The Journal of Experimental Biology 209, 3685-3694
Published by The Company of Biologists 2006
doi:10.1242/jeb.02418

Noradrenergic modulation of respiratory motor output during tadpole development: role of α-adrenoceptors

Stéphanie Fournier and Richard Kinkead

Department of Pediatrics, Université Laval, Centre de Recherche du Centre Hospitalier Universitaire de Québec, 10 rue de l'Espinay, Québec City, QC G1L 3L5, Canada

*Author for correspondence (e-mail: Richard.Kinkead@crsfa.ulaval.ca)

Accepted 28 June 2006

Summary

Noradrenaline (NA) is an important modulator of respiratory activity. Results from *in vitro* studies using immature rodents suggest that the effects exerted by NA change during development, but these investigations have been limited to neonatal stages. To address this issue, we used *in vitro* brainstem preparations of an ectotherm, *Rana catesbeiana*, at three developmental stages: pre-metamorphic tadpoles, metamorphic tadpoles and fully mature adult bullfrogs. We first compared the effects of NA bath application (0.02–10 μmol l^{-1}) on brainstem preparations from both pre-metamorphic (Taylor-Köllros stages VII–XI) and metamorphic tadpoles (TK stages XVIII–XXIII) and adult frogs. The fictive lung ventilation frequency response to NA application was both dose- and stage-dependent. Although no net change was observed in the pre-metamorphic group, NA application decreased fictive lung burst frequency in preparations from more mature animals. These effects were attenuated by application of α-adrenoceptor antagonists. Conversely, NA application elicited dose- and stage-dependent increases in fictive buccal ventilation frequency. We then assessed the contribution of α-adrenoceptors towards these responses by applying specific agonists (α_1: phenylephrine; α_2: clonidine; concentration range from 10 to 200 μmol l^{-1} for both). Of the two agonists used, only phenylephrine application consistently mimicked the lung burst frequency response observed during NA application in each stage group. However, both agonists decreased buccal burst frequency, thus suggesting that other (β) adrenoceptor types mediate this response. We conclude that modulation of both buccal and lung-related motor outputs change during development. NA modulation affects both types of respiratory activities in a distinct fashion, owing to the different adrenoceptor type involved.

Key words: control of breathing, development, amphibian, *Rana catesbeiana.*

Introduction

Noradrenaline (NA) is a key modulator of respiratory activity. During hypoxia or transitions between arousal states, activation of pontine noradrenergic neurons such as the locus coeruleus and the A5 group contributes to changes in respiratory activity associated with these conditions. Much of our current knowledge about noradrenergic modulation of respiratory activity stems from electrophysiological studies performed on *in vitro* brainstem preparations from neonatal rats showing that NA predominantly reduces fictive breathing frequency, owing mainly to α_2 adrenoceptor activation (Al-Zubaidy et al., 1996; Arata et al., 1998; Errchidi et al., 1991; Hilaire et al., 1989) (for a review, see Hilaire et al., 2004). However, the functional organization of noradrenergic modulation is not homogeneous amongst species since in *in vitro* preparations from neonatal mice, exogenous NA (either bath-applied or locally injected near medullary respiratory neurons) elicits the opposite effect; that is, an increase in fictive breathing frequency mediated by α_1-adrenoceptor activation (Viemari and Hilaire, 2002).

During development, noradrenergic neurons and their projections, along with the postsynaptic receptors undergo substantial reorganization (Winzer-Serhan et al., 1996). Thus, it is not surprising that the effects of NA on fictive breathing frequency observed in preparations from neonatal rodents are age-dependent. For instance, NA bath application to *in vitro* preparations from mice foetuses (embryonic day 16) does not reduce but increases phrenic burst frequency (Viemari et al., 2003). In preparations from newborn rats and mice, α_2-mediated inhibition of respiratory activity is strongest at birth, and the strength of this inhibition subsides progressively during the first postnatal days (Errchidi et al., 1991; Viemari et al., 2003) (for a review, see Hilaire et al., 2004). α_2-related modulation of respiratory activity probably persists until adulthood since in anesthetized rats, these adrenoceptors contribute to depression of breathing frequency following

hypoxia (Bach et al., 1999). However, proper comparison of α_2-mediated modulation of breathing frequency across developmental stages is difficult since these data were obtained with different experimental approaches (*in vivo versus in vitro*).

Developmental change in α_2-related modulation is especially remarkable in newborn mice in which the balance between α_1- and α_2-mediated influences is ultimately dominated by α_1-related facilitation of breathing frequency (Hilaire et al., 2004). Relatively little is known about the potential role of α_1-related modulation of respiratory activity in more mature developmental stages. However, data obtained from brainstem preparations from adult turtles suggest that this aspect of noradrenergic modulation persists until adulthood (Johnson et al., 1998).

Although we currently have a good understanding of how noradrenergic modulation affects breathing frequency during early life, especially in rodents (Hilaire et al., 2004), our current knowledge remains limited by the absence of a study addressing noradrenergic modulation at distinct developmental stages (including adults) using a single model system. To address this shortcoming, the main objective of the present study was to take advantage of the *in vitro* brainstem preparation from *Rana catesbeiana* tadpoles and adult bullfrogs to test the hypothesis that noradrenergic modulation of fictive breathing frequency (both buccal and lung) changes during development. In a second series of experiments, the use of α_1- and α_2-adrenoceptor agonists allowed us to address how these receptor types contribute to noradrenergic modulation over the course of development and the transition from aquatic to aerial breathing in this species. Parts of this work have been reported in abstract form (Fournier and Kinkead, 2005).

Materials and methods

Animals

Experiments were performed on 108 bullfrog (*Rana catesbeiana* Shaw) brainstem preparations from tadpoles (mass range: 4.5–22.8 g) and adult frogs (mass range: 18–325.5 g) obtained from a commercial supplier (Charles D. Sullivan, Nashville, TN, USA). Animals were housed in aquaria supplied with flowing, filtered and dechlorinated Québec City water maintained between 21° and 24°C (12 h:12 h light:dark photoperiod). Tadpoles were fed a mixed diet of spinach and Nutrafin pellets for turtles and amphibians. Adult frogs were fed live crickets. All experiments complied with the guidelines of the Canadian Council on Animal Care. The institutional animal care committee approved the specific protocols used in this study.

In vitro brainstem preparations

Animals were anesthetized by immersion in a solution of tricaine methane sulfonate (0.06 g l^{-1}) buffered to pH 7 with $NaHCO_3$. For adult frogs, the beaker was placed on ice for 40–60 min to slow metabolism and ensure adequate anaesthesia throughout the dissection (Winmill and Hedrick, 2003). Once unresponsive to body pinch, tadpoles and frogs were decerebrated by a transection just rostral to the eyes. In frogs (*N*=39), a hole was drilled in the cranium to allow decerebration. Tadpoles were then placed under the dissection microscope for determination of the developmental stage based on the criteria of Taylor and Kollros (Taylor and Kollros, 1946), and assigned to one of two groups: pre- (stages VII–XI; *N*=39) or metamorphic tadpoles (stages XVIII–XXIII; *N*=30). The cranium was opened to expose the brainstem and rostral spinal cord and allow dissection of the cranial nerves. The brain was irrigated with ice-cold (0–5°C) artificial cerebrospinal fluid (aCSF) to reduce axonal conductance throughout the dissection procedure. The composition of the aCSF was identical to the one developed for tadpoles (Liao et al., 1996) and adult frogs (Kinkead et al., 1994) and consisted of (in mmol l^{-1}): 104 NaCl; 4 KCl; 1.4 $MgCl_2$; 10 D-glucose; 25 $NaHCO_3$; 2.4 $CaCl_2$ for tadpoles and 75 NaCl; 4.5 KCl; 1 $MgCl_2$; 7.5 D-glucose; 40 $NaHCO_3$; 2.5 $CaCl_2$; 1 NaH_2PO_4 for adult bullfrogs. The superfusate was equilibrated with a 98% O_2/2% CO_2 gas mixture and had a pH of 7.9±0.1 for tadpoles and 7.8±0.1 for adult bullfrogs. The brainstem was transected between the optic tectum and the forebrain and then caudal to the hypoglossal nerve before being transferred to a small Petri dish coated with Sylgard (Dow Corning, Midland, MI, USA) where it was immobilized with insect pins. The arachnoid and pia membranes were carefully removed, and the brain was moved to the recording chamber where it was placed ventral side up.

