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Developmental changes in central O_2 chemoreflex in *Rana catesbeiana*: the role of noradrenergic modulation

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Summary

The *in vitro* brainstem preparation from *Rana catesbeiana* shows a functional central O_2 chemoreflex. Acute brainstem exposure to hypoxic superfusate elicits lung burst frequency responses that change over the course of development. Based on studies suggesting that brainstem noradrenergic neurons are involved in this reflex, we tested the following two hypotheses *in vitro*: (1) activation of adrenoceptors is necessary for the expression of the fictive lung ventilation response to hypoxia, and (2) changes in fast, Cl^--dependent neurotransmission (GABA/glycine) contribute to developmental changes in noradrenergic modulation. Experiments were performed on preparations from pre-metamorphics tadpoles (TK stages V–XIII) and adult bullfrogs. Acute exposure to hypoxic superfusate (98% N_2, 2% CO_2) increased fictive lung ventilation frequency in the pre-metamorphic group, whereas a decrease was observed in adults. Buccal burst frequency was unchanged by hypoxia. Noradrenaline (NA; 5 μmol l^{-1}) bath application mimicked both fictive breathing responses and application of the α_1-antagonist prazosine (0.5 μmol l^{-1}) blocked the lung burst response to hypoxia in both groups. Blocking $GABA_A$/glycine receptors with a bicuculine/strychnine mixture (1.25 μmol l^{-1}/1.5 μmol l^{-1}, respectively) or activation of $GABA_B$ pre-synaptic autoreceptors with baclofen (0.5 μmol l^{-1}) prevented the lung burst response to hypoxia and to the α_1-agonist phenylephrine (25 μmol l^{-1}) in both stage groups. We conclude that NA modulation contributes to the central O_2 chemoreflex in bullfrog, which acts *via* GABA/glycine pathways. These data suggest that maturation of GABA/glycine neurotransmission contributes to the developmental changes in this chemoreflex.

Key words: control of breathing, amphibian, GABA, chloride, bicarbonate.

Introduction

Noradrenaline (NA) is produced by several distinct groups of neurons within the central nervous system, where it exerts important modulatory influence on respiratory motor output. Several factors can activate NA neurons to elicit neurotransmitter release and data show that during hypoxia, activation of pontine noradrenergic neurons such the locus coeruleus (A6) and the A5 group contributes to the changes in respiratory activity in response to this condition. For instance, activation of LC neurons is necessary for the ventilatory depression observed during hypoxia in newborn lamb as brainstem transections or focal cooling of LC neurons abolished this response (Dawes et al., 1983; Moore et al., 1996). However, the mechanisms linking noradrenergic modulation and hypoxic ventilatory depression in newborn animals are not well understood (Bissonnette, 2000).

This ventilatory chemoreflex is well conserved amongst vertebrates, as exposing adult frogs to hypoxia also leads to ventilatory depression (Rose and Drotman, 1967). Furthermore, reducing O_2 levels of the artificial cerebrospinal fluid (aCSF) superfusing *in vitro* brainstems preparations decreases fictive lung ventilation frequency in *Rana catesbeiana* (Winmill et al., 2005) and newborn rat (Brockhaus et al., 1993). As this preparation is completely devoid of peripheral (sensory) inputs, these data show that this chemoreflex is of central origin. At this point, little is known about the neural mechanisms underlying the central O_2 chemoreflex in amphibians. While we have recently shown that, under standard (hyperoxic) conditions, NA bath application onto bullfrog brainstem preparations elicits fictive lung ventilation responses that are similar to those observed during hypoxia (Fournier and Kinkead, 2006), a direct involvement of noradrenergic modulation in the O_2 chemoreflex remains to be demonstrated in this species. With that in mind, the main objective of the present study was to better understand the mechanisms underlying the central O_2 chemoreflex by testing the hypothesis that noradrenergic receptor activation is necessary to observe a fictive lung ventilation response to hypoxia in the brainstem preparation from *Rana catesbeiana*. With this aim, we used selective pharmacological agents to block adrenoceptors prior to exposing brainstems to hypoxic aCSF.

An important aspect of the work of Winmill and colleagues (Winmill et al., 2005) is that the response to central hypoxia is stage-dependent, since the frequency change recorded from

brainstems originating from pre-metamorphic tadpoles was not as strong as the one from adult frogs. Given the lack of knowledge regarding this intriguing aspect of respiratory control development, our second objective was to address the mechanisms underlying maturation of the central O_2 chemoreflex. In bullfrogs, GABA is an another key modulator of neural activity that shows important stage-dependent effects on respiratory motor output that are similar to those observed during hypoxia (Broch et al., 2002). Based on the knowledge that (1) noradrenergic modulation of rhythmic motor behaviours such as locomotion and breathing can occur *via* indirect GABAergic pathways (Arata et al., 1998; Merrywest et al., 2002) and (2) the post-synaptic response following $GABA_A$ receptor activation changes substantially during development (Ben-Ari, 2002), we tested the hypothesis that developmental changes in noradrenergic modulation of fictive lung ventilation are due to maturation of (indirect) GABAergic pathways. This hypothesis was addressed using bath application of pharmacological agents interfering with GABAergic neurotransmission during selective adrenoceptor activation or hypoxia.

Materials and methods

Animals

Experiments were performed on 114 bullfrog *Rana catesbeiana* Shaw brainstem preparations, from tadpoles (mass range: 4.8–14.3 g) and adult frogs (mass range: 7.1–438 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 (photoperiod: 12 h:12 h light: dark). 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 anesthetised by immersion in a solution of tricaine methane sulfonate (MS-222: 0.06 g l^{-1}) buffered to pH 7 with $NaHCO_3$. For frogs, the beaker containing the MS-222 solution was placed on ice for 30–60 min to slow metabolism and ensure adequate anesthesia 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, 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), which range between stages I to XXV. According to these criteria, metamorphosis begins at stage XVIII. Animals between stages V and XIII were assigned to the pre-metamorphic group (N=57). 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 used in our previous studies (Kinkead et al., 1994; Kinkead et al., 2002; Fournier and Kinkead, 2006). For tadpoles, the aCSF consisted of (in mmol l^{-1}): 104 NaCl; 4 KCl; 1.4 $MgCl_2$; 10 D-glucose; 25 $NaHCO_3$; 2.4 $CaCl_2$, and for adult bullfrogs: 75 NaCl; 4.5 KCl; 1 $MgCl_2$; 7.5 D-glucose; 40 $NaHCO_3$; 2.5 $CaCl_2$; 1 NaH_2PO_4. The use of a higher bicarbonate concentration in the aCSF for adult bullfrogs is common (e.g. Winmill et al., 2005) and is based on the fact that (1) during bullfrog development, the transition from water to air breathing is associated with a respiratory acidosis, which is compensated by renal HCO_3^- retention (Just et al., 1973), (2) the blood–brain barrier of frog is permeable to HCO_3^- (Wright, 1972) and (3) increasing aCSF [HCO_3^-] results in a more stable respiratory activity in preparations from adult bullfrogs (Kinkead et al., 1994). The superfusate was equilibrated with a 98% O_2/2% CO_2 gas mixture to pH 7.90±0.15 for tadpoles and pH 7.8±0.15 for adult bullfrogs (Kinkead et al., 1994; Torgerson et al., 1997). 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 immobilised with insect pins. The dura matter and parts of the arachnoid (where possible) 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 i.d.) pulled to a fine tip with a vertical microelectrode puller (Stoelting Instrument, Wood Dale, IL, USA). The tip was broken and beveled 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; CWE, 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 analog 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, hyperoxic) 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 stabilise for 30–45 min, until stable rhythmic neural activity was recorded from both nerves. Since most of the drugs used are light-sensitive, drug preparation and experiments were conducted with dim lights. All drugs were obtained from Sigma/RBI Aldrich (St Louis, MO, USA).

