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Development of the respiratory response to hypoxia in the isolated brainstem of the bullfrog *Rana catesbeiana*

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Summary

The aim of this study was to examine the effects of cellular hypoxia, and the contribution of anaerobic metabolism, on respiratory activity in bullfrogs at different stages of development. Respiratory-related neural activity was recorded from cranial nerve rootlets in isolated brainstem preparations from pre-metamorphic (Taylor–Köllros (T-K) stages VIII-XVI) and post-metamorphic tadpoles (T-K stages XXIV-XXV) and adults. Changes in fictive gill/lung activity in brainstems from pre-metamorphic tadpoles and lung activity in post-metamorphic tadpoles and adults were examined during superfusion with control (98% O_2/2% CO_2) or hypoxic (98% N_2/2% CO_2) artificial cerebrospinal fluid (aCSF). Iodoacetate (IAA; 100 μmol l^{-1}) was used in conjunction with hypoxic aCSF to inhibit glycolysis. Gill burst frequency in pre-metamorphic brainstems did not change over a 3 h exposure to hypoxia and fictive lung burst frequency slowed significantly, but only after 3 h hypoxia. Blockade of glycolysis with IAA during hypoxia significantly reduced the time respiratory activity could be maintained in pre-metamorphic, but not in adult, brainstems. In brainstems from post-metamorphic tadpoles and adults, lung burst frequency became significantly more episodic within 5–15 min hypoxic exposure, but respiratory neural activity was subsequently abolished in every preparation. The cessation of fictive breathing was restored to control levels upon re-oxygenation. Neither tadpole nor adult brainstems exhibited changes in neural bursts resembling 'gasping' that is observed in mammalian brainstems exposed to severe hypoxia. There was also a significant increase in the frequency of 'non-respiratory' bursts in hypoxic post-metamorphic and adult brainstems, but not in pre-metamorphic brainstems. These results indicate that pre-metamorphic tadpoles are capable of maintaining respiratory activity for 3 h or more during severe hypoxia and rely to a great extent upon anaerobic metabolism to maintain respiratory motor output. Upon metamorphosis, however, hypoxia results in significant changes in respiratory frequency and pattern, including increased lung burst episodes, non-ventilatory bursts and a reversible cessation of respiratory activity. Adults have little or no ability to maintain respiratory activity through glycolysis but, instead, stop respiratory activity until oxygen is available. This 'switch' in the respiratory response to hypoxia coincides morphologically with the loss of gills and obligate air-breathing in the post-metamorphic frog. We hypothesize that the cessation of respiratory activity in post-metamorphic tadpoles and adults is an adaptive, energy-saving response to low oxygen.

Key words: amphibian, bullfrog, *Rana catesbeiana*, development, tadpole, respiratory rhythm generation, iodoacetate

Introduction

Many ectothermic vertebrates, including fish, turtles and amphibians, have evolved a remarkable tolerance to bouts of severe hypoxia or anoxia, an adaptation that allows for overwintering in hypoxic, ice covered ponds for several months (Hochachka and Lutz, 2001). The crucian carp *Carassius carassius* and freshwater turtle *Trachemys scripta* have brains that are capable of surviving days of anoxia at room temperature, and months of anoxia at temperatures near 0°C (Nilsson, 2001; Hochachka and Lutz, 2001). A number of cellular mechanisms are known to account for the extreme hypoxia tolerance of carp and turtles including a switch to anaerobic metabolism to maintain activity in carp (Nilsson, 2001) and extreme downregulation of neuronal processes to reduce ATP demand in turtles (Lutz and Nilsson, 2004). Some of the reductions in cellular processes include, but are not limited to, downregulation of specific ion channels (Pérez-Pinzón et al., 1992; Bickler et al., 2000) and 'spike arrest' (Sick et al., 1993) of neuronal electrical activity. Indeed, brain electrical activity in the turtle brain is reduced by about tenfold after 1 h exposure to anoxia (Fernandes et al., 1997). These strategies would be expected to affect all neuronal processes in the brain, but little attention has been focused on the effects of hypoxia on respiratory motor activity as an index of brain function. Recent studies indicate that, at least in the short term,

respiratory activity in carp (Stecyk and Farrell, 2002) and respiratory motor output in the isolated turtle brainstem (Johnson et al., 1998) is maintained during severe hypoxia, despite the reduction in electrical activity (Fernandes et al., 1997).