Electrophysiological recordings

Bursts of respiratory-related motor activity were recorded simultaneously from the rootlets of cranial nerves V and X using suction electrodes. The pipettes were constructed from borosilicate glass (0.84 mm internal diameter) pulled to a fine tip with a vertical microelectrode puller (Stoelting Instrument, Wood Dale, IL, USA). The tip was broken and bevelled to achieve appropriate tip diameter. Neural activity signals recorded from the suction electrodes were amplified (gain=10 000) and filtered (low cut-off: 10 Hz; high cut-off: 1 kHz) using a differential AC amplifier (model 1700; A-M systems, Everett, WA, USA). Vagal and trigeminal signals were then full-wave rectified and integrated (time constant: 100 ms) using a moving averager (model MA-821; CEW, Ardmore, PA, USA). The raw and integrated nerve signals were viewed on an oscilloscope and digitized for recording with a data acquisition system (model DI-720; Dataq Instruments, Akron, OH, USA). The sampling rate of the analogue to digital conversion for the raw signal was 2500 Hz.

Experimental protocol

Once the recording electrodes were in place, the brainstem preparation was superfused with control (drug-free) aCSF at room temperature (20–22°C) delivered at a rate ranging between 4 and 6 ml min^{-1}. The preparation was allowed to return to ambient temperature and stabilize for 30–60 min, until stable rhythmic neural activity was recorded from both nerves.

The first series of experiments compared the effects of noradrenaline (NA) bath application on fictive breathing

frequencies (both buccal and lung) between developmental stages. For this series, the protocol began by recording respiratory-related motor output for 10 min before NA was added in increasing concentrations to a second aCSF reservoir (pre-metamorphic stages: N=12; metamorphic stages: N=10; adult=9). The brainstem was exposed to the first NA concentration for 10 min before a higher NA concentration was delivered to the preparation. Other studies have shown that an equilibration period of at least 5 min is necessary to obtain measurements that do not reflect a transient effect of the drug (Belzile et al., 2002; Kinkead et al., 2002; Onimaru et al., 1998). Brainstem preparations were exposed, in succession, to seven increasing NA concentrations: 0.02, 0.05, 0.1, 0.5, 1, 5 and 10 μmol l^{-1}. This concentration range was based on values reported in the literature (Arata et al., 1998) and preliminary experiments, which suggested that tadpole preparations were slightly more sensitive to NA than mammals. The final application was followed by a 'wash-out' period during which the preparation was superfused with drug-free aCSF for a period ranging from 30 to 90 min before a final recording of respiratory-related motor output was made. Preliminary experiments were performed to confirm that, as in mammals, changes in respiratory activity (especially lung-related motor output) observed during NA application are mainly associated with α-adrenoceptor activation. In these distinct experiments, only one NA concentration (5 μmol l^{-1}) was bath-applied in the presence of prazosine (0.5 μmol l^{-1}; α_1-adrenoceptor antagonist) or RX821002 (25 μmol l^{-1}; α_2-adrenoceptor antagonist). Being concerned about potential carry over effects, each preparation was exposed to one antagonist only. These preliminary experiments were performed on preparations from pre-metamorphic tadpoles (N=5 for each drug) and adult bullfrogs (N=4 for each drug) only. Since NA active drugs are light sensitive, drug preparation and experiments were conducted with dim lights. Drug reservoirs were covered to minimize light exposure. All drugs were obtained from Sigma/RBI Aldrich (St Louis, MO, USA).

The second series of experiments addressed the contribution of α_1- and α_2-adrenoceptors in the modulation of fictive breathing frequencies across developmental stages by comparing the effects of α_1 and α_2 agonist application between stage groups. In these experiments, brainstem preparations were superfused for 10 min with aCSF containing increasing concentrations of the α_1 receptor agonist phenylephrine (10, 25, 100 and 200 μmol l^{-1}; pre-metamorphic: N=8; metamorphic: N=13; adult: N=10) or the α_2 receptor agonist clonidine (10, 25, 100 and 200 μmol l^{-1}; pre-metamorphic: N=9; metamorphic: N=7; adult: N=12) according to the protocol described previously.

Data analysis

Fictive breathing frequency values for respiratory burst activity were obtained by analysing the last 3 min of application of each NA concentration (including baseline). *In vitro* tadpole and frog brainstem preparations typically produce two patterns of respiratory-related neural activity: (1) high frequency, low amplitude, and (2) low frequency, high amplitude, reflecting fictive buccal and lung ventilation, respectively (Liao et al., 1996; Torgerson et al., 1998). Cranial nerve burst amplitude from a single electroneurogram is not always sufficient to adequately identify fictive lung and buccal bursts (Sanders and Milsom, 2001; Torgerson and al., 1998). Thus, two nerve signals were analysed simultaneously, here vagal nerve activity was used as a sensitive marker of fictive lung activity to distinguish between lung- and buccal-related signals (Kogo et al., 1994; Kogo and Remmers, 1994).

Lung and buccal burst frequencies were obtained by counting the number of lung- and buccal-related bursting events for the 3-min segment analysed, and averaged for a 1-min period.

Under baseline conditions, fictive breathing frequency differed between stage groups (stage effect: P<0.0001; Fig. 1 and Fig. 2A). Fictive breathing frequency data were therefore expressed as a percentage change from baseline to allow between-group comparisons of the fictive lung ventilation responses to drug application.

All measurements are reported as the mean ± s.e.m. The results were analysed statistically using a two-way analysis of variance (ANOVA; Statview version 5.01; SAS Institute, Cary, NC, USA) followed by Fisher's protected least significant difference (PLSD) test (P<0.05). A repeated-measures design was used when appropriate.

Results

Stage-dependent effects of noradrenaline bath application on fictive lung ventilation frequency

The representative neurograms shown in Fig. 1 illustrate the effects of applying NA (5 μmol l^{-1}) on the respiratory-related motor output produced by brainstem preparations from the different developmental stages investigated. Addition of NA to the aCSF changed fictive lung burst frequency in a stage-dependent manner (stage×drug: P<0.0001; Fig. 2A). In the pre-metamorphic group, 9 out of 12 (75%) brainstem preparations showed an increase in fictive lung burst frequency with increasing NA concentration up to 5 μmol l^{-1}. The other three preparations showed a slight frequency decrease. Overall, ANOVA results for the entire pre-metamorphic group indicate that NA application had no significant effect on fictive lung burst frequency (drug effect: P=0.35; Fig. 2A). Application of 5 μmol l^{-1} NA in the presence of the selective α_1-adrenoceptor antagonist prazosine had no effect on fictive lung ventilation (drug effect: P=0.4; Fig. 3A); however, a decrease in lung burst frequency was observed when NA was applied with RX821002 (selective α_2-adrenoceptor antagonist) simultaneously (drug effect: P=0.05; Fig. 3C).

