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Regulation of respiratory-related hypoglossal motor output by α_1 adrenergic and serotonin 5-HT₃ 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-HT₃ 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.

Keywords

reptile; chelonian; turtle; episodic breathing; respiratory regularity; α_1 adrenergic; 5-HT₃

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

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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 amphibians is 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-HT₂ 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-HT₃ 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 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-HT₃ 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 PO₂ 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 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.*, 1998b). 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 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 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 bubbled with 5% CO₂-95% O₂; 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, α₁ adrenergic agonist), prazosin hydrochloride (prazosin, α₁ adrenergic antagonist), 3-Tropanyl-indole-3-carboxylate hydrochloride (tropisetron, 5-HT₃ antagonist), and 1-(3-Chloro-phenyl)biguanide hydrochloride (mCPBG, 5-HT₃ 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- to 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 $\pm$ 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.

3. Results

3.1 Phenylephrine increased burst frequency and altered burst pattern

To test for time-dependent changes in respiratory motor output, spontaneous respiratory-related hypoglossal bursts were recorded for 3.5 h in control solution ($n=29$), following a 3–6 h equilibration time period. No changes were observed in burst frequency (Figs. 2A, 2B). Burst amplitude decreased from 1.0 to 0.91 ± 0.02 by 90 min ($P<0.001$) and to 0.84 ± 0.03 at 210 min (Fig. 2C, $P<0.001$). Bursts/respiratory event did not change for 150 min, but decreased by 0.21 ± 0.07 bursts/respiratory event at 180 min (Fig. 2D, $P=0.001$). Respiratory event interval standard deviation and coefficient of variation were unchanged over 3.5 h (Fig. 3). Thus, the frequency and regularity of respiratory motor output were stable for 3.5 h, while the number of bursts/respiratory event was stable for 2.5 h.

To test for α_1 -adrenergic-dependent changes in respiratory motor output, phenylephrine was applied at 20 μM ($n=11$), 50 μM ($n=21$), 100 μM ($n=9$) or 200 μM ($n=9$) for 60 min, followed by a 120-min washout (Fig. 1C). Phenylephrine application resulted in acute (during drug application) and long-lasting (>90 min post-drug application) increases in burst frequency (Fig. 2A). Since there were no significant differences between baseline burst frequency values ($P>0.125$ for all comparisons), frequency data were graphed as delta burst frequency to improve clarity. With respect to the acute increase in burst frequency during drug application, 50 μM phenylephrine produced the largest increase of 0.74 ± 0.12 bursts/min ($P<0.001$), while 100 μM produced the smallest increase of 0.20 ± 0.04 bursts/min ($P=0.02$, Fig. 2B). The acute frequency response to phenylephrine was not dose-dependent, as 20 and 200 μM phenylephrine produced intermediate frequency increases (Fig. 2B). During the washout period, phenylephrine (20 μM , 50 μM and 200 μM) application resulted in long-lasting burst frequency increases of 0.18 ± 0.04 , 0.40 ± 0.08 , and 0.17 ± 0.06 bursts/min at the 210-min time point ($P=0.032$, $P<0.001$, $P<0.001$ for drug effects, respectively; Fig. 2B). Burst frequency returned to baseline values following the 100 μM application (Fig 2B).

Burst amplitude increased to 1.04 ± 0.05 from baseline with 20 μM phenylephrine at 90 min ($P=0.017$ compared to controls), and remained elevated through the 120–180 min time points ($P=0.023$ compared to controls, Fig. 2C). Burst amplitude during the 50 μM and 100

μM phenylephrine applications roughly matched the gradual decrease in amplitude observed in control brainstems. Phenylephrine at $200 \mu\text{M}$ decreased burst amplitude from 1.0 to 0.75 ± 0.07 at 90 min ($P=0.031$ compared to control). No changes in burst shape were observed with phenylephrine application except for $50 \mu\text{M}$ phenylephrine, which elicited acute and long-lasting decreases in burst duration ($P<0.05$, Table 1).

Phenylephrine at all four concentrations converted episodic discharge to mostly singlet bursts (Fig. 1C). Phenylephrine at concentrations of $20 \mu\text{M}$ and $50 \mu\text{M}$ acutely decreased bursts/respiratory event from 1.59 ± 0.19 to 1.23 ± 0.11 and from 1.42 ± 0.09 to 1.03 ± 0.01 , respectively, ($P<0.001$, Fig. 2D), but both concentrations approached control values during washout at the 210-min time point. In contrast, phenylephrine at $100 \mu\text{M}$ and $200 \mu\text{M}$ application acutely decreased bursts/respiratory event to 1.01 ± 0.01 at 90 min for both concentrations, and remained in a singlet burst pattern during washout, with values of 1.06 ± 0.06 and 1.09 ± 0.09 bursts/respiratory event at 210 min, respectively ($P<0.001$; Fig. 2D).