Series I: the role of α-adrenoceptors in the hypoxic chemoreflex in vitro

To demonstrate that our preparations produced a hypoxic response similar to the one reported previously in this species

(Winmill et al., 2005), these experiments first compared the effects of acute brainstem superfusion with hypoxic aCSF on fictive breathing frequencies (both lung and buccal) between two distinct developmental stages groups: adult bullfrogs and pre-metamorphic tadpoles. For this series, the protocol began by recording respiratory-related motor output for 10 min. Meanwhile, a second aCSF reservoir was bubbled with a hypoxic gas mixture (98% N_2, 2% CO_2), which was then delivered to the preparation for 10 min. Hypoxia was followed by a recovery period, during which the preparation was superfused with drug-free, hyperoxic aCSF for a period ranging between 50 to 70 min before a final recording of respiratory-related motor output was made.

To determine whether activation of noradrenergic receptors is necessary for the central hypoxic chemoreflex, brainstrem preparations were superfused with a selective α-adrenoceptor antagonist prior to hypoxic exposure. Following 10 min of drug-free baseline recording, brainstem preparations were superfused with aCSF containing either the α_1 receptor antagonist prazosine (Pr; 0.5 μmol l^{-1}; pre-metamorphic, *N*=5; adult, *N*=6) or the α_2 receptor antagonist RX821002 (RX; 25 μmol l^{-1}; pre-metamorphic, *N*=10; adult, *N*=6) for 20 min to obtain a second baseline value in the presence of the antagonist. Following this equilibration period, the preparation was subjected to hypoxia for 10 min in the presence of antagonist before a 50 min 'drug-free' recovery period. Because we were concerned about potential carry-over effects, each preparation was exposed to one antagonist only. The choice of these pharmacological agents was based on our previous work showing that these receptors play a key role in the modulation of fictive lung ventilation (Fournier and Kinkead, 2006); doses were selected from other studies (e.g. Errchidi et al., 1991) and preliminary experiments.

Series II: developmental changes in noradrenergic modulation of fictive ventilation: the role of Cl^- inhibition

This series of experiments first established the stage-dependent effects of NA bath application on fictive breathing frequencies, as shown previously (Fournier and Kinkead, 2006). The protocol began by recording baseline (drug-free) respiratory-related motor output for 10 min before the preparation was superfused with aCSF from a second reservoir containing 5 μmol l^{-1} NA for 10 min (pre-metamorphic, *N*=6; adult, *N*=6). This procedure was followed by a 50–70 min wash out period under control conditions.

We then assessed the potential contribution of indirect GABAergic pathways in the noradrenergic modulation of respiratory activity across developmental stages. GABA and glycine are commonly co-released (Jonas et al., 1998; O'Brien and Berger, 1999), such that simultaneous application of bicuculline ($GABA_A$ antagonist) and strychnine (glycine antagonist) is necessary for efficient blockade of this inhibitory pathway (Jonas et al., 1998). Preliminary experiments confirmed that this was the case for our system also. Following baseline recording, we applied aCSF with a bicuculline/strychnine mixture for 30 min (concentration: 1.25 μmol l^{-1}/1.5 μmol l^{-1}, respectively) and obtained a second 'baseline' recording (in the presence of the antagonist mixture). The selection of these concentrations was based on other studies (Broch et al., 2002) and preliminary experiments, which confirmed that our preparations could still produce a motor output that was respiratory-like when the drugs were applied simultaneously. We then added NA (5 μmol l^{-1}; 10 min) to the aCSF in the presence of the bicuculline/strychnine mixture before a wash out period of 50–70 min was made with control aCSF.

The involvement of GABAergic/glycinergic pathways in NA modulation of fictive breathing and hypoxic chemoreflex was also tested using selective α-adrenoceptor agonists and hypoxic aCSF. In those experiments, preparations were superfused with aCSF containing the bicuculline/strychnine mixture before the α_1 receptor agonist phenylephrine (Phe; 25 μmol l^{-1}), the α_2 receptor agonist clonidine (Clo; 25 μmol l^{-1}) or hypoxia was applied to the brainstem (pre-metamorphic, *N*=6; adult, *N*=6, for all), according to the protocol described previously. The selection of the agonist concentration was based on dose–response curves performed previously (Fournier and Kinkead, 2006). Although $GABA_A$ and glycine receptors are highly selective Cl^- channels, HCO_3^- current (*via* $GABA_A$ receptors) affects GABA responses, especially in mature neurons (Yamada et al., 2004). To determine whether the use of a higher [HCO_3^-] in the aCSF used in adult frogs affected our results (especially experiments involving the bicuculline/strychnine mixture), these experiments were repeated by superfusing adult brainstems with the aCSF used in tadpoles (low [HCO_3^-]; *N*=4).

The effects of the bicuculline/strychnine mixture on the baseline bursting pattern were minimal (e.g. Fig. 4); however, the potential caveats associated with the fact that bicuculline blocks voltage-activated K^+ currents that help to set the resting potential and thus control spontaneous cell firing (Johansson et al., 2001; Druzin et al., 2004), brought us to consider an alternate approach. For these experiments, the $GABA_B$ receptor agonist baclofen (0.5 μmol l^{-1}) was added to the aCSF to activate presynaptic autoreceptors (and thus reduce endogenous GABA/glycine release). Following baseline measurements, the preparation was superfused with baclofen for 30 min before preparations were exposed to phenylephrine or hypoxia for 10 min as described previously (pre-metamorphic: Phe, *N*=5; hypoxia, *N*=6; adults: Phe, *N*=4; hypoxia, *N*=6). Note that because of results obtained in our previous work (Fournier and Kinkead, 2006) and other data showing that, unlike α_2-adrenoceptors, α_1-adrenoceptors are consistently involved in NA modulation of fictive lung ventilation and their activation is necessary for the O_2 chemoreflex in all developmental stages (Fig. 2), the baclofen experiments were performed with phenylephrine only. These procedures were followed by a 50 min recovery period under control (drug free) conditions. For these experiments, the baclofen concentration was based on the one reported by Straus et al. (Straus et al., 2000) and our preliminary experiments.

Data analysis

Fictive breathing frequency values for respiratory burst activity were obtained by analysing the last 3 min of activity for each condition (baseline, drug and/or hypoxia). *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 identify fictive lung and buccal bursts adequately (Sanders and Milsom, 2001). 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 within the 3-min segment analysed, and averaged for a 1-min period. Buccal burst frequency could not be quantified during bicuculline and strychnine application because they abolish the buccal ventilation (Galante et al., 1996; Broch et al., 2002).

All measurements are reported as the mean ± 1 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

Unlike mammals, frogs do not rely on an aspiration pump to ventilate their lungs. Instead, these amphibians use a buccal force pump to 'push' the gas exchange medium (water or air) over their gills and lungs. During early life, tadpoles can exploit both water and air to meet their gas exchange requirements, and both types of breathing movements are produced by the same buccal pump (Burggren and West, 1982). Although the sequence for recruiting the glottal and narial valves are distinct for water and air breathing, the buccal muscles activated for pumping are essentially the same for gill and lung ventilation; however, the neural commands driving both types of movements are different. The motor output driving gill ventilation is characterised by its high frequency and low amplitude. In contrast, the command driving air breathing is less frequent but has greater amplitude as lung ventilation requires more forceful contraction of the buccal pump to push air into the lungs (Gans et al., 1969; West and Jones, 1975). These patterns of neural activity are produced by the *in vitro* brainstem preparations from both preparations (brainstems from tadpoles and adult frogs), even though air breathing is infrequent in pre-metamorphic tadpoles (McLean et al., 1995; Liao et al., 1996; Broch et al., 2002; Fournier and Kinkead, 2006). Although adult frogs no longer ventilate their gills, the motor output driving this activity remains, and the buccal oscillations thus produced are hypothesised to play a role in olfaction and/or 'refreshing' gas contents in the buccal cavity prior to the next lung inflation. The trigeminal neurograms shown in Fig. 1 (lower traces) illustrate the bursting patterns associated with these types of breathing movements.