Amphibians are clearly less tolerant of severe hypoxia than turtles and carp, but are capable of surviving bouts of anoxia for 4–5 h at room temperature (Knickerbocker and Lutz, 2001) and more than a day at 5°C (Hermes-Lima and Storey, 1996). Brain ATP levels in the hypoxic frog gradually decline and when ATP reaches about 30% of normoxic levels, there is an increase in extracellular K^+, indicative of a loss of ionic homeostasis signaling the onset of neuronal death (Lutz and Nilsson, 2004). The progressive changes in brain ATP levels and changes in ionic homeostasis are also likely to compromise respiratory motor activity in amphibians, but this has not been examined in any detail. In the adult grass frog *Rana pipiens*, anoxia results in a progressive decline in respiratory activity and a complete cessation of breathing in about 30 min (Rose and Drotman, 1967).

Most studies in amphibians that have examined the effect of hypoxia on ventilation have used more moderate levels of hypoxia (10–15% O_2). At these levels of oxygen, both larval and adult amphibians increase ventilation to maintain oxygen homoestasis (Burggren and Doyle, 1987; Kruhøffer et al., 1987; Smatresk and Smits, 1991). Larval (tadpole) amphibians increase gill ventilation and lung ventilation, a response that changes with development (West and Burggren, 1982; Burggren and Doyle, 1987). For example, early stage tadpoles show large increases in gill ventilation in response to hypoxia, but as development proceeds, larger increases in lung ventilation occur relative to gill ventilation (Burggren and Doyle, 1987). These shifts in the response to hypoxia presumably reflect the increasing reliance on lung ventilation for oxygen acquisition prior to metamorphosis.

Following metamorphosis, when the gills involute and the post-metamorphic frog becomes an obligate air-breather, the response to hypoxia is an overall increase in lung ventilation frequency and amplitude, but also a distinct change in the respiratory pattern to include an increase in episodic breathing (Torgerson et al., 1998; Kinkead and Milsom, 1994). Preventing oscillations in blood gases does not prevent the production of lung episodes (Smatresk and Smits, 1991; Kinkead and Milsom, 1994), suggesting that episodic breathing is an intrinsic feature of the amphibian brainstem respiratory oscillator. However, the mechanisms that stimulate the increase in episodic breathing during hypoxia are unclear.

The *in vitro* amphibian brainstem model has recently been used in a number of studies examining developmental aspects of respiratory rhythm generation (Gdovin et al., 1999; Broch et al., 2002; Kinkead et al., 2002; Winmill and Hedrick, 2003a,b). The amphibian model provides the unique advantage of direct developmental comparisons, since spontaneous respiratory motor output can be recorded at all developmental stages. In addition, the presence of spontaneously generated respiratory activity provides an index of efferent motor output from the brainstem, thereby allowing the effects of brain hypoxia to be directly examined without the influence of peripheral oxygen chemoreceptors. Thus, this model may provide useful insight into the mechanisms and development of the respiratory response to hypoxia. The aim of the present study was to characterize the respiratory response to hypoxia in the *in vitro* bullfrog brainstem at different stages of development, and to determine the contribution of anaerobic metabolism to fictive breathing during hypoxia.

Materials and methods

Animals

Experiments were performed on a total of 12 pre-metamorphic (mean body mass, 8.1 g), 5 post-metamorphic (mean body mass, 4.9 g) and 13 adult (mean body mass, 227 g) North American bullfrogs *Rana catesbeiana* Shaw of either sex. Tadpoles were classified according to the staging criteria of Taylor and Köllros (1946) (T-K). Pre-metamorphic tadpoles ranged from T-K stages VIII-XVI; post-metamorphic tadpoles were T-K stages XXIV-XXV. Animals were purchased from a commercial supplier (Charles D. Sullivan Co., Inc.; Nashville, TN, USA). Tadpoles were kept in fiberglass aquaria with aerated, dechlorinated tapwater; adults were maintained in plastic tanks with continuous access to water. All animals were maintained at room temperature (20–23°C). All experimental procedures were approved by the CSUH Institutional Animal Care and Use Committee.