With respect to respiratory event regularity, 20 , 50 and $100 \mu\text{M}$ phenylephrine decreased respiratory event interval standard deviation and coefficient of variation. Phenylephrine ($100 \mu\text{M}$) produced the largest decrease in respiratory event interval standard deviation of 43.3 ± 19.0 s ($P<0.001$) at 90 min, while $200 \mu\text{M}$ produced the smallest decrease of 25.6 ± 6.7 s ($P=0.08$, Fig. 3B). During the washout period, $20 \mu\text{M}$ and $50 \mu\text{M}$ phenylephrine application resulted in long-lasting respiratory event interval standard deviation decreases of 30.9 ± 8.4 s and 27.1 ± 8.2 s at the 210-min time point ($P<0.001$ compared to baseline, $P=0.026$, $P=0.005$ for drug effects, respectively; Fig. 3B). Respiratory event interval standard deviation returned to baseline values following the $100 \mu\text{M}$ and $200 \mu\text{M}$ applications (Fig. 3B). Phenylephrine rapidly increased burst frequency and decreased bursts/respiratory event during drug application, resulting in an initial increase in respiratory event interval coefficient of variation at the 60-min time point for 50 – $200 \mu\text{M}$ phenylephrine, followed by a decrease in respiratory event interval coefficient of variation at the 90-min time point (Figs. 3C, 3D). For example, at the 90-min time point, phenylephrine at $20 \mu\text{M}$ and $50 \mu\text{M}$ decreased respiratory event interval coefficient of variation from 0.31 ± 0.04 to 0.15 ± 0.03 and 0.23 ± 0.02 to 0.14 ± 0.01 , respectively ($P=0.003$, $P=0.015$ compared to baseline; Fig. 3C). Based on this study and previous work (Bartman *et al.*, 2010), a highly regular respiratory event burst pattern has a respiratory event interval coefficient of variation of <0.2 . Since there was some variability in baseline respiratory event interval coefficient of variation values ($P>0.177$), data were graphed as delta respiratory event interval coefficient of variation to improve clarity (Fig. 3D). Similar to the data in Fig. 3C, phenylephrine at $20 \mu\text{M}$ and $50 \mu\text{M}$ decreased respiratory event interval coefficient of variation at the 90-min time point ($P<0.004$ and $P=0.01$ compared to controls) with one other significantly lower value at the 150-min time point for $20 \mu\text{M}$ (Fig. 3D). There was a strong non-significant trend for a drug effect ($P=0.05$). The $50 \mu\text{M}$ phenylephrine concentration was chosen for subsequent experiments because it produced the maximum increase in frequency and decrease in burst timing variables (respiratory event interval standard deviation and interval coefficient of variation, bursts/respiratory event) with minimal depression of burst amplitude.

3.2 Endogenous α_1 -adrenergic receptor activation regulated burst amplitude

To test for endogenous α_1 -adrenergic receptor activation, prazosin was bath-applied for 2 h at $0.25 \mu\text{M}$ ($n=13$), $0.5 \mu\text{M}$ ($n=15$) and $1.0 \mu\text{M}$ ($n=8$). Prazosin did not change burst frequency or bursts/respiratory event (Figs. 4A, 4C). Prazosin application decreased burst amplitude in a dose-dependent manner, with the largest decrease from 1.0 to 0.62 ± 0.05 of baseline with $1.0 \mu\text{M}$ prazosin at the 150-min time point ($P=0.018$ for drug effect, Fig. 4B). Prazosin ($1.0 \mu\text{M}$) increased respiratory event interval standard deviation by 22.9 ± 10.7 s ($P=0.025$, Fig. 4D) at the 60-min time point, while $0.5 \mu\text{M}$ prazosin increased respiratory

event interval coefficient of variation by 0.12 ± 0.05 ($P=0.008$, Fig. 4E) at the 60-min time point; however, both values returned to baseline levels by the 90-min time point.