Series I

Stage-dependent effects of hypoxia on fictive lung frequency only

The trigeminal neurograms shown on Fig. 1 illustrate the effects of reducing P_{O_2} levels in the aCSF on the respiratory-related motor output produced by brainstem preparations from two distinct developmental stages. By the end of the hypoxic period, fictive lung burst frequency recorded from the pre-metamorphic group increased by 97% whereas a 31% decrease was observed in the adult group (hypoxia effect: P=0.005 and P=0.04, respectively; Fig. 1A). Statistical analysis confirmed that this response is stage-dependent (stage×hypoxia: P=0.001).

Hypoxia did not change buccal burst frequency in either group stage (hypoxia effect: P=0.79 and P=0.70, in pre-metamorphic and adult group, respectively; Fig. 1B). These data show that, in both groups, hypoxia had no effect on buccal burst frequency. Consequently, only data describing the effects of the pharmacological agents on 'baseline' buccal burst frequency are presented for conciseness.

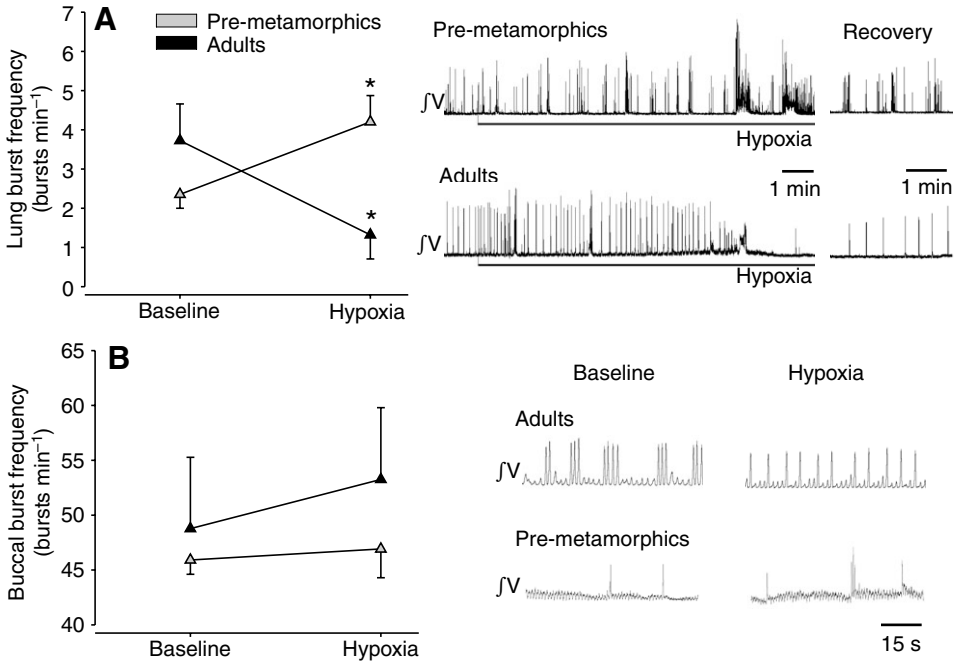

Fig. 1. The effects of exposing brainstem preparations to acute hypoxia on (A) lung burst frequency (N=7 in each group) and (B) buccal burst frequency. These experiments were performed on brainstems from pre-metamorphic tadpoles (grey) and adult bullfrogs (black). Representative trigeminal neurograms (∫V, trigeminal nerve) showing changes in fictive breathing during hypoxia are shown on the right. Note that the neurograms shown in B correspond to parts of those in A but on shorter time scale, to illustrate the two types of fictive breathing movements produced by this preparation. For these neurograms, the y-axis scales are the same in both panels. Values are means ± s.e.m. *Value statistically different from baseline at P<0.05.

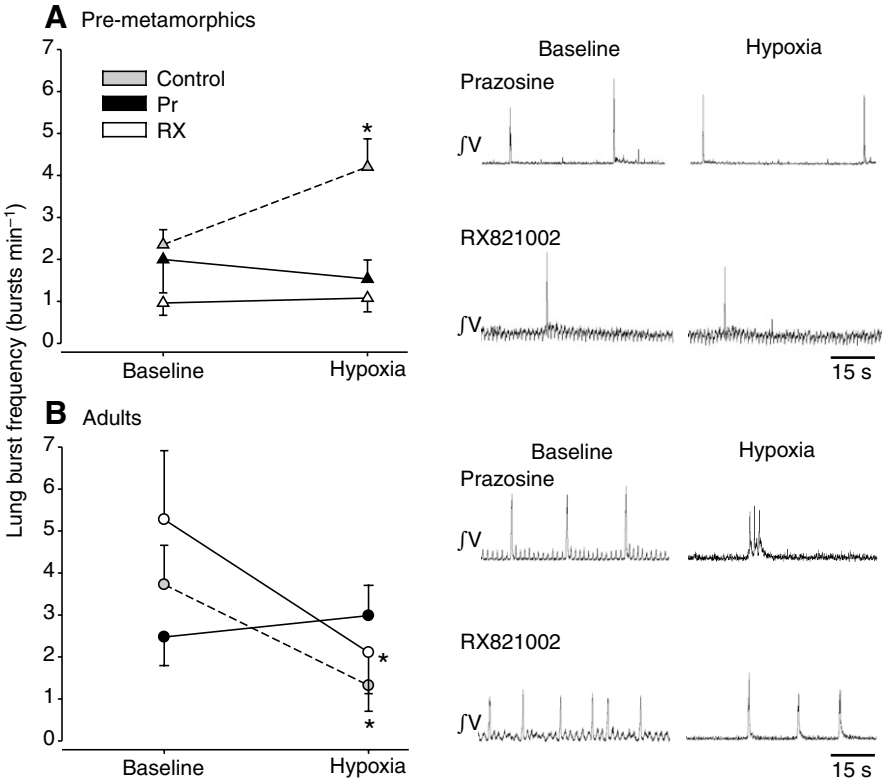

Fig. 2. (A,B) The effects of the selective α_1-adrenoceptor antagonist prazosine (Pr; 0.5 μmol l^{-1}) and the selective α_2-adrenoceptor antagonist RX821002 (RX; 25 μmol l^{-1}) on the fictive lung ventilation frequency response to hypoxia measured in (A) the pre-metamorphic (control, *N*=7; Pr, *N*=5; RX, *N*=10) and (B) adult groups (control, *N*=7; Pr, *N*=6; RX, *N*=6). Note that in these figures, the control data (grey symbols; broken line) was transposed from Fig. 1 to facilitate comparisons. Trigeminal neurograms (∫V, trigeminal nerve) showing changes in respiratory-related motor output under baseline and hypoxic conditions in the presence of the antagonists prazosine or RX 821002 in both stage groups are shown on the right. Values are means ± s.e.m. *Value statistically different from baseline at $P<0.05$.