Conversely, application of NA to brainstems from metamorphic tadpoles decreased fictive lung burst frequency at concentrations greater than 1 μmol l^{-1}. In the adult group, the fictive lung burst frequency decreased with a concentration of 0.02 μmol l^{-1} and higher than 1 μmol l^{-1}. These effects were reversed during the wash-out period (Fig. 2). Expressing

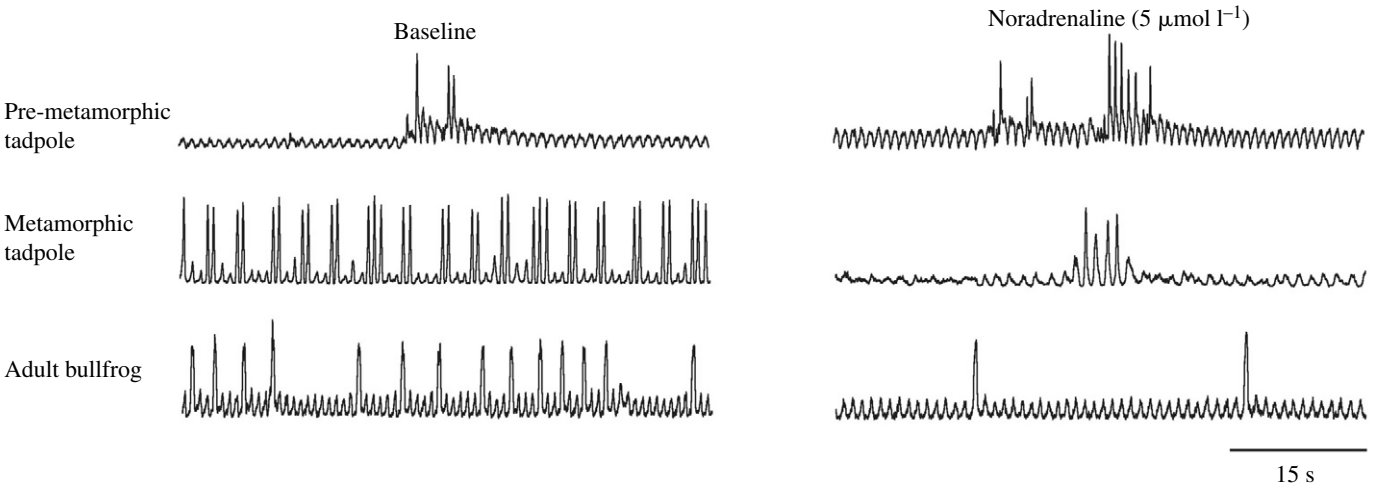

Fig. 1. Trigeminal neurograms comparing changes in lung- and buccal-related motor output with bath application of 5 μmol l^{-1} noradrenaline (NA) concentrations. These recordings illustrate the distinction between the two patterns of respiratory-related neural activity, and show the stage-dependent effects of NA on the neural correlates of respiratory activity. Recordings were obtained from isolated brainstem preparations from pre- and metamorphic tadpoles, and an adult bullfrog.

frequency values as a percentage change from baseline yielded results similar to those reported with absolute values (drug effect: P=0.013; Fig. 2B). That is, NA bath application had no net effect on the fictive lung burst frequency measured in preparations from the pre-metamorphic group (P=0.19) but decreased it in preparations from metamorphic tadpoles and adult frogs (P<0.001 and P=0.0002, respectively). Furthermore, expressing data this way confirmed that the fictive lung burst frequency response is stage-dependent (stage×drug: P=0.0003; Fig. 2B).

Stage-dependent effects of noradrenaline bath application on fictive buccal ventilation frequency

Under baseline conditions, the buccal burst frequency was similar in all three groups (stage effect: P=0.14; Fig. 4A). Unlike preparations from pre-metamorphic tadpoles, fictive buccal ventilation was not always observed in brainstems from more mature animals. For instance, only two preparations from adult frogs produced a signal in which fictive buccal ventilation frequency could be quantified reliably at more than two NA concentrations. Although the mean buccal frequency values obtained for this group are reported in Fig. 4, these data could not be included in the ANOVA for repeated measures. Data suggest, however, that in the adult group, NA application had no effect on fictive buccal ventilation frequency. In preparations from both tadpole groups, NA bath application enhanced fictive buccal ventilation frequency (drug effect: P<0.0001; Fig. 4A). Both tadpole groups began to respond to NA at the same dose (5 μmol l^{-1}), but the fictive buccal ventilation frequency increase observed in brainstems from more mature tadpoles was greater than the one reported for the pre-metamorphic group (stage×drug: P=0.029; Fig. 4A). Expressing data as a percentage change from baseline produced results similar to those reported with absolute values (stage×drug: P=0.013; Fig. 4B). In both tadpole groups, the wash-out period restored the fictive buccal ventilation frequency values back to their initial (baseline) values. Note that neither adrenoceptor antagonist could prevent the increase in fictive buccal ventilation observed when 5 μmol l^{-1} NA was applied to preparations from either pre-metamorphic tadpoles or adult bullfrogs (drug effect: P<0.05 for all; Fig. 3B,D).

Effects of α_1-adrenoceptor activation on fictive lung ventilation frequency

Addition of the selective α_1-adrenoceptor agonist phenylephrine to the aCSF altered fictive lung ventilation frequency in all stage groups, and these effects were stage-dependent (stage×drug: P<0.0001; Fig. 5A). In the pre-metamorphic group, application of low phenylephrine concentrations (10 and 25 μmol l^{-1}) increased fictive lung burst frequency; however, this response was not maintained when higher concentrations of phenylephrine were applied (Fig. 5A). These results contrast with those obtained in the metamorphic group in which phenylephrine application decreased fictive lung burst frequency, and preparations from adult frogs in which phenylephrine had no significant effect (P=0.29; Fig. 5A). Fictive lung ventilation frequency returned to baseline values during the wash-out period.

Effects of α_1-adrenoceptor activation on fictive buccal ventilation frequency

Again, few preparations from adult frogs produced a reliable fictive buccal ventilation signal; these data are displayed but could not be included in the ANOVA for repeated measures. Application of phenylephrine to tadpole brainstem preparations affected buccal burst frequency (drug effect: P=0.0007); however, the responses were not stage-dependent (stage ×