3.3 Phenylephrine-induced increase in burst frequency and decrease in burst amplitude blocked by prazosin pre-treatment

To determine which phenylephrine-induced changes may be due to α_1 adrenergic receptor activation, brainstems ($n=12$) were pretreated with prazosin ($0.5 \mu\text{M}$) for 30 min, followed by 1.0 h co-application of phenylephrine ($50 \mu\text{M}$) and prazosin ($0.5 \mu\text{M}$). Prazosin at $0.5 \mu\text{M}$ was chosen to avoid a large decrease in burst amplitude. The phenylephrine-induced increase in burst frequency was blocked by prazosin application ($P<0.001$ for drug effect, Fig. 5A), and prazosin reversed the phenylephrine-dependent decrease in burst amplitude ($P=0.032$ for drug effect, Fig. 5B). However, phenylephrine still decreased bursts/respiratory event by 0.35 ± 0.11 at the 90-min time points ($P<0.001$, Fig. 5C). In addition, phenylephrine application reduced respiratory event interval standard deviation from $73.6 \pm 14.5 \text{ s}$ to $23.3 \pm 4.5 \text{ s}$ ($P<0.001$, Fig. 5D), and reduced the respiratory event interval coefficient of variation by 0.18 ± 0.06 ($P=0.004$, Fig. 5E). The acute increase in respiratory event interval coefficient of variation at the 30-min time point was abolished because there was no phenylephrine-induced increase in burst frequency (Fig. 5A). Thus, only the phenylephrine-induced increase in burst frequency and decrease in amplitude appeared to require α_1 adrenergic receptor activation.

3.4 Phenylephrine-induced changes in respiratory event timing are not due to 5-HT₃ receptor activation alone

Since the changes in respiratory event timing elicited by phenylephrine were not due to α_1 -adrenergic receptor activation, it is possible that phenylephrine crossed over to activate serotonin 5-HT₃ receptors, which decreases respiratory event interval coefficient of variation and bursts/respiratory event similar to phenylephrine (Bartman *et al.*, 2010). To test this hypothesis, brainstems ($n=14$) were pretreated with $50 \mu\text{M}$ tropisetron (5-HT₃ antagonist) for 30 min, followed by a 1.0 h co-application of phenylephrine ($50 \mu\text{M}$) and tropisetron ($50 \mu\text{M}$). Tropisetron pre-treatment did not block the phenylephrine-induced 0.41 ± 0.10 decrease in bursts/respiratory event ($P<0.001$, Fig. 6A) or the phenylephrine-induced decrease in respiratory event interval standard deviation, which decreased from $70.9 \pm 11.3 \text{ s}$ to $28.1 \pm 11.9 \text{ s}$ ($P=0.010$ compared to baseline, Fig. 6B). Tropisetron also did not appear to alter the effects of phenylephrine on respiratory event interval coefficient of variation. There was a significant drug effect ($P=0.009$) and difference at the 90-min time point ($P=0.028$; Fig. 6C), but the data were nearly identical when graphed as delta respiratory event interval coefficient of variation ($P=0.263$ at 90-min time point; Fig. 6D). Taken together, these data suggest that tropisetron was unable to block the phenylephrine-induced changes in respiratory burst timing.

3.5 Phenylephrine-induced decrease in bursts/respiratory event did not require α_1 adrenergic or 5-HT₃ receptor activation

Since independent α_1 adrenergic receptor or 5-HT₃ receptor activation may be sufficient to change burst timing, both prazosin ($0.5 \mu\text{M}$) and tropisetron ($50 \mu\text{M}$) were co-applied to brainstems ($n=13$) for 30 min with phenylephrine ($50 \mu\text{M}$) added for 1.0 h. The decrease in bursts/respiratory event was not blocked by pretreatment with both receptor antagonists, as bursts/respiratory event decreased from 1.41 ± 0.13 to 1.08 ± 0.05 after a 1.0 h phenylephrine application ($P<0.001$, Fig. 7A). However, phenylephrine-induced changes in respiratory event regularity were blocked by both receptor antagonists. In the presence of prazosin and tropisetron, phenylephrine was unable to significantly decrease respiratory event interval standard deviation or coefficient of variation (Figs. 7B, 7C). Thus, the phenylephrine-induced decrease in bursts/respiratory event did not appear to require α_1

adrenergic and 5-HT₃ receptor activation, whereas α_1 adrenergic and 5-HT₃ receptor blockade abolished the phenylephrine-induced increase in respiratory event regularity.

3.6 Convergence of α_1 adrenergic and 5-HT₃ signaling pathways regulating burst frequency

Independent activation of either α_1 adrenergic or 5-HT₃ receptors increases burst frequency (Fig. 2A; Bartman *et al.*, 2010). To determine if the effects of these receptors might converge on a common pathway, phenylephrine (50 μ M) and mCPBG (5-HT₃ agonist; 100 μ M) were applied sequentially to test if their effects were additive. These concentrations were chosen because they produced the maximum acute frequency increase (Fig. 2B for phenylephrine; data not shown for n=8 cumulative dose-response curves for mCPBG). Thus, phenylephrine was bath-applied for 30 min, followed by a 30-min co-application of phenylephrine and mCPBG (n=13). Phenylephrine application increased burst frequency by 0.33 ± 0.04 bursts/min at 30 min ($P=0.003$ compared to baseline), with no additional increase following co-application of mCPBG and phenylephrine (Fig. 8). Reversing the order of drug application was not possible because a 1.0 h application of 100 μ M mCPBG caused burst frequency to drift over time to higher values without establishing a steady-state (n=7, data not shown).