In vitro *brainstem preparation*

Animals were anesthetized prior to surgery. Tadpoles were anesthetized by submersion in an aqueous solution of ethyl-*m*-aminobenzoate (MS-222; Sigma Chemical Co., St Louis, MO, USA; 0.5 g l^{-1}) buffered to pH 7.8 with sodium bicarbonate. Adults were anesthetized by placing them in a sealed container (1.5 l) with a cotton gauze that held approximately 1 ml of the volatile anesthetic 2-bromo-2-chloro-1,1,1-trifloroethane (halothane; Webster Veterinary Supply, Inc., Sterling, MA, USA). In a previous study with adult bullfrogs, we found that halothane results in the rapid induction of a surgical plane of anesthesia, and fictive breathing is produced much faster than with MS-222 anesthesia (Hedrick and Winmill, 2003). When breathing movements ceased and the withdrawal and corneal reflexes were abolished (adults: 15–20 min; tadpoles: 2–5 min), animals were removed from anesthesia. Tadpoles were placed in iced water for 1 h prior to surgery in order to slow metabolism and maintain anesthesia for subsequent dissection.

A small opening was made in the cranium allowing for the transection of the brainstem at the rostral border of the optic lobes and subsequent removal of the forebrain. During decerebration and subsequent dissection, the brainstem was constantly perfused with cold (5–10°C) artificial cerebrospinal fluid (aCSF) with the following composition: (in mmol l^{-1}) adult: NaCl 75.0, KCl 4.0, $MgCl_2$ 1.0, NaH_2PO_4 1.0, $NaHCO_3$ 40.0, $CaCl_2$ 2.5, glucose 5.0; tadpole: NaCl 104.0, KCl 4.0,

$MgCl_2$ 1.4, $NaHCO_3$ 25.0, $CaCl_2$ 2.4, glucose 10.0 (adapted from Kinkead et al., 1994; Torgerson et al., 2001).

The brainstem was removed and placed in a recording chamber where the dura and arachnoid were removed. Throughout this process and during all subsequent experiments, the recording chamber was continuously perfused with oxygenated aCSF (pH 7.8, 20°C) from a gravity-fed reservoir (350 ml) at a flow rate of 5–10 ml min^{-1}. Glass suction electrodes were attached to the nerve roots of cranial nerve (CN) V (trigeminal), X (vagus) and XII (hypoglossal) in the adult preparation and CN V, VII (facial) and XII in the tadpole, for the recording of respiratory-related neural activity. These cranial nerves innervate elevator and depressor muscles in the oropharyngeal region and are responsible for controlling glottal airflow, and for generating water flow over the gills in tadpoles (Gradwell, 1972) and airflow during ventilatory and non-ventilatory behaviors in the adult (DeJongh and Gans, 1969). Previous studies have verified that neural activity from CN V, VII, X and XII are associated with breathing movements in tadpoles (Gdovin et al., 1998) and adults (Sakakibara, 1984). Neural activity was amplified 10,000 times with a differential AC amplifier (A-M systems model 1700; Everett, WA, USA), filtered (100–1 kHz) and recorded on a computer that interfaced with a data acquisition system sampling at 2 kHz (Powerlab 8/S; AD Instruments, Milford, MA, USA).

Effects of hypoxia on respiratory-related motor output

Pre-metamorphic tadpole, post-metamorphic tadpole and adult brainstem preparations were superfused with oxygenated aCSF (20°C; pH 7.8), bubbled with 98% O_2/2% CO_2 for approximately 1 h, before a 10 min control recording was made. Following the control recording, superfusate was switched to a reservoir containing aCSF (20°C; pH 7.8) bubbled with anoxic, isocapnic gas (98% N_2/2% CO_2) from an electronic mixing flowmeter (Cameron Instruments Co., Port Aransas, TX, USA; model GF-3MP). In early experiments with pre-metamorphic (N=3) and adult (N=3) brainstems, we monitored the P_{O_2} of the hypoxic aCSF and brain tissue with a polarographic oxygen microelectrode (30 μm tip diameter; Diamond General Corp., Ann Arbor, MI, USA). We found that within 5 min of switch to the hypoxic aCSF, P_{O_2} near the brain surface and within the brain tissue at any depth was near 0 kPa. Thus, we are confident that the brain tissue in all experiments underwent sustained, severe hypoxia.