α_1-adrenoceptor activation is necessary for manifestation of the fictive lung ventilation response to hypoxia

In the pre-metamorphic group, addition of the selective α_1-adrenoceptor antagonist prazosine to the aCSF did not alter baseline fictive lung ventilation frequency (drug effect: P=0.44; Fig. 2A). While this drug tended to decrease baseline frequency in the adults, the effect was not statistically significant (drug effect: P=0.33; Fig. 2B). During hypoxia, prazosine prevented the fictive lung burst frequency increase observed in the pre-metamorphic group (hypoxia×drug: P=0.007; Fig. 2A) whereas in adult frogs, this antagonist prevented the frequency decrease as this variable remained unchanged during the hypoxic stimulation period (hypoxia×drug: P=0.02; Fig. 2B). Statistical analysis confirmed that prazosine had a stage-dependent effect on the response to hypoxia (stage×hypoxia×drug: P=0.0007).

α_2-adrenoceptor activation is necessary for manifestation of the fictive lung ventilation response to hypoxia in pre-metamorphic group only

Addition of the selective α_2-adrenoceptor antagonist RX821002 to the aCSF did not alter baseline fictive lung ventilation frequency in either group (drug effect: P=0.43 and P=0.30, in pre-metamorphic and adult group respectively; Fig. 2). In the pre-metamorphic group, the increase in fictive lung burst frequency by hypoxia was blocked by application of RX821002 (hypoxia×drug: P=0.003; Fig. 2A). However, the decrease of fictive lung burst frequency caused by hypoxia was not blocked in the adult group (hypoxia×drug: P=0.60) and statistical analysis confirmed that this effect is stage-dependent (stage×hypoxia×drug: P<0.001; Fig. 2B).

Series II

GABA/glycine receptor activation is necessary for manifestation of the fictive lung ventilation response to noradrenergic receptor activation

In the pre-metamorphic group, application of NA (5 μmol l^{-1}) onto brainstem preparations increased fictive lung burst frequency (drug effect: P=0.003; Fig. 3A). Conversely, the same NA concentration applied onto brainstems from adults decreased fictive lung burst frequency (drug effect: P=0.02). Statistical analysis confirmed that, as we have shown previously (Fournier and Kinkead, 2006), the effects of NA on fictive lung ventilation are stage-dependent (stage×drug: P=0.0002). In both groups, the recovery period restored the fictive lung ventilation frequency values back to their initial (baseline) values (data not shown).

Addition of the bicuculline/strychnine mixture to the aCSF altered baseline fictive lung ventilation frequency in a stage-dependent manner (stage×drug: P<0.0001). In the pre-metamorphic group, lung burst frequency increased, whereas in the adult group, bath application of the mixture decreased lung burst frequency (drug effect: P=0.002 and P=0.03, respectively; Fig. 3B,C).

Addition of NA to the aCSF in the presence of the bicuculline/strychnine mixture decreased fictive lung ventilation frequency in the pre-metamorphic group (drug effect: P=0.04; Fig. 3B). Conversely, addition of the bicuculline/strychnine mixture prior to NA application prevented the decrease in fictive lung burst frequency normally observed in adults (drug effect: P=0.26; Fig. 3C). For both groups, fictive lung ventilation frequency returned to baseline values during the wash-out period (data not shown).

Addition of the selective α_1-adrenoceptor agonist Phe to the aCSF altered baseline fictive lung ventilation frequency in a stage-dependent manner (stage×drug: P=0.0003; Fig. 4A). In the pre-metamorphic group, application of Phe (25 μmol l^{-1}) onto brainstem preparations increased fictive lung burst frequency whereas application of the same concentration onto

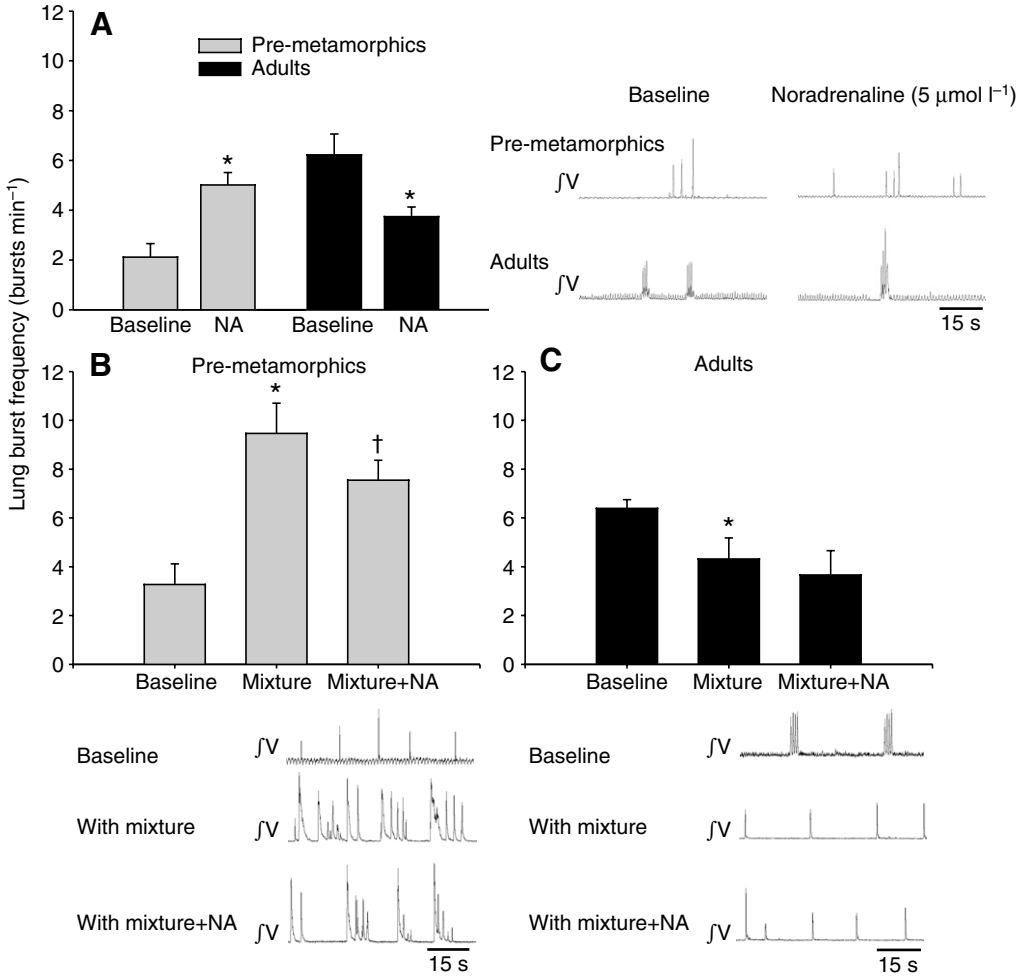

Fig. 3. (A) Stage-dependent changes in fictive lung burst frequency during noradrenaline (NA; 5 μmol l^{-1}) application. The histograms (left) show the mean data and the trigeminal neurograms (∫V, trigeminal nerve; right) show representative recordings obtained under baseline conditions and following NA bath application. Data are reported for both stage groups (pre-metamorphic: grey bars, *N*=6; and adults: black bars, *N*=6). (B,C) The same experiment was performed in the presence of the $GABA_A$/glycine antagonist mixture (bicuculline 1.25 μmol l^{-1}/strychnine 1.5 μmol l^{-1}) in both stage groups. For these experiments, representative trigeminal neurograms showing respiratory-related activity observed under each condition are shown below the histograms. Values are means ± s.e.m. *Values statistically different from baseline at $P<0.05$; †value statistically different from corresponding mixture value at $P<0.05$.

adult preparations decreased it (drug effect: $P=0.005$ and $P=0.016$, respectively; Fig. 4A). Fictive lung ventilation frequency returned to baseline values during the wash-out period (data not shown).