3.7 Burst regularity, episodicity, and amplitude were pattern-sensitive

To test for pattern sensitivity of phenylephrine application in our preparation, phenylephrine was applied for four, 5-min applications (20 min total drug exposure Fig. 9A, n=13), separated by 20-min washout periods. For comparison, phenylephrine was applied in a sustained manner for 20 min (Fig. 9B, n=13). Data from baseline, 20 min post-application ("Post Drug"), and the last 20 min of the 2.0 h washout period ("Washout") were compared. Burst frequency increased by 0.25 ± 0.08 and 0.23 ± 0.05 bursts/min post-application following intermittent and sustained phenylephrine application, respectively ($P<0.001$ compared to baseline for both patterns), but both returned to baseline during washout (Fig. 9C). Intermittent application of phenylephrine did not change burst amplitude, while sustained phenylephrine decreased amplitude to 0.90 ± 0.05 during the washout period ($P=0.02$, Fig. 9D). Similarly, intermittent phenylephrine did not decrease bursts/respiratory event, while sustained phenylephrine decreased bursts/respiratory event by 0.42 ± 0.19 post-application ($P=0.003$), an effect that was reversed during washout (Fig. 9E). Finally, intermittent phenylephrine did not decrease respiratory event interval standard deviation while sustained phenylephrine decreased respiratory event interval standard deviation from 58.3 ± 11.3 s to 28.3 ± 4.5 s ($P=0.002$), reaching 27.2 ± 4.2 s at the end of the washout period ($P=0.004$; Fig. 9F).

4. Discussion

This is the first study to investigate phenylephrine-induced acute and long-lasting changes in respiratory motor output in isolated adult turtle brainstems. Phenylephrine application induced acute and long-lasting increases in hypoglossal burst frequency by a mechanism that may require α_1 adrenergic receptor activation. However, the changes in respiratory timing elicited by phenylephrine application did not require only α_1 adrenergic receptor activation. The phenylephrine-induced increase in regularity required either α_1 adrenergic or serotonin 5-HT₃ receptor activation, while the decrease in burst episodicity was independent of both receptor subtypes. Sequential activation of both α_1 adrenergic and 5-HT₃ receptors did not result in an additive burst frequency increase, suggesting the possibility of convergent neuronal pathways, or co-localization of receptors. There were no long-lasting changes in burst amplitude with intermittent phenylephrine application, suggesting that turtle brainstems do not express α_1 adrenergic-dependent hypoglossal long-term facilitation.

4.1 Adrenergic modulation of respiratory frequency

Adrenergic modulation of respiration is complex, and depends on age, species, method of drug delivery, and presence or absence of the pons (Corcoran and Milsom, 2009). In mammals, central adrenergic receptor activation can increase or decrease respiratory frequency depending on receptor subtype. In rodents, activation of the A5 region located in the caudal ventrolateral pons (Errchidi *et al.*, 1991; Dawid-Milner *et al.*, 2001) or A1/C1 region located in the ventral medulla decreases burst frequency by an α_2 adrenergic-dependent mechanism (Zanella *et al.*, 2006). On the other hand, activation of the A6 region (locus coeruleus) increases respiratory burst frequency by augmenting inspiratory activity in the pre-Bötzinger Complex by an α_1 adrenergic-dependent mechanism (Hakuno *et al.*, 2004; Viemari *et al.*, 2004). Finally, bath-applied phenylephrine increases respiratory-related spinal burst frequency in isolated neonatal rat brainstem-spinal cord preparations, an effect that is blocked by prazosin pretreatment (Makino *et al.*, 2006). Thus, α_1 adrenergic receptor activation in mammalian brainstems results in an acute increase in respiratory frequency.