Inhibition of glycolysis with iodoacetate

Pre-metamorphic (N=6) and adult (N=6) preparations were used to examine the role of anaerobic metabolism during hypoxia. Procedures were identical to those described above, with the following exceptions. After recording activity with superfusion of the control aCSF, the superfusate was switched to hypoxic aCSF containing 100 μmol l^{-1} iodoacetate (IAA) to inhibit anaerobic metabolism. Activity was recorded while preparations were superfused with IAA in hypoxic aCSF for up to 4 h in pre-metamorphic tadpoles (average 3 h), and 1.5 h in post-metamorphic tadpoles and adults. All preparations were allowed to recover in oxygenated aCSF following IAA in hypoxic aCSF.

Data analysis

Respiratory-related neural discharges were defined by previously described criteria for fictive gill and lung bursts (Reid and Milsom, 1998; Torgerson et al., 1998; Hedrick and Winmill, 2003; Winmill and Hedrick, 2003a,b). Burst frequency is defined as neural bursts per minute. Lung episodes are defined as two or more bursts occurring in succession separated by a pause not longer than the average duration of two ventilatory cycles. Non- respiratory bursts were defined as bursts with duration longer than 1 s and having a burst shape not resembling that of normal lung breaths (see Hedrick and Winmill, 2003). Burst duration was measured from the onset of deviation from the baseline to the return to baseline in the integrated neural trace. Rise time was measured from the onset of deviation from the baseline to the peak value in the integrated neural trace and analyzed as a percentage of burst duration. Burst amplitude was measured in arbitrary units and analyzed as a percentage of control from the integrated neural trace.

For each experiment a minimum of five bursts were analyzed from the control recording and during 10 min intervals throughout the period of hypoxic exposure until respiratory activity ceased (post-metamorphic and adult preparations). Fictive breathing during hypoxia in post-metamorphic and adult preparations was analyzed during the 5–15 min period after the switch to hypoxic aCSF. All preparations exhibited respiratory activity during this period and superfusate/tissue P_{O_2} was near 0 kPa (see above).

A one-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparison test (Zar, 1974) was used for evaluation of statistical significance between fictive breaths during anoxia compared with that in the control period, within each experimental group. Percentage data were converted to their arcsine values prior to statistical analysis (Zar, 1974). The minimal level of statistical significance was taken as $P<0.05$. All values are mean ± S.E.M., unless otherwise specified. All statistical and graphical analyses were carried out using commercially available software programs (Graphpad Prism, v. 3.0.1, San Diego, CA, USA; Igor Pro v. 4.01, Wavemetrics, Inc., Lake Oswego, OR, USA).

Results

Hypoxia-induced changes in respiratory rhythm

The respiratory response to hypoxia differed dramatically between developmental stages. Pre-metamorphic tadpole preparations exhibited rhythmic respiratory related motor activity in the form of low amplitude, high frequency fictive gill bursts and high amplitude, low frequency fictive lung bursts (Fig. 1A). Both gill and lung rhythm persisted in all pre-metamorphic preparations throughout a 3 h period of superfusion with hypoxic aCSF. In one preparation superfused