Similar to previous experiments (Fig. 3B,C), bath application of the bicuculline/strychnine mixture alone had stage-dependent effects on baseline lung burst frequency (stage×drug: $P<0.0001$; Fig. 4B,C). In both groups, this treatment prevented changes in lung burst frequency related to subsequent Phe application (drug effect: $P=0.63$ and 0.82, in pre-metamorphic and adult groups, respectively; Fig. 4A,B). Fictive lung ventilation frequency returned to baseline values during the wash-out period in the pre-metamorphic group; however, this was not the case for the adults in which a frequency increase was observed (data not shown).

Bath application of the selective α_2-adrenoceptor agonist Clo increased fictive lung burst frequency in both stage groups (drug effect: $P=0.017$ and $P=0.002$, respectively; Fig. 5A); however, this response was prevented by pre-treatment with the bicuculline/strychnine mixture. Addition of Clo to the aCSF containing bicuculline/strychnine decreased fictive lung burst frequency in pre-metamorphic group, but had no further effect in preparations from adult bullfrogs ($P=0.004$ and $P=0.77$ respectively; Fig. 5A,B). Fictive lung burst frequency returned to baseline values during the wash-out period in both groups (data not shown).

Stage-dependent effects of GABA/glycine receptor blockade on the fictive lung ventilation response to hypoxia

Unlike control (drug-free) conditions (Fig. 1), acute exposure to hypoxia in presence of the bicuculline/strychnine mixture decreased fictive lung burst frequency in preparations from pre-metamorphic animals. In the adults, however, these agents failed to prevent the lung burst frequency depression normally observed during hypoxia (hypoxia effect: $P=0.001$ and $P=0.005$, respectively; Fig. 6A,B). Thus in these experiments,

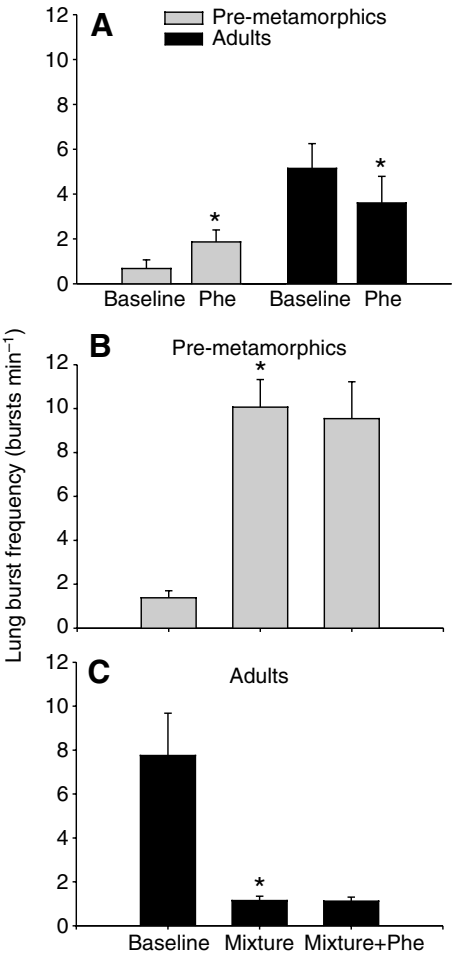

Fig. 4. (A) Stage-dependent changes in fictive lung burst frequency during bath application of the α_1-adrenoceptor agonist phenylephrine (Phe; 25 μmol l^{-1}). (B,C) The effects of Phe application on lung burst frequency in the presence of the $GABA_A$/glycine antagonist mixture (bicuculline 1.25 μmol l^{-1}/strychnine 1.5 μmol l^{-1}) in (B) the pre-metamorphic and (C) adult groups (N=6, each group). Values are means ± s.e.m. *Values statistically different from baseline at P<0.05.

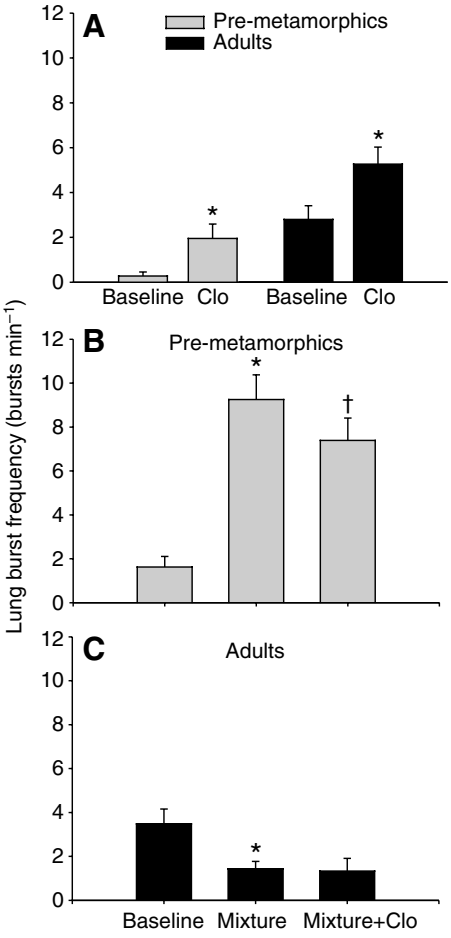

Fig. 5. (A) Stage-dependent changes in fictive lung burst frequency during bath application of the α_2-adrenoceptor agonist clonidine (Clo; 25 μmol l^{-1}). (B,C) The effects of Clo application on lung burst frequency in the presence of the $GABA_A$/glycine antagonist mixture (bicuculline 1.25 μmol l^{-1}/strychnine 1.5 μmol l^{-1}) in (B) the pre-metamorphic and (C) adult groups (N=6, each group). Values are means ± s.e.m. *Values statistically different from baseline at P<0.05; †value statistically different from corresponding mixture value at P<0.05.

the lung burst frequency response was not stage-dependent (hypoxia×drug×stage: P=0.13). In adults, repeating these experiments with low bicarbonate (tadpole) aCSF produced opposite effects: exposing adult brainstems to hypoxia in the presence of the bicuculline/strychnine mixture had no effect on lung burst frequency (hypoxia effect: P=0.66). ANOVA confirmed that, in adult frogs, the hypoxic response observed in the presence of the strychnine/bicuculline mixture was influenced by aCSF HCO_3^- concentration (hypoxia×[HCO_3^-]: P=0.014). From these results, we needed to ensure that under control conditions, developmental changes in the lung burst frequency response to hypoxia is not related to the aCSF used for each stage group (low *versus* high [HCO_3^-] for pre-metamorphics and adults, respectively). To do so, two adult brainstems were superfused with control (drug-free) tadpole aCSF (low [HCO_3^-]) under baseline and hypoxic condition. Both preparations showed a lung burst frequency decrease (85% and 54%, respectively) that is well within the response range observed when adult preparations are superfused with the appropriate aCSF (high [HCO_3^-]).

Baclofen blocks lung burst frequency response to α_1-adrenoceptor activation and hypoxia

Application of a bicuculline/strychnine mixture often disrupts basal bursting pattern, which makes data analysis difficult. Although these effects on the baseline bursting pattern were minimal (e.g. Fig. 4), the potential caveats associated with the fact that bicuculline blocks voltage-activated K^+ currents, which help to set the resting potential and thus control spontaneous cell firing (Johansson et al., 2001; Druzin et al., 2004), led us to consider an alternative approach. For these experiments, the $GABA_B$ receptor agonist baclofen (0.5 μmol l^{-1}) was added to the aCSF to activate presynaptic autoreceptors (and thus reduce endogenous GABA/glycine release).

Despite suggestive trends, application of low concentrations of the selective $GABA_B$ agonist baclofen had no significant

effects on lung burst frequency in either stage group (drug effect: P=0.09 and P=0.19, in pre-metamorphic and adult groups, respectively; Fig. 7A,B). However, this drug effectively blocked the lung burst response to Phe application in both groups as preparations pre-treated with baclofen maintained the same fictive lung frequency (Phe×drug: P=0.004 and 0.01, in pre-metamorphic and adult group, respectively; Fig. 7).