In ectothermic vertebrates, tyrosine hydroxylase-immunoreactive regions corresponding to A5 and A6 are located in reptiles (López *et al.*, 2008; Smeets and Reiner, 1994) and amphibians (Marín *et al.*, 1996; González *et al.*, 1994). While these anatomical regions appear similar to those found in mammals, the projections of these regions and type of adrenergic receptors activated by these regions are unknown. Furthermore, the role of brainstem α_1 adrenergic receptors in regulating respiratory burst frequency is complex. While bath-applied phenylephrine increases respiratory burst frequency in adult turtle brainstems (Johnson *et al.*, 1998a; Fig. 2 in this paper), phenylephrine had no effect on burst frequency in adult bullfrog brainstems even though the norepinephrine-induced frequency decrease was blocked by an α_1 adrenergic receptor antagonist (Fournier and Kinkead, 2006). Thus, there are potentially significant differences in adrenergic control of breathing in different classes of vertebrates.

In the previous study on adult turtle brainstems (Johnson *et al.*, 1998a), cumulative dose response curves were performed for phenylephrine. Cumulative dose-response curves, however, are vulnerable to drug tachyphylaxis because of the prolonged drug exposures. For example, this study showed that 1.0 h of 20 μ M phenylephrine application increased respiratory burst frequency by an α_1 adrenergic-dependent mechanism, whereas in cumulative dose-response experiments, no significant frequency increase was observed until 200 μ M phenylephrine was applied (Johnson *et al.*, 1998a). Further, individual dose-response experiments in this study showed that burst frequency changes were not dose-dependent, as 100 μ M phenylephrine resulted in the smallest increase in burst frequency while 50 μ M produced the largest increase. The complex relationship between frequency response and phenylephrine concentration is not easily explained. It's possible that phenylephrine activated several different pathways that converge onto the respiratory control system in the brainstem with the net result being an increase in burst frequency, but with large variability in the magnitude of the response. Therefore, as in mammals, α_1 adrenergic receptor activation in turtles increases respiratory burst frequency, but the site of action of these receptors (pons vs. medulla) is still unknown.

4.2 Adrenergic modulation of respiratory burst amplitude

Sustained phenylephrine application in neonatal rodent rhythmically active medullary slices results in a reversible dose-dependent increase in the amplitude of hypoglossal motor output (Selvaratnam *et al.*, 1998; Neverova *et al.*, 2007). In contrast, intermittent local application of phenylephrine to hypoglossal motoneurons in similar preparations causes a long-lasting (>60 min) increase in hypoglossal motor output amplitude (Neverova *et al.*, 2007). The pattern sensitivity of the adrenergic-dependent, long-lasting increase in hypoglossal

motoneuron excitability is similar to hypoxia-induced long-term facilitation, which is induced by intermittent rather than sustained stimuli (Mitchell *et al.*, 2001).

In this study, sustained bath application of phenylephrine to isolated turtle brainstems increased burst amplitude at 20 μM , but decreased burst amplitude when phenylephrine was $>50 \mu\text{M}$. It is not clear why the larger phenylephrine concentrations decreased hypoglossal burst amplitude. Changing phenylephrine application pattern from sustained to intermittent did not result in long-lasting increases in hypoglossal burst amplitude as it does in rat preparations (Neverova *et al.*, 2007), nor was intermittent phenylephrine application sufficient to elicit long-term changes in respiratory event interval standard deviation, coefficient of variation or bursts/respiratory event (unlike sustained phenylephrine application in turtles). However, it is possible that the 5-min application was not sufficient to activate all possible α_1 -adrenergic receptors located in the center of the brainstem that regulate respiratory burst variables. Thus, isolated turtle brainstems do not exhibit pattern sensitivity with respect to α_1 adrenergic-dependent hypoglossal long-term facilitation. One possible explanation may be that our turtle experiments were performed in isolated brainstems that contained the entire pons and medulla. Intermittent phenylephrine application may have activated or inhibited competing signaling pathways whose simultaneous activation prevented or masked long-term facilitation in hypoglossal burst amplitude.

4.3 Adrenergic modulation of respiratory burst timing

In mammals, breathing irregularity is observed in preparations with decreased adrenergic function in the brainstem. Mutant *Mecp2* mice have decreased number of tyrosine hydroxylase-positive neurons in A1/C1 and A2/C2 regions in the medulla and have decreased medullary norepinephrine levels (Viemari *et al.*, 2005). These anatomical defects are correlated with a highly variable respiratory cycle period and frequent apneas in mutant *Mecp2* mice (Viemari *et al.*, 2005). Similarly, *Phox2a* expression in mice is required for the proper development of the A6 region (Morin *et al.*, 1997) and *Phox2a* mutant fetuses have increased cycle variability compared to control mice (Viemari *et al.*, 2004). Thus, the link between loss of adrenergic modulation and irregular breathing is well established in mammals.