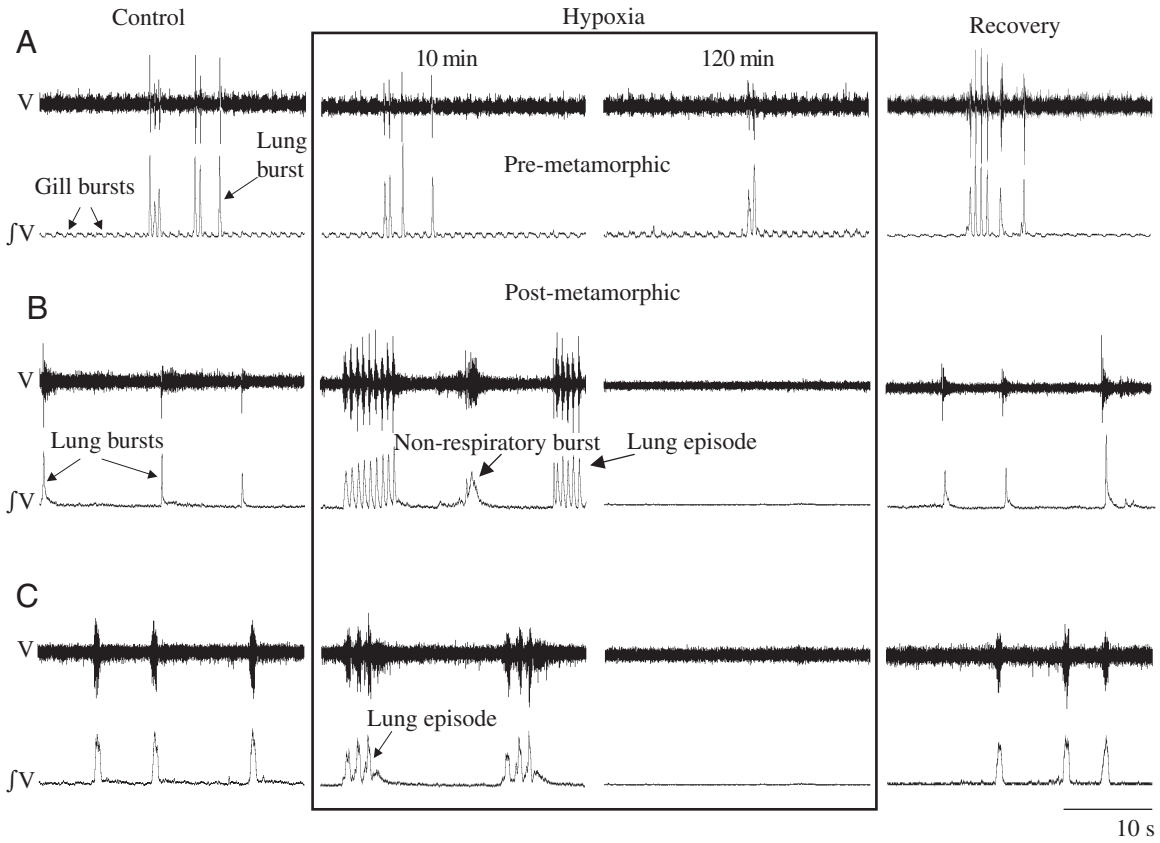

Fig. 1. Effects of 2 h hypoxia on respiratory-related neural activity from pre-metamorphic, post-metamorphic and adult brainstems. Raw (V) and integrated (∫V) activity recorded from the trigeminal cranial nerve (CN V) preparations from (A) stage XIV pre-metamorphic tadpole, (B) stage XXIV post-metamorphic tadpole and (C) adult brainstem during control conditions, 10 min and 120 min hypoxia, and recovery. Fictive gill and lung burst activity are present in the pre-metamorphic tadpole after 2 h, but neural activity ceased in the post-metamorphic and adult preparations. An example of a single non-respiratory burst is shown for the post-metamorphic brainstem during hypoxia.

with anoxic aCSF for >4 h, respiratory-related neural output persisted throughout the period of hypoxia. During the period of hypoxic exposure, there was no detectable change in burst characteristics compared with control recordings (Fig. 1A).

Fictive breathing in post-metamorphic tadpole and adult preparations consisted solely of low frequency, high amplitude lung bursts (Fig. 1B,C). High frequency, low amplitude bursts, characteristic of non-ventilatory fictive buccal activity, were present in two of seven adult preparations and were not analyzed for the purpose of this study. In contrast to the response of pre-metamorphic tadpoles, post-metamorphic tadpole and adult preparations exposed to hypoxia within 5–15 min became very episodic (Fig. 1B,C), and exhibited an increase in the number of 'non-respiratory' neural bursts (Fig. 1B) (cf. Hedrick and Winmill, 2003); this was followed by a complete cessation of respiratory activity after 20–40 min. The cessation of respiratory activity was sustained throughout the period of hypoxic exposure, but was reversible upon re-oxygenation with control aCSF (Fig. 1B,C).