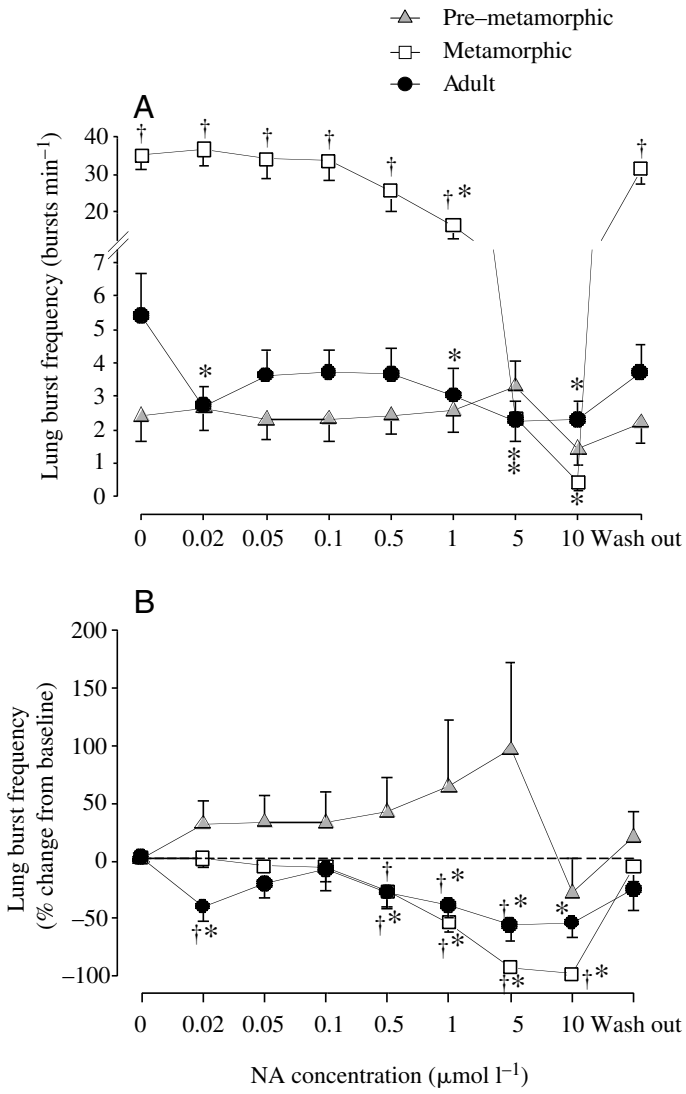

Fig. 2. Stage-dependent changes in fictive lung burst frequency during noradrenaline (NA) application at different concentration. NA application was followed by a 30–90 min wash-out period. (A) Absolute lung burst frequency, (B) data expressed as a percentage change from baseline values. In A and B, responses were measured in pre- (triangles; *N*=12) and metamorphic tadpoles (squares; *N*=10), and adult frogs (circles; *N*=9). Values are expressed as means ± s.e.m. *Values statistically different from baseline at $P<0.05$. †Values statistically different from corresponding pre-metamorphic value at $P<0.05$.

concentration: $P=0.72$; Fig. 5B). In the pre-metamorphic group, phenylephrine application elicited a biphasic response: an initial frequency decrease (phenylephrine concentrations of 10 and 25 μmol l^{-1}) followed by an increase at the highest dose (200 μmol l^{-1}). In the metamorphic group, fictive buccal burst frequency decreased when lower concentrations of phenylephrine were applied (10 and 25 μmol l^{-1}); however, this response was not sustained when bath concentrations were increased further. Some of these effects were persistent, as a 90-min wash-out period was not sufficient for the preparation to return to its initial fictive buccal ventilation frequency value, an effect that was especially noticeable in the pre-metamorphic group.

Effects of α_2-adrenoceptor activation on fictive lung ventilation frequency

α_2-adrenoceptor activation with clonidine had stage-dependent effects on fictive lung burst frequency (stage×drug: $P<0.0001$; Fig. 6A). In the pre-metamorphic group, clonidine bath application increased fictive lung ventilation frequency in a dose-dependent manner; however, the response was statistically significant at the highest dose only. In the adult group, the increase in fictive lung ventilation frequency was significant at a lower dose (25 μmol l^{-1}) but then remained relatively stable when higher clonidine concentrations were applied (Fig. 6A). These results differ from the response observed in the metamorphic group in which application of low clonidine concentrations (10 and 25 μmol l^{-1}) decreased fictive lung ventilation frequency. These effects were reversed during the wash-out period in both tadpole groups but persisted beyond the recovery period in the adult group (Fig. 6A).

Effects of α_2-adrenoceptor activation on fictive buccal ventilation frequency

Clonidine decreased buccal burst frequency in a dose- and stage-dependent manner (drug effect: $P<0.0001$; stage × drug: $P=0.02$; Fig. 6B). Brainstems from adult frogs appeared to show the greatest decreased followed by the pre-metamorphic group; however, the small number of adults exhibiting buccal ventilation (*N*=2) did not allow us to confirm these results statistically. Thus, the clonidine dose necessary to elicit a significant decrease in buccal burst frequency appeared to be lowest in the adult group (10 μmol l^{-1}), followed by the pre- and metamorphic groups (25 μmol l^{-1} and 100 μmol l^{-1}, respectively). These effects were not reversed during the wash-out period.

Discussion

This study addressed developmental changes in noradrenergic modulation of fictive lung and buccal ventilation that take place from early (pre-metamorphic) stages to fully mature adult animals within a single model system. Overall, the data support our hypothesis that noradrenergic modulation of fictive lung and buccal ventilation frequency changes substantially over the course of development. Based on results obtained in foetal and newborn mammals (Al-Zubaidy et al., 1996; Arata et al., 1998; Errchidi et al., 1991; Viemari and Hilaire, 2002) (for a review, see Hilaire et al., 2004) we focused our work on the role of α-adrenoceptors in these developmental changes, and data indicate that the changes in fictive lung ventilation frequency that follow NA bath application are probably related to these receptor types because these responses were mimicked or prevented by selective agonists or antagonists, respectively. Conversely, the increase in fictive buccal ventilation frequency observed during NA application