Two respiratory motor burst timing characteristics were quantified in this study – respiratory event regularity (respiratory event interval standard deviation and coefficient of variation) and episodicity (bursts/respiratory event), which were not characterized in an earlier study (Johnson *et al.*, 1998a). This study found that respiratory event regularity and episodicity can be uncoupled from one another because drugs can alter one variable, but not the other (see below).

Episodic breathing is found in all classes of vertebrates depending on developmental stage or level of respiratory drive (Fong *et al.*, 2009); however, the mechanisms that control episodic breathing in mammals are not well-understood. Neurotransmitters that increase respiratory drive, such as catecholamines, can transform singlet breathing into episodic breathing and then to continuous breathing at high respiratory drive (Fong *et al.*, 2009). In this study, phenylephrine application increased respiratory burst frequency and event regularity, and decreased bursts/respiratory event in turtle brainstems to the extent that the motor output approached continuous breathing *in vivo* (Fig. 1C, middle panels). Thus, our data are consistent with the hypothesis that increased respiratory drive converts episodic breathing to continuous breathing. In isolated turtle brainstems, phenylephrine and 5-HT₃ receptor agonist drugs decrease bursts/respiratory events (Bartman *et al.*, 2010), while nociceptin/orphanin receptor activation and 5-HT₃ receptor blockade increase bursts/respiratory event (Bartman *et al.*, 2010; Johnson *et al.*, 2010). In isolated amphibian

brainstems, bursts/respiratory event are decreased by activation of NK1 (substance P), mu-opioid, and GABA_B receptors (Straus *et al.*, 2000; Davies *et al.*, 2009), and by blocking nitric oxide synthase (Harris *et al.*, 2002).

With respect to pharmacology, the phenylephrine-induced increase in respiratory event regularity was not by blocked by prazosin or tropisetron application alone, but was prevented by co-application of both antagonists. In contrast, the decrease in bursts/respiratory event was not prevented by blockade of α_1 adrenergic and 5-HT₃ receptors. Thus, as the increase in respiratory event regularity was blocked, but not the decrease in bursts/respiratory event, these two variables were “uncoupled” experimentally. The mechanisms underlying this uncoupling are not known and could be due to poorly understood actions of these drugs on turtle neurotransmitter receptors. Episodic breathing in vertebrates is proposed to be produced by several “elements” or neural networks within the brainstem that produce the burst pattern (single breath), respiratory rhythm (intraepisode oscillatory motor output), and breathing pattern (which turns the respiratory rhythm on or off) (Milsom *et al.*, 1999; Fong *et al.*, 2009). Our findings support this general model because these variables can be altered independently in isolated turtle brainstems by applying various drugs. Our data extend this model by suggesting that the network controlling breathing pattern or respiratory event production (subsequently referred to as the “respiratory event oscillator”) regulates respiratory event regularity in addition to respiratory event frequency. In isolated turtle brainstems, α_1 - and 5-HT₃-dependent mechanisms appear to regulate the respiratory event oscillator by altering frequency and regularity (Figs. 4–5; Bartman *et al.*, 2010). The respiratory rhythm within respiratory events in turtle brainstems appears to be regulated by a 5-HT₃-dependent mechanism because 5-HT₃ receptor activation and blockade changes bursts/respiratory event (Bartman *et al.*, 2010). The phenylephrine-induced decrease in bursts/respiratory event was not blocked by co-application of α_1 and 5-HT₃ receptor antagonist drugs (Fig. 6A), suggesting that phenylephrine acts by an unknown mechanism (see discussion above).

mCPBG application against a background phenylephrine application did not change burst frequency (Fig. 8), suggesting that α_1 adrenergic and 5-HT₃ receptor activation stimulates convergent neuronal pathways with respect to neurons regulating burst frequency. Alternatively, α_1 adrenergic and 5-HT₃ receptor may be co-localized on the same neurons. One potential site of convergence is intracellular Ca²⁺ levels. α_1 adrenergic receptors are coupled to the G_{q/11} proteins, which release Ca²⁺ from intracellular stores (Michelotti *et al.*, 2000) and 5-HT₃ receptors are permeable to Ca²⁺ (Barnes *et al.*, 2009). Thus, activation of either α_1 adrenergic or 5-HT₃ receptors could increase intracellular Ca²⁺ levels in a manner that then leads to changes in burst frequency.

4.4 Significance of adrenergic modulation of breathing in turtles

This study suggests that α_1 adrenergic modulation of respiratory motor burst frequency and respiratory event regularity in red-eared slider turtles is more similar to that observed in mammals rather than amphibians. α_1 adrenergic receptor activation increases respiratory burst frequency and respiratory event regularity in both mammals (Viemari *et al.*, 2005; Viemari, 2008) and turtles. In both mammals and red-eared slider turtles, catecholamines increase respiratory drive and change the respiratory motor output pattern to near continuous breathing (Fong *et al.*, 2009). A detailed analysis of α_1 adrenergic activation on lung burst regularity and episodicity in adult amphibians is needed to make a more complete comparison between vertebrates.