A summary of the changes in gill/lung burst frequency in pre-metamorphic tadpoles is provided in Fig. 2A. In pre-metamorphic tadpoles, fictive gill frequency was 57.2±1.0 min^{-1} in control aCSF and decreased slightly during hypoxia to 45.2±3.0 min^{-1} at 180 min hypoxic exposure (Fig. 2A; $P>0.05$). Fictive lung burst frequency decreased significantly from 2.5±0.4 min^{-1} to 0.7±0.1 min^{-1} ($P<0.05$), but only after 180 min hypoxia (Fig. 2A; $P<0.05$). Lung frequency returned to control values when preparations were returned to oxygenated aCSF. Gill activity ceased in three of six preparations and continued at a decreased level in the remaining preparations, but was not significantly different from the control recordings ($P>0.05$).

Hypoxic exposure in post-metamorphic tadpoles resulted in a cessation of respiratory-related activity in four of five preparations within 20 min after the onset of hypoxia and neural activity ceased in all preparations by 30 min. The average time to cessation for all five preparations was 18 min in hypoxia. Fictive apnea persisted during prolonged hypoxic exposure of up to 2 h and was reversible in all preparations upon return to oxygenated aCSF. Control lung burst frequency in the post-metamorphic preparation was 5.8±1.8 min^{-1} and did not change significantly during the initial 20 min exposure to hypoxia (Fig. 2B; $P>0.05$).

In adult preparations, lung burst frequency was 4.6±1.2 min^{-1} in control aCSF (Fig. 2B). Exposure to hypoxia resulted in a complete cessation of neural activity in all seven

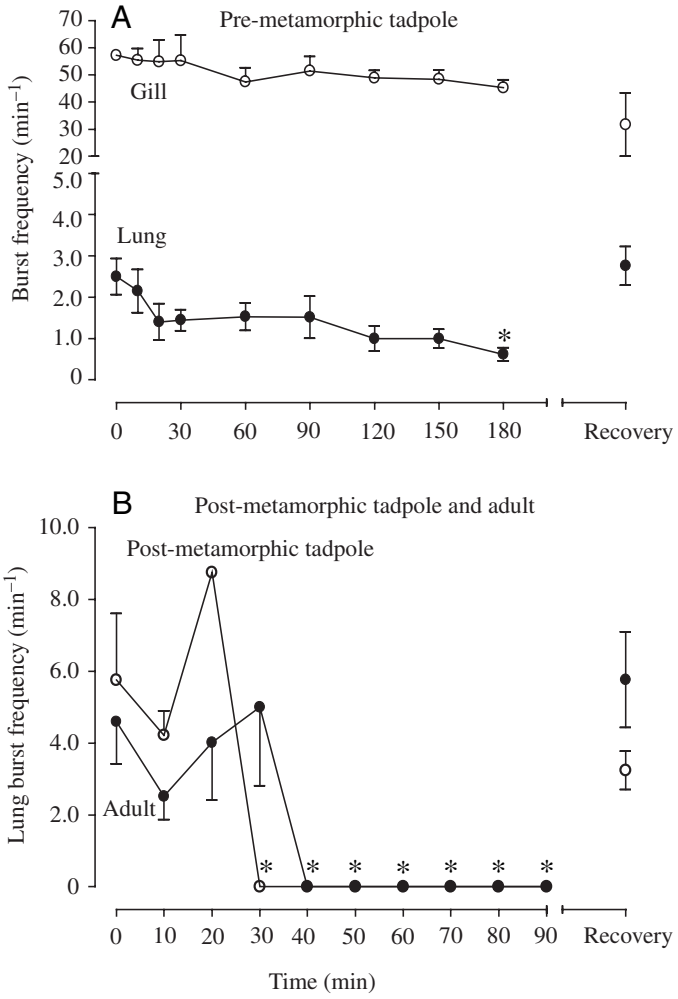

Fig. 2. Summary of effects of hypoxia on burst frequency in (A) fictive gill (open circles) and lung (solid circles) in pre-metamorphic tadpoles and (B) on lung burst frequency in the post-metamorphic tadpole (open circles), and adult (solid circles). *P<0.05 compared with control values.