In the next experiment, baclofen application did not change baseline lung burst frequency in either group (drug effect: P=0.82 and P=0.75, in pre-metamorphic and adult groups, respectively) but blocked the lung burst frequency response to hypoxia (hypoxia×drug: P=0.001 and P=0.03, in pre-metamorphic and adult group, respectively; Fig. 8).

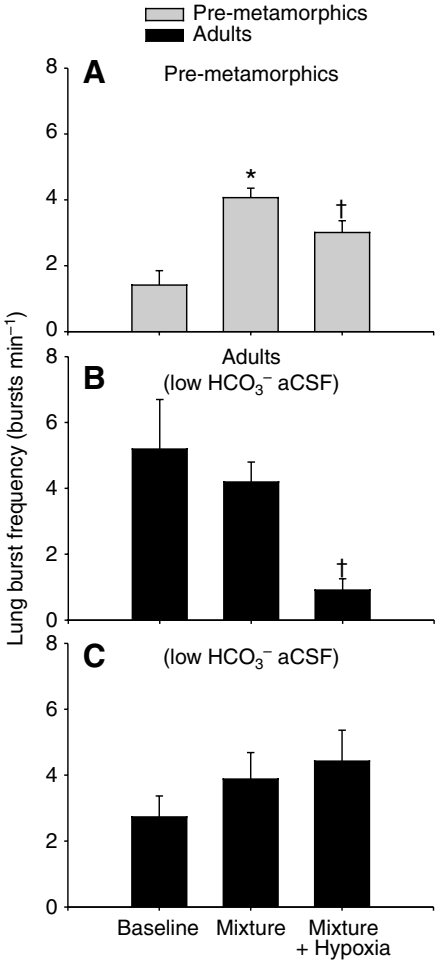

Fig. 6. Effects of $GABA_A$/glycine antagonist mixture (bicuculline 1.25 μmol l^{-1}/strychnine 1.5 μmol l^{-1}) bath application on lung burst frequency under 'baseline' and hypoxic conditions. The histograms show lung burst frequency measured at the end of the 10 min hypoxic period in the presence of the antagonist mixture. These experiments were performed on preparations from pre-metamorphic tadpoles (A; grey bars, N=6) and adult bullfrogs (B; black bars, N=6). (C) In adults, these experiments were also performed using low [HCO_3^-] aCSF (tadpole) (N=4) to determine whether the composition of the aCSF contributes to the effect observed in B. Values are means ± s.e.m. *Value statistically different from baseline at P<0.05; †values statistically different from corresponding mixture values at P<0.05.

Fictive buccal ventilation response to pharmacological agents

NA bath application (5 μmol l^{-1}) onto brainstem preparations from pre-metamorphic tadpoles increased fictive buccal burst frequency (drug effect: P=0.10; Fig. 9A) whereas application of the same NA concentration onto brainstems from adult bullfrogs had no effect on this variable (drug effect: P=0.29; Fig. 9A). These effects were stage-dependent (stage×drug: P=0.05); however, these data should be interpreted with care since not all preparations from adults could produce a reliable buccal-related signal. In both groups, the wash out period restored the fictive lung ventilation frequency values back to their initial (baseline) values (data not shown).

As expected (Broch et al., 2002), addition of the bicuculline/strychnine mixture to the aCSF abolished buccal burst frequency in both groups (drug effect: P=0.0002 and P=0.008, pre-metamorphic and adult respectively; Fig. 9B), and subsequent addition of NA had no effect (data not shown).

Addition of Phe (25 μmol l^{-1}) to the aCSF did not alter fictive buccal ventilation frequency in either stage group (drug effect: P=0.93 and P=0.49, pre-metamorphic and adult, respectively; Fig. 9C). Fictive buccal ventilation frequency returned to baseline values during the wash out period (data not shown). Moreover, Clo application (25 μmol l^{-1}) to the aCSF decreased fictive buccal ventilation frequency in both stage groups (drug effect: P=0.025 and 0.004, respectively; Fig. 9D). Effects on fictive buccal ventilation were not stage-dependent (stage×drug: P=0.95) and were reversed in both groups (data not shown). Addition of Clo in the presence of the bicuculline/strychnine mixture did not restore fictive buccal related activity (data not shown). Hypoxia alone had no effect on fictive buccal activity in either group (Fig. 9E); however, addition of the bicuculline/strychnine mixture to the aCSF abolished fictive buccal activity in both groups (drug effect: P=0.0001) and the hypoxia period could not initiate this activity (data not shown).

Discussion

We used *in vitro* brainstem preparations from *Rana catesbeiana* tadpoles and adult bullfrogs to address development of the neural mechanisms underlying the fictive ventilatory responses to brainstem hypoxia. Our results show that the fictive lung ventilation response to central hypoxia exhibits the same developmental changes as those seen with noradrenergic modulation, and that noradrenergic modulation (*via* α-adrenoceptors) is necessary to central O_2 chemoreflex function (Fig. 2). However, the modulatory influence that NA exerts onto the neural network generating fictive lung ventilation likely acts indirectly *via* GABAergic/glycinergic pathways, as drugs interfering with this neurotransmission prevent the effects of noradrenergic agonists as well as the lung burst frequency response to hypoxia in both stage groups (Figs 3–8). With development, the effects of GABA/glycine change from excitatory to inhibitory owing to the progressive establishment of Cl^- gradients in target neurons (Ben-Ari, 2002), and our data strongly suggest that this maturation process contributes to the developmental change of the central hypoxic

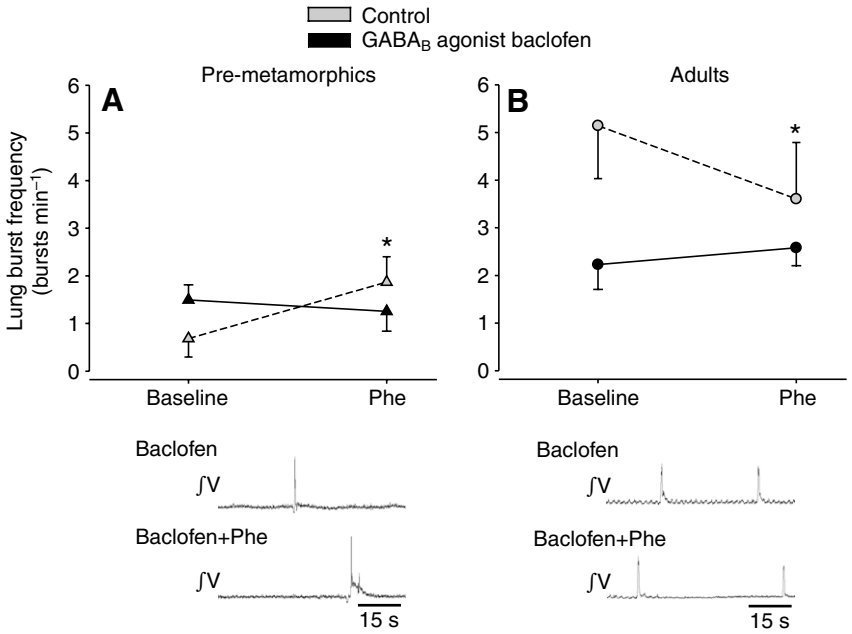

Fig. 7. Effects of bath application of the selective $GABA_B$ agonist baclofen (0.5 μmol l^{-1}) on lung burst frequency response to application of the α_1-adrenoceptor agonist phenylephrine (Phe; 25 μmol l^{-1}) in (A) the pre-metamorphic (*N*=5) and (B) adult groups (*N*=4). To facilitate comparisons, control data from both stage groups (grey symbols, broken lines; *N*=6 in each group) were transposed from Fig. 4. Trigeminal neurograms (∫V, trigeminal nerve) presented below show representative respiratory-related activity recorded under each condition. Values are means ± s.e.m. *Values statistically different from baseline at $P<0.05$.

chemoreflex also. Although the present work provides new information pertaining to the mechanisms underlying this chemoreflex and its maturation, it is not possible at this point to determine whether NA neurons are chemosensors *per se* or whether they are simply part of the neural pathway generating this response.