One novel finding in this study is the pharmacological ‘uncoupling’ of respiratory event regularity and episodicity. Phenylephrine application modulated respiratory event regularity by a mechanism that required α_1 adrenergic or 5-HT₃ activation, while the decrease in

bursts/respiratory event persisted during simultaneous α_1 adrenergic and 5-HT₃ receptor blockade. This showed that a decrease in respiratory event interval standard deviation and coefficient of variation does not necessarily go hand in hand with a decrease in bursts/respiratory event. This finding is consistent with the hypothesis that there are multiple synaptically-coupled rhythm generators in turtles that control when a respiratory event occurs, the frequency and number of breaths during an respiratory event, and breathing frequency during continuous breathing. Developing pharmacological methods for uncoupling these rhythm generators, similar to the quantal slowing observed in mammals (Mellen *et al.*, 2003), will be valuable tools for testing this hypothesis.

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Highlights

$\alpha 1$ adrenergic receptor activation altered respiratory pattern in turtle brainstems.

The increase in burst frequency and amplitude required $\alpha 1$ adrenergic receptors.

The increase in burst regularity required $\alpha 1$ adrenergic and 5-HT₃ receptors.

The decrease in bursts/episode persisted during $\alpha 1$ adrenergic and 5-HT₃ blockade.

Intermittent $\alpha 1$ adrenergic receptor activation did not induce neuroplasticity.

Fig. 1. Phenylephrine application alters respiratory motor output. (A) Drawing of turtle brainstem with cranial nerves labeled. A suction electrode attached to the hypoglossal nerve roots records spontaneous respiratory-related motor output. (B) Integrated and rectified traces of hypoglossal motor output show triplet, doublet and singlet respiratory events (indicated with brackets). (C) Changes in respiratory-related hypoglossal motor bursts before, during and after application of four phenylephrine concentrations.

Fig. 2.

Phenylephrine alters hypoglossal burst discharge. Data are shown for control brainstems (small circles), and brainstems exposed to phenylephrine at 20 μ M (triangles), 50 μ M (large circles), 100 μ M (squares), and 200 μ M (diamonds). All phenylephrine concentrations increased burst frequency, with the increase persisting during washout for 20 μ M, 50 μ M and 200 μ M (A, B). Burst amplitude increased with 20 μ M phenylephrine compared to controls, while 50 μ M and 100 μ M decreased amplitude at the same rate as control brainstems. Phenylephrine at 200 μ M produced acute and long-lasting decreases in burst amplitude (C). All phenylephrine concentrations acutely decreased bursts/respiratory event with long-lasting decreases observed for 100 and 200 μ M (D). Symbols: * ($P < 0.05$ compared to controls at that time point); filled in symbol ($P < 0.05$ compared to baseline for that drug dose); † ($P < 0.05$ for drug effect).

Fig. 3.

Phenylephrine alters respiratory event regularity. Data are shown for control brainstems (small circles), and brainstems exposed to phenylephrine at 20 μ M (triangles), 50 μ M (large circles), 100 μ M (squares), and 200 μ M (diamonds). All phenylephrine concentrations decreased respiratory event interval standard deviation, with the decrease persisting during washout for 20, 50, and 200 μ M (A, B). Phenylephrine (50, 100, and 200 μ M) increased respiratory event interval coefficient of variation immediately following application at the 60-min time point and 20 μ M and 50 μ M phenylephrine decreased respiratory event interval coefficient of variation at the 90-min time point (C, D). Symbols: * ($P < 0.05$ compared to controls at that time point); filled in symbol ($P < 0.05$ compared to baseline for that drug dose); † ($P < 0.05$ for drug effect).

Fig. 4. Endogenous α_1 -adrenergic receptor activation. Data are shown for prazosin at 0.25 μM (triangles), 0.5 μM (squares) and 1.0 μM (circles). There were no consistent effects on burst frequency, bursts/respiratory event, respiratory event interval standard deviation or coefficient of variation (A, C, D, E), but all concentrations of prazosin decreased amplitude (B). Symbols: * ($P < 0.05$ compared to controls at that time point); filled in symbol ($P < 0.05$ compared to baseline); † ($P < 0.05$ for drug effect).

Fig. 5.

