet' burst patterns had a baseline average of <1.25 bursts/respiratory event. 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. All measurements were averaged into 20- or 30-min bins and reported as the mean ± S.E.M. Two-way ANOVA with repeated measures design were calculated (Sigma Stat, Jandel Scientific Software, San Rafael, CA). In many cases, normality and equal variance assumptions were not satisfied. Several data transformations were attempted but none were successful in completely eliminating normality or equal variance assumption violations. For percent time-to-peak, data were transformed with arcsine square root to pass normality test before a one-way ANOVA with repeated measures design was calculated.