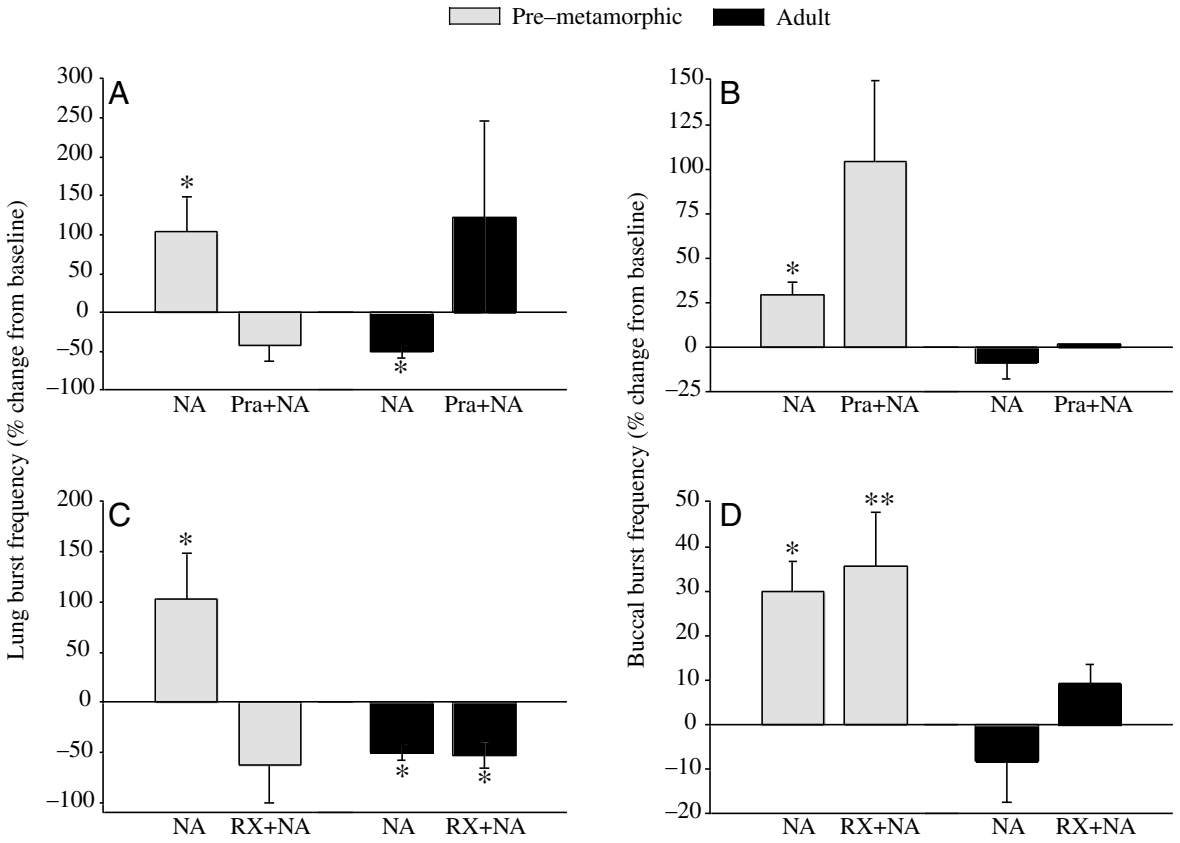

Fig. 3. The effects of NA bath application (5 μmol l^{-1}) on fictive lung (A,C) and buccal (B,D) ventilation frequencies in the presence of the selective α_1-adrenoceptor antagonist prazosine (Pra; 0.5 μmol l^{-1}; A,B) or the selective α_2-adrenoceptor antagonist RX821002 (RX; 25 μmol l^{-1}; C,D). These experiments were performed on brainstem preparations from pre-metamorphic tadpoles (grey bars) and adult bullfrogs (black bars). *Values statistically different from baseline at $P<0.05$. **Values statistically different from baseline at $P<0.10$.

could not be reproduced or blocked by α-agonists or antagonists, thus suggesting that these effects involve β-adrenoceptors. Although both types of respiratory activities require activation of essentially the same motoneurone pools, these differences further substantiate the functional distinctions between the neural circuits that drive lung and buccal ventilation (Wilson et al., 2002), as different adrenoceptor types are involved in the modulation of fictive lung and buccal ventilation.

Critique of method

Although the value of the amphibian model for electrophysiological investigations of respiratory control development is acknowledged (Belzile et al., 2004; Hedrick, 2005; Kinkead, 1997; Milsom et al., 1999; Vasilakos et al., 2005), we are well aware of the limitations inherent to the use of bath application of pharmacological agents onto brainstem preparations (Kinkead et al., 2002). The results obtained from such approach must be interpreted cautiously since the sites of action of the drugs are unknown, and the pharmacological specificities of the agents used have been characterized in mammals and may not apply equally in amphibians. Furthermore, clonidine, in addition to being a selective α_2-adrenoceptor agonist, also has affinity for imidazole binding sites. Despite these limitations, this approach nonetheless allows an initial assessment of the neural mechanisms contributing to the maturation of the respiratory control system. However, the fact that the changes in lung ventilation observed following *in vivo* clonidine application in *Bufo marinus* and systemic injection of adrenaline and noradrenaline in *Rana temporaria* were similar to those found in the present study help validate our approach and data interpretation (Niechaj, 1971; Rives and Bernard, 2001).

The experimental design used to assess the effects of increasing NA concentrations could be a confounding factor since the time between application of the first and last dose is relatively long (over 1 h) and may be sufficient to allow dynamic changes in the response owing to adrenoceptor internalization. However, comparison of the results obtained in the dose–response experiments (Fig. 2) in which 5 μmol l^{-1} of NA was the sixth concentration applied (i.e. 60 min later) with those obtained in the preliminary experiments in which 5 μmol l^{-1} of NA was the first dose applied show that the system's responsiveness is similar and thus maintained over time.

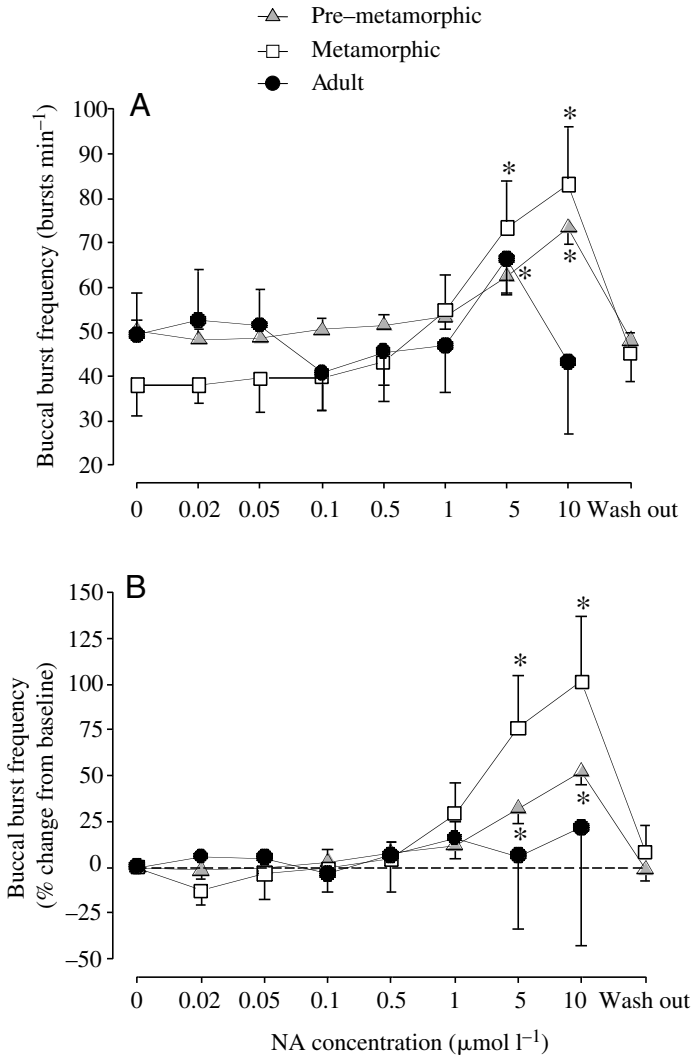

Fig. 4. Dose-dependent changes in fictive buccal burst frequency during noradrenaline (NA) application at different concentrations. NA application was followed by a 30–90 min wash-out period. (A) Absolute buccal burst frequency, (B) frequency data expressed as a percentage change from baseline values. In A and B, data were obtained in pre- (triangles; N=11) and metamorphic tadpoles (squares; N=7), and adult frogs (circles; N=4). Values are expressed as means ± s.e.m. *Values statistically different from baseline at P<0.05.