Critique of method

Frogs encounter severe hypoxic conditions, for example during estivation and overwintering. However, such conditions are infrequent and animals rarely encounter hypoxia levels similar to the one used in our study. Although the use of less severe hypoxia may be more physiologically relevant, we chose this level mainly to reproduce previous *in vitro* studies (Brockhaus et al., 1993; Winmill et al., 2005). Even so, results obtained in adult bullfrog brainstems were similar to the hypoxic responses reported in intact frogs (Rose and Drotman, 1967), newborn lambs (Dawes et al., 1983; Moore et al., 1996) and newborn rats *in vitro* (Brockhaus et al., 1993). This protocol allowed us to note that the fictive breathing response to central hypoxia is restricted to lung ventilation as fictive buccal movements were not affected by this stimulus. This is a key observation because this result further distinguishes the mechanisms regulating these two types of respiratory related motor outputs (Vasilakos et al., 2005; Janczewski and Feldman, 2006). It also indicates that in adults, decrease in fictive lung ventilation is not related to a non-specific depression of CNS function.

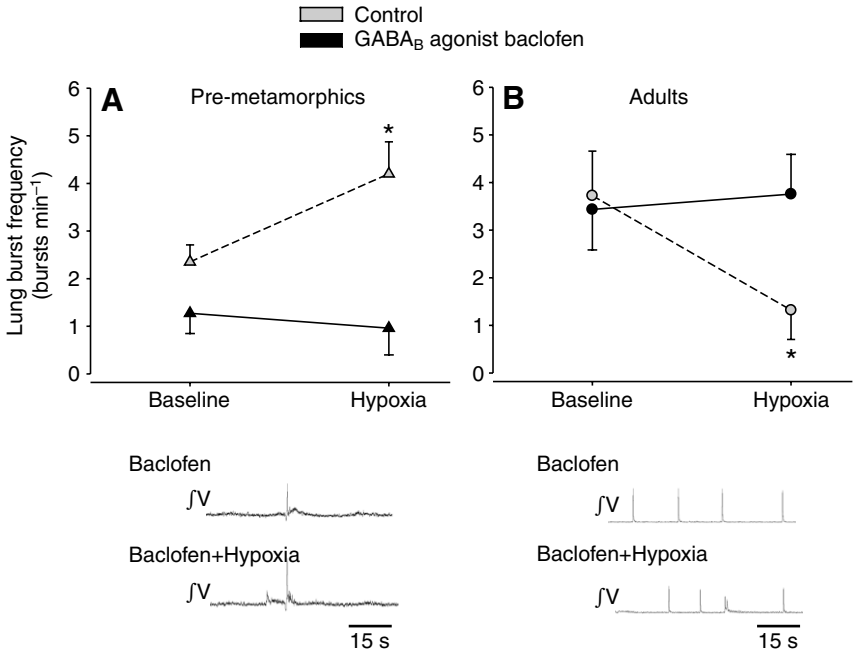

Fig. 8. Effects of the selective $GABA_B$ agonist baclofen (0.5 μmol l^{-1}) on the lung burst frequency responses to hypoxia in (A) the pre-metamorphic group and (B) adult group. Responses were measured under control (drug-free) conditions (*N*=7, in each group) and in the presence of baclofen (*N*=6, in each group). Note that in these figures, the control data (grey symbols, broken lines) were transposed from Fig. 1 to facilitate comparisons. Trigeminal neurograms (∫V, trigeminal nerve) presented below show representative respiratory-related activity recorded under each condition. Values are means ± s.e.m. *Values statistically different from baseline at $P<0.05$.

The role of α-adrenoceptors in the hypoxic chemoreflex in bullfrog brainstems

Overall, our results are consistent with those reported by Winmill and collaborators (Winmill et al., 2005) as we showed that hypoxia affects fictive lung ventilation in a stage-dependent manner. Hypoxia decreased fictive lung ventilation frequency in the adult group, but caused a modest increase in lung burst frequency in pre-metamorphic brainstems. For reasons that are unclear to us, the latter response differs slightly from the one reported by these authors who observed no increase in lung burst frequency during hypoxia (Winmill et al., 2005). However, the stage-dependent lung burst frequency responses to hypoxia in the present study were similar to those observed following NA bath application (Fournier and Kinkead, 2006), which constitutes circumstantial evidence to support the hypothesis that NA is involved in the central hypoxic chemoreflex. But given that prazosine or RX821002 application was sufficient to block the increase in fictive lung burst frequency in the pre-metamorphic group, the sum of these data lead us to conclude that manifestation of the central hypoxic chemoreflex requires α-adrenoceptor activation. These results contrast with those observed in adults in which only prazosine (not RX821002) effectively blocked lung burst frequency depression during hypoxia, thus indicating that only α_1-adrenoceptor activation mediates the hypoxic response in this group. Reduction in α_2-adrenoceptor expression with maturation may explain why these receptors no longer contribute to this response in the adults; however, we have no direct evidence in that regard. These data are nonetheless consistent with the lung burst frequency response to α-adrenoceptor agonist application because only in pre-metamorphic brainstems can the α_2-agonist clonidine mimic the lung burst frequency change observed during NA application (Fournier and Kinkead, 2006).