α_1 adrenergic receptor activation required for phenylephrine-induced frequency increase and amplitude decrease. Data are shown for prazosin (0.5 μ M) pretreatment (squares) and for phenylephrine (50 μ M) application alone (grey circles; same data as in Figs. 2 and 3). Prazosin prevented the phenylephrine-induced increase in burst frequency (A), but not the decrease in bursts/respiratory event (C) or respiratory event interval standard deviation or coefficient of variation (D, E). Prazosin pre-treatment blocked the phenylephrine-induced decrease in burst amplitude (B). * ($P < 0.05$ compared to phenylephrine at that time point); filled in symbol ($P < 0.05$ compared to baseline); † ($P < 0.05$ for drug effect).

Fig. 6.

5-HT₃ receptor blockade does not alter phenylephrine-induced changes in burst timing. Data are shown for tropisetron (50 μ M) pretreatment (squares) and for phenylephrine (50 μ M) application alone (grey circles; same data as in Figs. 2 and 3). Tropisetron pretreatment did not prevent the phenylephrine-induced decrease in bursts/respiratory event (A), respiratory event interval standard deviation (B) or coefficient of variation (C, D). Symbols: * ($P < 0.05$ compared to phenylephrine at that time point); filled in symbol ($P < 0.05$ compared to baseline); † ($P < 0.05$ for drug effect).

Fig. 7. Phenylephrine-induced increase in regularity, but not decrease in bursts/respiratory event, blocked by co-application of α_1 adrenergic and 5-HT₃ receptor antagonists. Data are shown for prazosin (0.5 μ M) and tropisetron (50 μ M) pretreatment (squares) and for phenylephrine (50 μ M) application alone (grey circles; same data as in Figs. 2 and 3). In the presence of prazosin and tropisetron, phenylephrine decreased bursts/respiratory event (A), but not respiratory event interval standard deviation (B) or coefficient of variation (C). Symbols: * ($P < 0.05$ compared to phenylephrine at that time point); filled in symbol ($P < 0.05$ compared to baseline); † ($P < 0.05$ for drug effect).

Fig. 8.

α_1 adrenergic and 5-HT₃ receptor-dependent increases in burst frequency are not additive. Data are shown for phenylephrine (50 μ M) and mCPBG (100 μ M) co-application (squares). No further increase in burst frequency is observed when mCPBG is applied against a background concentration of phenylephrine. Symbols: filled in symbol ($P < 0.05$ compared to baseline)

Fig. 9.

Pattern sensitivity of phenylephrine application. For intermittent phenylephrine applications, 20 min of control aCSF was followed by four, 5-min phenylephrine (50 μ M) applications separated by 20 min of control aCSF, followed by a 120 min of control aCSF (A). For sustained phenylephrine applications, 20 min of control aCSF was followed by one 20-min phenylephrine (50 μ M) application, followed by a 120 min of control aCSF (B). Both intermittent and sustained phenylephrine increased burst frequency during drug application, but returned to baseline values after washout (C). Sustained, but not intermittent, phenylephrine application decreased burst amplitude (D) and bursts/respiratory event (E). Both sustained and intermittent phenylephrine application decreased respiratory event interval standard deviation, but only the sustained application resulted in long-lasting effects (F). Symbols: *($P < 0.05$ compared to baseline)

Table 1 **α -1 agonists; acute and long-lasting changes in burst duration and percent time-to-peak**

	20 μ M Phenylephrine	50 μ M Phenylephrine	100 μ M Phenylephrine	200 μ M Phenylephrine
<i>Burst Duration (sec)</i>				
Baseline	17.2 $\pm$ 2.1	17.9 $\pm$ 1.3	20.2 $\pm$ 3.0	16.1 $\pm$ 1.9
Drug Application	17.1 $\pm$ 1.5	*15.4 $\pm$ 1.0	21.1 $\pm$ 2.3	15.4 $\pm$ 1.6
Washout	16.7 $\pm$ 1.8	*14.9 $\pm$ 1.1	19.1 $\pm$ 1.9	16.4 $\pm$ 1.8
<i>Percent Time-to-Peak (time to peak divided by peak duration)</i>				
Baseline	36.6 $\pm$ 4.2	39.4 $\pm$ 3.0	39.3 $\pm$ 2.0	42.3 $\pm$ 3.5
Drug Application	40.8 $\pm$ 4.2	45.7 $\pm$ 1.6	45.7 $\pm$ 2.9	52.5 $\pm$ 2.4
Washout	38.2 $\pm$ 4.5	43.2 $\pm$ 1.9	39.7 $\pm$ 1.9	48.7 $\pm$ 2.9

*
P<0.05 compared to baseline.