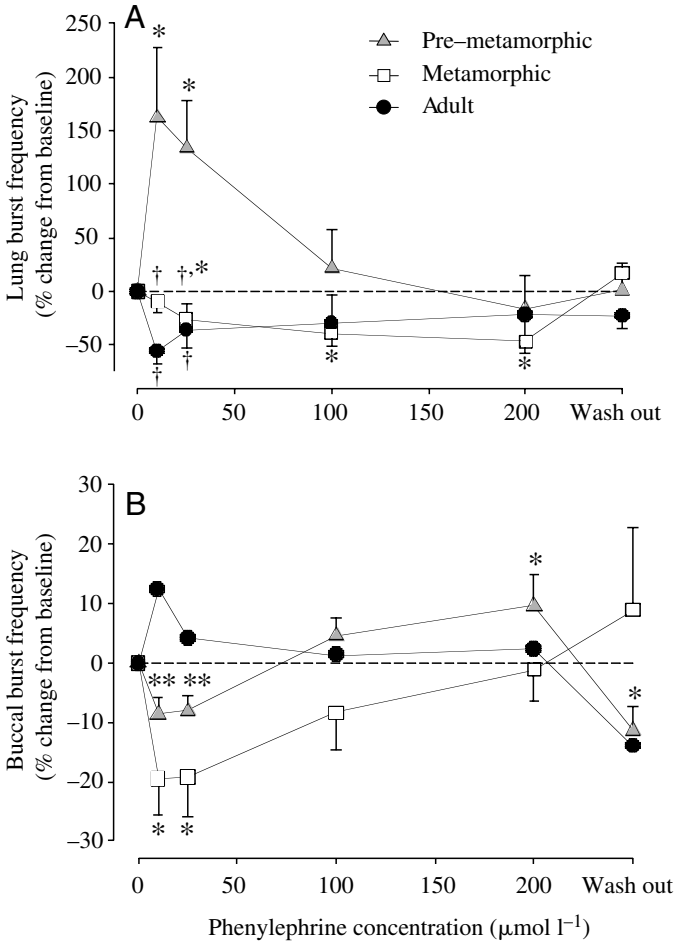

Fig. 5. Dose-dependent changes in fictive lung and buccal burst frequency during application of increasing concentration of the α_1-adrenoceptor agonist phenylephrine (Phe). Phe application was followed by a 30–90 min wash-out period. (A) Data expressed as a percentage change from lung baseline values, and (B) data expressed as a percentage change from buccal baseline values. In A, lung burst frequency responses were measured in pre- (triangles; N=6) and metamorphic tadpoles (squares; N=12), and adult frogs (circles; N=10). For fictive buccal burst frequency responses (B), mean data were obtained in pre- (N=8) and metamorphic tadpoles (N=13), but not in adult frogs (N=1). Adults were excluded from the statistical analysis since the number of replicates was too low; for this group, data are displayed without error bars. Values are expressed as means ± s.e.m. *Values statistically different from baseline at P<0.05. **Values statistically different from baseline at P<0.10. †Values statistically different from corresponding values from the pre-metamorphic group at P<0.05.

Neuroanatomical considerations

In neonatal rodents, different groups of noradrenergic neurons are responsible for the heterogeneous modulation of fictive breathing (Hilaire et al., 2004). For instance, activation of the pontine A5 noradrenergic neurons exerts an inhibitory modulation of respiratory rhythm (*via* α_2-adrenoceptors), whereas A6 neurons (locus coeruleus) facilitate fictive breathing frequency (Hilaire et al., 2004). Our understanding of amphibian noradrenergic system in relation to respiratory neurons is sketchy in comparison with that of mammals, and this shortcoming makes it difficult to ascribe the effects reported in the present study to specific groups of neurons. We know that the organization of noradrenergic neurons of amphibians shares many features with that of mammals, but no studies have reported the existence of A5-like neurons in amphibians. Conversely, neuroanatomical studies have shown that, on the basis of location, neurotransmitter content and efferent projections, the isthmic noradrenergic cell group of

amphibians is homologous to the locus coeruleus of amniotes (Marin et al., 1996). These neurons are present in our preparation, and probably contribute to the changes in the respiratory motor output discussed below.

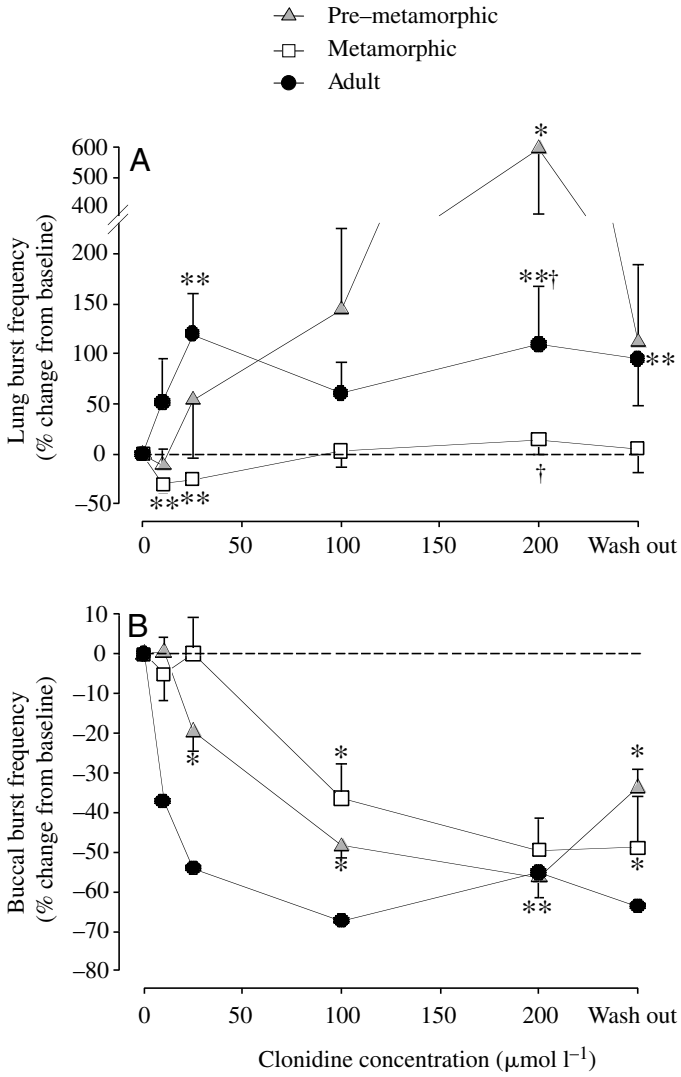

Fig. 6. Dose- and stage-dependent changes in fictive lung and buccal burst frequency during application of increasing concentration of the α_2-adrenoceptor agonist clonidine (Clo). Clo application was followed by a 30–90 min wash-out period. (A) Data expressed as a percentage change from baseline lung values, and (B) data expressed as a percentage change from baseline buccal values. In A, lung burst frequency responses were measured in pre- (triangles; *N*=9) and metamorphic tadpoles (squares; *N*=7), and adult frogs (circles; *N*=12). For fictive buccal burst frequency responses (B), mean data were obtained in pre- (*N*=9) and metamorphic tadpoles (*N*=6), and adult frogs (*N*=2). Adults were excluded from the statistical analysis since the number of replicates was too low; for this group, data are displayed without error bars. Values are expressed as means ± s.e.m. *Values statistically different from baseline, $P<0.05$. **Values statistically different from baseline, $P<0.10$. †Values statistically different from corresponding values from the pre-metamorphic group, $P<0.05$.

Developmental changes in noradrenergic modulation of fictive lung ventilation frequency

As we mentioned previously, these results are consistent with our hypothesis since each of the agonists applied elicited stage-dependent changes in fictive lung ventilation frequency. Unlike the other stage groups, the lung burst frequency response to NA application measured from pre-metamorphic brainstems was not homogeneous, as two distinct responses were observed. Most preparations increased lung burst frequency in response to moderate NA concentrations (between 0.5 and 5 μmol l^{-1}), but we have no data that allows us to explain why other preparations responded differently. For instance, the nature of the response (increase *versus* decrease) did not correlate with the developmental stage. Sex could affect this response, however, male and female tadpoles were not distinguished prior to brainstem dissection. Given that a low phenylephrine concentration was sufficient to elicit a comparable (but significant) increase in fictive lung ventilation frequency, we propose that, in this stage group, the response observed during NA bath application is mainly mediated by α_1-adrenoceptor activation. The fact that the response to low clonidine concentration was small in the pre-metamorphic group suggests that the contribution of α_2-adrenoceptors towards this response is relatively modest.