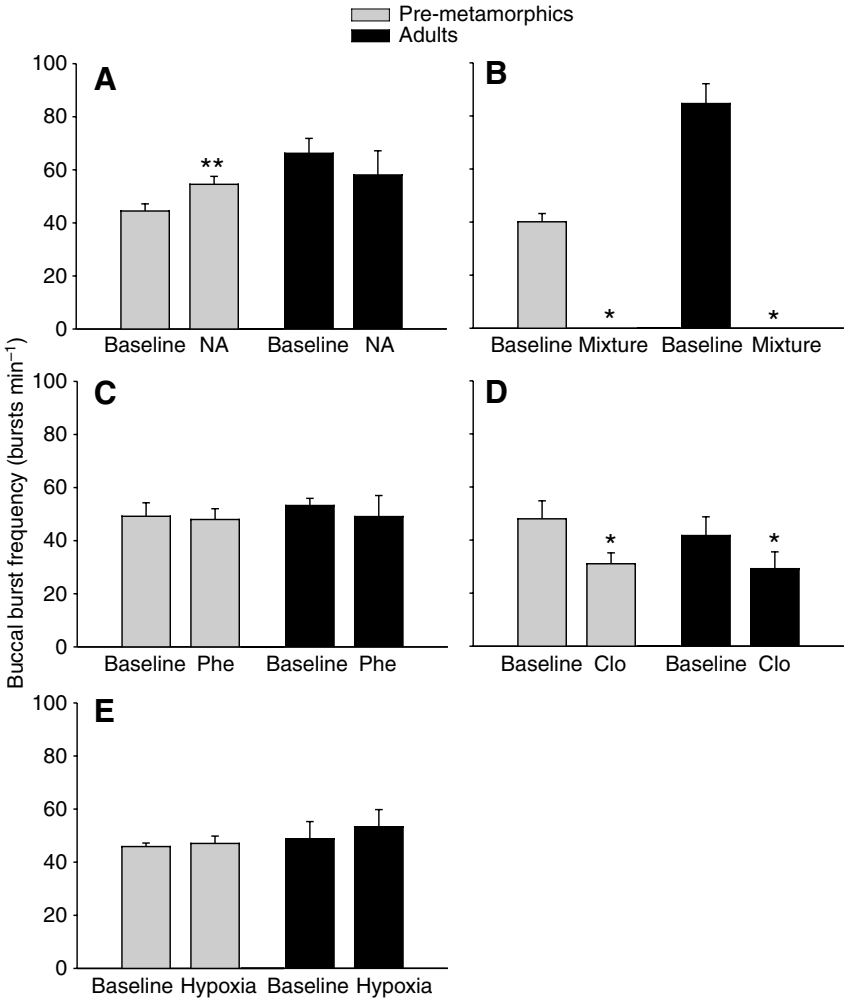

Fig. 9. Buccal burst frequency data obtained during each series of experiments for brainstem preparations from pre-metamorphic tadpoles (grey bars) and adult bullfrogs (black bars). Data were obtained before and after application of (A) noradrenaline (NA; 5 μmol l^{-1}), (B) $GABA_A$/glycine antagonist mixture (bicuculline 1.25 μmol l^{-1}/ strychnine 1.5 μmol l^{-1}), (C) the α_1-adrenoceptor agonist phenylephrine (Phe; 25 μmol l^{-1}), (D) the α_2-adrenoceptor agonist clonidine (Clo; 25 μmol l^{-1}), or (E) aCSF hypoxia. Note that application of the bicuculine/strychnine mixture abolished fictive buccal burst frequency in all preparations (only data from NA experiments are shown), and this could not be restored by any experimental treatment. Values are means ± s.e.m. *Values statistically different from baseline at $P<0.05$; **values statistically different from baseline at $P<0.10$.

Indirect GABAergic/glycinergic pathways mediate NA modulation of fictive lung ventilation

Our hypothesis that NA modulation of fictive lung ventilation acts *via* indirect (GABAergic/glycinergic) pathways is based on previous work showing that such interaction effectively modulates rhythmic motor behaviours. For instance, Arata and collaborators (Arata et al., 1998) showed that in the medulla-spinal cord preparation from newborn rat, NA depressed Pre-I rhythm and rhythmic C4 respiratory output in a standard perfusate. However, the direct effect of NA on Pre-I neuron firings in Cl^--free solution was excitatory, suggesting that the respiratory rhythm depression in normal conditions was mediated by another inhibitory system. This interpretation was confirmed by using a $GABA_A$ antagonist that attenuated the depression observed following NA application (Arata et al., 1998). Such organisation seems highly conserved amongst vertebrates since in *Xenopus laevis*, blocking $GABA_A$ and glycine receptors prior to α-adrenoceptor activation prevents changes in the spinal locomotor output recorded *in vitro*. These results indicate that GABAergic/glycinergic pathways are necessary for NA to modulate fictive swimming in this species (Merrywest et al., 2002).

Our results are consistent with those previous reports since $GABA_A$ and glycine receptor blockade prior to NA agonist application prevented changes in lung burst frequency. Moreover, the use of baclofen to attenuate endogenous

GABA/glycine release corroborated these results. But more importantly in the present context, both approaches were effective in all stage groups. Together, these results support our hypothesis that developmental changes in GABA/glycine neurotransmission are involved in developmental changes in noradrenergic neuromodulation and fictive lung ventilation response to central hypoxia.

GABAergic/glycinergic pathways are involved in central hypoxic chemoreflex

Hypoxia increases GABA concentration in brain tissues as a function of the severity and duration of hypoxia (Wood et al., 1968). This response affects ventilatory activity because $GABA_A$ receptor blockade prior to hypoxic exposure attenuated ventilatory depression (Miller et al., 2000). Despite species and preparation differences, our results showing that the lung burst frequency increase observed during hypoxia is counteracted by the bicuculline/strychnine mixture are consistent with these studies and the hypothesis that GABAergic/glycinergic neurotransmission is involved in the central O_2 chemoreflex. However, this interpretation must be made cautiously since in the present study, the hypoxic response obtained in the adult group was influenced by aCSF [HCO_3^-] concentration. Our experiments do not allow us to explain why in the adult group, application of the bicuculline/strychnine mixture could block the hypoxic response under low aCSF [HCO_3^-] condition only. This result was surprising because under standard (high [HCO_3^-]) conditions, this antagonist mixture effectively blocked all lung burst responses to NA agonist application. The bicuculline/strychnine concentration used may not have been sufficient to prevent GABA/glycine receptor activation that occurs during hypoxia; however, using a higher bicuculline/strychnine concentration was not possible given the effects on bursting pattern. Incomplete GABA/glycine receptor blockade, combined with the fact that severe hypoxia commonly causes intracellular acidosis, would favor inward HCO_3^- current *via* $GABA_A$ receptors and cell hyperpolarisation. Such a situation would not occur under low [HCO_3] aCSF condition. This explanation is speculative but consistent with the fact that HCO_3^- currents affect GABA responses, especially in mature neurons (Yamada et al., 2004). Accordingly, this suggests that during metamorphosis, the renal compensation of respiratory acidosis (*via* HCO_3^- retention) provoked by the transition from water to air breathing plays an important role in respiratory control maturation in this species.

The limitations inherent to the use of bicuculline/strychnine for such studies are well documented. For instance, bicuculline disrupts lung bursting pattern, abolishes all buccal-related activity, and blocks voltage-activated K^+ currents (Johansson et al., 2001; Broch et al., 2002; Druzin et al., 2004). Based on this, we used an alternate approach by applying the $GABA_B$ agonist baclofen to activate pre-synaptic autoreceptors and attenuate GABA (and glycine) release from nerve terminals (Harrison et al., 1988). Unlike the bicuculline/strychnine mixture, baclofen did not disrupt baseline bursting pattern, had minimal effects on buccal activity, and effectively prevented the hypoxic response in both stage groups under experimental conditions that mimic physiological CSF [HCO_3^-]. In light of these results, we conclude that, in this preparation, activation of GABAergic/glycinergic pathways is necessary to elicit a reflexive lung burst frequency response to central hypoxia.

Perspectives

Studies using brainstem preparations from newborn mammals have shown that activation of noradrenergic neurons can exert opposite effects on phrenic burst frequency, depending on which group of neurons were activated (A5: inhibitory *versus* A6: excitatory) (Hilaire et al., 2004). These observations are difficult to reconcile given that both groups of NA neurons converge on the same neural circuits that generate respiratory rhythm (Dobbins and Feldman, 1994). There are distinctions in CNS organisations between amphibians and mammals; however, our demonstration that NA acts *via* indirect pathways provides clues to the NA paradox reported in mammals. It is possible that, in mammals, one pathway (e.g. A5) acts *via* GABAergic interneurons whereas A6 neurons act directly. Clearly, more work needs to be done to address this issue.

The functional significance of the central O_2 chemoreflex is not intuitive. In pre-metamorphic tadpoles, for instance, increasing lung ventilation frequency during hypoxia appears futile because lungs are not fully developed. However, lung inflation is a stimulus that facilitates lung development. As such, the central O_2 chemoreflex could contribute (albeit indirectly) to lung development. On the other hand, most mature air breathing animals tend to increase lung ventilation during hypoxia, a response that is mainly mediated by peripheral chemoreceptors. Given the energy cost associated with hyperventilation, this response may not be optimal under conditions of reduced O_2 availability. It is therefore possible that the central inhibitory response to hypoxia aims to counterbalance the excitatory input from peripheral chemosensory structures to produce a more cost efficient response.

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