These results differ from those observed in preparations from more mature animals in which NA application caused a net decrease in fictive lung ventilation frequency. In the metamorphic group, this response probably involves both α-adrenoceptor subtypes since both phenylephrine and clonidine elicited similar decreases in fictive lung ventilation frequency. However, none of the doses used were sufficient to elicit a response of the same magnitude, thereby suggesting that the response observed with NA reflects simultaneous activation of more than one receptor type. In the adult group, however, the decrease in fictive lung ventilation frequency observed during NA application was blocked by prazosine but not by RX821002. Furthermore, the response observed during phenylephrine application was similar to the one reported with NA, thus indicating that α_1-adrenoceptors contribute to this response.

Most electrophysiological studies addressing noradrenergic modulation of fictive lung ventilation have been performed on immature rodents. However, brainstem preparations from turtles are an excellent model system for *in vitro* investigation of respiratory control in a mature animal. In their experiments, Johnson et al. (Johnson et al., 1998) reported that both NA and phenylephrine increase the lung burst frequency generated by turtle brainstems. These results differ substantially from those obtained in adult frogs in which opposite effects were observed, but are similar to those obtained in the pre-metamorphic group. In the absence of data from immature turtles, it is difficult to determine whether such interspecies differences are related to different developmental trajectories or whether turtles simply do not show much change in the organization of the neural pathways involved in noradrenergic modulation during development.

It is noteworthy that α_1-adrenoceptor activation increases fictive lung ventilation frequency in neonatal rodents also. Although more species need to be investigated, α_1-mediated stimulation of lung ventilation during early life could be a common configuration amongst vertebrates. Although fewer studies have addressed the role of β-adrenoceptors in the modulation of respiratory rhythm *in vitro*, data indicate that activation of these receptors has little effect on the fictive breathing frequency in mammalian preparations (Arata et al., 1998).

To the best of our knowledge, no other preparations besides amphibians and neonatal rodents have been used to investigate α_2-mediated modulation of respiratory activity *in vitro*. In mammals, α_2-adrenoceptor activation typically decreases fictive breathing frequency (Al-Zubaidy et al., 1996; Arata et al., 1998; Errchidi et al., 1991; Viemari and Hilaire, 2002) (for a review, see Hilaire et al., 2004). Whereas clonidine application elicited a modest lung burst frequency depression in the amphibian metamorphic group, more convincing responses were observed in the other two groups in which α_2-adrenoceptor activation augments fictive lung ventilation in this species. Again, the lack of mammalian data from other developmental stages makes is difficult to explain these interspecies differences.

Using low Ca^{2+}, high Mg^{2+} solutions to block chemical synaptic transmission, Arata et al. (Arata et al., 1998) showed that, in neonatal rats, the direct effect of adrenaline on pre-motor respiratory neurons is mainly excitatory, whereas the inhibitory actions arise from indirect (GABAergic) pathways. A similar configuration in *Rana catesbeiana* could contribute to the developmental changes in lung burst frequency response to NA and phenylephrine application because during early development, $GABA_A$ receptor activation depolarises neurons and increases their excitability. With maturation, Cl^- gradients are better established and the effects of GABA become mainly inhibitory (Ben-Ari, 2002). The role of indirect GABAergic pathways in noradrenergic modulation of respiratory activity during development is currently being addressed in our laboratory.

Developmental changes in noradrenergic modulation of fictive buccal ventilation frequency

In tadpoles, NA bath application elicited stage-dependent increases in buccal burst frequency. Although the minimum NA concentration necessary to elicit a response was the same in both groups (5 μmol l^{-1}), the response was stage-dependent, and the increase observed in metamorphic brainstems tended to be larger than the one observed in the pre-metamorphic group. Application of α-adrenoceptor agonists clearly showed that neither receptor type is involved in this response since both drugs decreased buccal burst frequency. Consequently, it would appear that β-adrenoceptors mediate the increase in fictive buccal ventilation frequency observed during NA application. This evidence suggests (albeit indirectly) that the noradrenergic circuitry modulating lung *versus* buccal ventilation is distinct, or that different receptor types are involved (α *versus* β).

Several factors can contribute to changes in buccal ventilation frequency. Since production of both lung- and buccal-related respiratory activity requires activation of the same motoneurone pools, it is clear the expression of one type of activity will reduce the time available for the expression of the other. Given that fictive lung ventilation frequency produced by pre- and metamorphic tadpole brainstems changed in opposite ways during NA application, it is unlikely that this activity interfered with the production of buccal-related activity. Thus, the increase in buccal burst frequency observed during NA application could reflect a change in the endogenous rhythm and/or facilitation of its expression by NA. These differences in the magnitude of the responses observed during NA *versus* selective agonist application likely reflect the opposite effects of α- and β-adrenoceptors on buccal burst frequency. Although these results provide no clue concerning the potential mechanisms underlying developmental changes in the magnitude of this response, the fact that its nature does not change (increase *versus* decrease) suggests that changes in adrenoceptor expression and/or the capacity for NA release in the vicinity of respiratory neurons are probably involved.

Perspectives

Noradrenergic modulation of respiratory motor output changes substantially over the course of development. In mammals, many noradrenergic neurons are hypoxia sensitive and have been implicated in the ventilatory response to hypoxia (Neubauer and Sunderram, 2004; Roux et al., 2000; Soulier et al., 1997). In pre-metamorphic tadpoles, activation of noradrenergic neurons by hypoxia would tend to facilitate both lung and buccal ventilation. These responses could help alleviate the reduction in O_2 availability and contribute to both air breathing behaviour and lung development. However, developmental change in fictive buccal ventilation frequency response to NA application is contrary to the changes described in intact bullfrog tadpoles in which the buccal hyperventilatory response to aquatic hypoxia decreases during development (Jia and Burggren, 1997). This change has been ascribed to progressive degeneration of the gills arches containing O_2 chemoreceptor (Jia and Burggren, 1997), and changes in noradrenergic modulation may occur as a reaction to the decrease in chemosensory afferent signal to help maintain adequate buccal ventilation during hypoxia.

The attenuation of fictive lung ventilation frequency during NA application observed in more mature animals is more difficult to explain because contribution of the lungs in gas exchange become increasingly important during development (Burggren and West, 1982), and decreasing lung ventilation frequency during hypoxia seems paradoxical, especially when, in intact frogs, enhancing pulmonary O_2 uptake is the primary adjustment to increased O_2 demand (Pinder and Burggren, 1986). However, this response may be part of a larger strategy aimed at minimizing energy expenditure when more mature (and less tolerant) tadpoles and frogs face hypoxia.

This research was supported by a discovery grant by the Natural Science and Engineering Research Council of Canada (R.K.) and the Canada Research Chair in Respiratory Neurobiology.

References

Al-Zubaidy, Z. A., Erickson, R. L. and Greer, J. J. (1996). Serotonergic and noradrenergic effects on respiratory neural discharge in the medullary slice preparation of neonatal rats. *Pflugers Arch.* **431**, 942-949.

Arata, A., Onimaru, H. and Homma, I. (1998). The adrenergic modulation of firings of respiratory rhythm-generating neurons in medulla-spinal cord preparation from newborn rat. *Exp. Brain Res.* **119**, 399-408.

Bach, K. B., Kinkead, R. and Mitchell, G. S. (1999). Post-hypoxia frequency decline in rats: sensitivity to repeated hypoxia and α_2-adrenoreceptor antagonism. *Brain Res.* **817**, 25-33.

Belzile, O., Gulemetova, R. and Kinkead, R. (2002). Role of 5-$HT_{2A/C}$ receptors in serotonergic modulation of respiratory motor output during tadpole development. *Respir. Physiol. Neurobiol.* **133**, 277-282.

Belzile, O., Simard, E., Gulemetova, R., Bairam, A. and Kinkead, R. (2004). Un modèle amphibien pour l'étude du développement du contrôle de la respiration. *Med. Sci.* **20**, 904-908.

Ben-Ari, Y. (2002). Excitatory actions of GABA during development: the nature of the nurture. *Nat. Rev. Neurosci.* **3**, 728-739.

Burggren, W. W. and West, N. H. (1982). Changing respiratory importance of gills, lungs and skin during metamorphosis in the bullfrog *Rana catesbeiana*. *Respir. Physiol.* **47**, 151-164.

Errchidi, S., Monteau, R. and Hilaire, G. (1991). Noradrenergic modulation of the medullary respiratory rhythm generator in the newborn rat: an in vitro study. *J. Physiol. Lond.* **443**, 477-498.

Fournier, S. and Kinkead, R. (2005). Noradrenergic modulation of respiratory motor output during bullfrog development. *Auton. Neurosci.* **119**, 153.

Hedrick, M. S. (2005). Development of respiratory rhythm generation in ectothermic vertebrates. *Respir. Physiol. Neurobiol.* **149**, 29-41.

Hilaire, G., Monteau, R. and Errchidi, S. (1989). Possible modulation of the medullary respiratory rhythm generator by the noradrenergic A5 area: an in vitro study in the newborn rat. *Brain Res.* **485**, 325-332.

Hilaire, G., Viemari, J. C., Coulon, P., Simonneau, M. and Bevengut, M. (2004). Modulation of the respiratory rhythm generator by the pontine noradrenergic A5 and A6 groups in rodents. *Respir. Physiol. Neurobiol.* **143**, 187-197.

Jia, X. and Burggren, W. W. (1997). Developmental changes in chemoreceptive control of gill ventilation in larval bullfrogs (*Rana catesbeiana*). I. Reflex ventilatory responses to ambient hyperoxia, hypoxia and NaCN. *J. Exp. Biol.* **200**, 2229-2236.

Johnson, R. A., Johnson, S. M. and Mitchell, G. S. (1998). Catecholaminergic modulation of respiratory rhythm in an in vitro turtle brain stem preparation. *J. Appl. Physiol.* **85**, 105-114.

Kinkead, R. (1997). Episodic breathing in frogs: converging hypotheses on neural control of respiration in air breathing vertebrates. *Am. Zool.* **37**, 31-40.

Kinkead, R., Filmyer, W. G., Mitchell, G. S. and Milsom, W. K. (1994). Vagal input enhances responsiveness of respiratory discharge to central changes in pH/CO_2 in bullfrogs. *J. Appl. Physiol.* **77**, 2048-2051.

Kinkead, R., Belzile, O. and Gulemetova, R. (2002). Serotonergic modulation of respiratory motor output during tadpole development. *J. Appl. Physiol.* **93**, 936-946.

Kogo, N. and Remmers, J. E. (1994). Neural organization of the ventilatory activity in the frog, *Rana catesbeiana*. II. *J. Neurobiol.* **25**, 1080-1094.

Kogo, N., Perry, S. F. and Remmers, J. E. (1994). Neural organization of the ventilatory activity in the frog, *Rana catesbeiana*. I. *J. Neurobiol.* **25**, 1067-1079.

Liao, G. S., Kubin, L., Galante, R. J., Fishman, A. P. and Pack, A. I. (1996). Respiratory activity in the facial nucleus in an in vitro brainstem of tadpole, *Rana catesbeiana*. *J. Physiol. Lond.* **492**, 529-544.

Marin, O., Smeets, W. J. and Gonzalez, A. (1996). Do amphibians have a true locus coeruleus? *NeuroReport* **7**, 1447-1451.

Milsom, W. K., Reid, S. G., Meier, J. T. and Kinkead, R. (1999). Central respiratory pattern generation in the bullfrog, *Rana catesbeiana*. *Comp. Biochem. Physiol.* **124A**, 253-264.

Neubauer, J. A. and Sunderram, J. (2004). Oxygen-sensing neurons in the central nervous system. *J. Appl. Physiol.* **96**, 367-374.

Niechaj, A. (1971). The effects of catecholamines on the slow rhythm of pulmonay respiration in the frog. *Folia Biol. Krakow* **19**, 371-384.

Onimaru, H., Shamoto, A. and Homma, I. (1998). Modulation of respiratory rhythm by 5-HT in the brainstem-spinal cord preparation from newborn rat. *Pflugers Arch.* **435**, 485-494.

Pinder, A. W. and Burggren, W. W. (1986). Ventilation and partitioning of oxygen uptake in the frog Rana pipiens: effects of hypoxia and activity. *J. Exp. Biol.* **126**, 453-468.

Rives, J. E. and Bernard, D. G. (2001). α_2-adrenergic receptors and the central control of breathing in the cane toad, *Bufo marinus*. *Respir. Physiol.* **125**, 213.

Roux, J. C., Peyronnet, J., Pascual, O., Dalmaz, Y. and Pequignot, J. M. (2000). Ventilatory and central neurochemical reorganization of O_2 chemoreflex after carotid sinus nerve transection in rat. *J. Physiol. Lond.* **522**, 493-501.

Sanders, C. E. and Milsom, W. K. (2001). The effects of tonic lung inflation on ventilation in the American bullfrog *Rana catesbeiana* Shaw. *J. Exp. Biol.* **204**, 2647-2656.

Soulier, V., Gestreau, C., Borghini, N., Dalmaz, Y., Cottet-Emard, J. M. and Pequignot, J. M. (1997). Peripheral chemosensitivity and central integration: neuroplasticity of catecholaminergic cells under hypoxia. *Comp. Biochem. Physiol.* **118A**, 1-7.

Taylor, A. C. and Kollros, J. J. (1946). Stages in the normal development of *Rana pipiens* larvae. *Anat. Rec.* **94**, 7-24.

Torgerson, C. S., Gdovin, M. J. and Remmers, J. E. (1998). Fictive gill and lung ventilation in the pre- and postmetamorphic tadpole brain stem. *J. Neurophysiol.* **80**, 2015-2022.

Vasilakos, K., Wilson, R. J., Kimura, N. and Remmers, J. E. (2005). Ancient gill and lung oscillators may generate the respiratory rhythm of frogs and rats. *J. Neurobiol.* **62**, 369-385.

Viemari, J. C. and Hilaire, G. (2002). Noradrenergic receptors and in vitro respiratory rhythm: possible interspecies differences between mouse and rat neonates. *Neurosci. Lett.* **324**, 149.

Viemari, J. C., Burnet, H., Bevengut, M. and Hilaire, G. (2003). Perinatal maturation of the mouse respiratory rhythm-generator: in vivo and in vitro studies. *Eur. J. Neurosci.* **17**, 1233-1244.

Wilson, R. J., Vasilakos, K., Harris, M. B., Straus, C. and Remmers, J. E. (2002). Evidence that ventilatory rhythmogenesis in the frog involves two distinct neuronal oscillators. *J. Physiol.* **540**, 557-570.

Winmill, R. E. and Hedrick, M. S. (2003). Developmental changes in the modulation of respiratory rhythm generation by extracellular K^+ in the isolated bullfrog brainstem. *J. Neurobiol.* **55**, 278-287.

Winzer-Serhan, U. H., Raymon, H. K., Broide, R. S., Chen, Y. and Leslie, F. M. (1996). Expression of α_2 adrenoceptors during rat brain development–I. α_{2A} messenger RNA expression. *Neuroscience* **76**, 241.