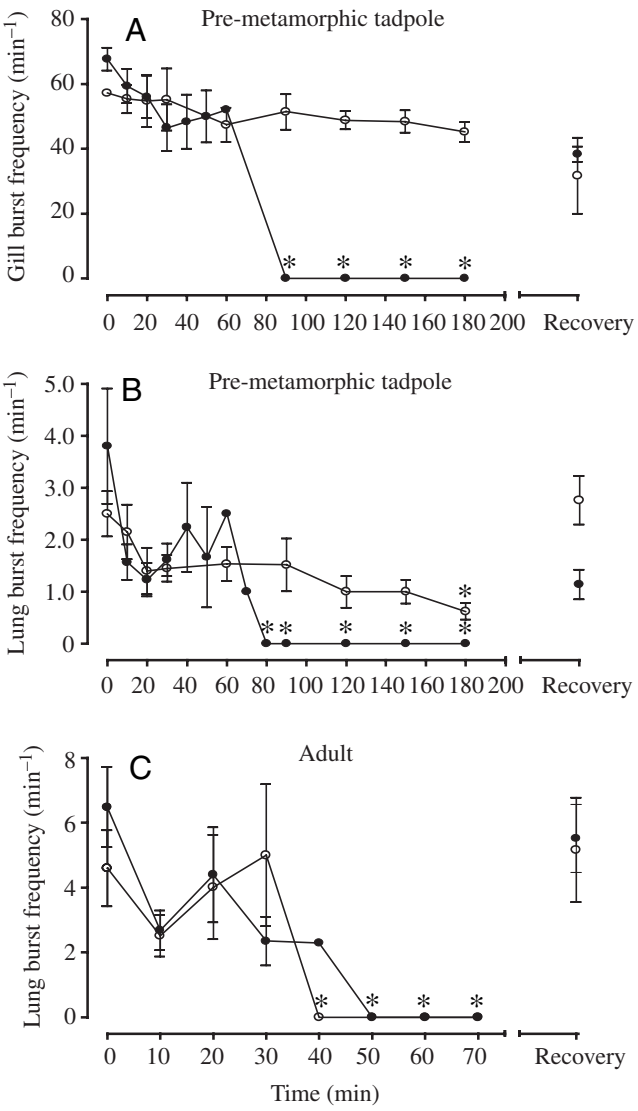

Fig. 3. Effects of hypoxia (open circles) and hypoxia + iodoacetate (solid circles) in aCSF on gill/lung burst frequencies in tadpole and adult brainstems. (A) pre-metamorphic tadpole gill cranial nerves frequency; (B) pre-metamorphic lung burst frequency; (C) adult lung burst frequency. *P<0.05 compared with control values.

preparations by 40 min, with an average time of cessation of 24 min. There was no significant change from control until all respiratory activity stopped. Fictive apnea persisted during prolonged hypoxic exposure, in some cases lasting for >2 h, and was fully reversible in six of seven preparations (Fig. 2B).

Contribution of anaerobic metabolism during extreme hypoxia

The role of anaerobic metabolism during extreme hypoxia was investigated by inhibiting glycolysis with IAA (Fig. 3). IAA inhibits glycolysis by blocking glyceraldehyde-3-phosphate dehydrogenase (Xia et al., 1992). These data were compared with the data obtained from pre-metamorphic and adult brainstems exposed to hypoxia alone (Fig. 2). In pre-metamorphic tadpoles, superfusion with hypoxic aCSF containing 100 μmol l^{-1} IAA significantly reduced the amount of time gill and lung burst activity could be maintained in hypoxia (Fig. 3A,B). After 40 min exposure to IAA in hypoxic aCSF, only three of six pre-metamorphic preparations exhibited fictive gill ventilation and by 60 min only one of six preparations showed any gill activity (Fig. 3A,B). After 90 min superfusion with hypoxia-IAA, fictive gill bursts were abolished in every preparation (Fig. 3A). Upon reoxygenation with control aCSF, three of six preparations recovered gill activity. By contrast, hypoxia alone had no effect on gill activity for 3 h (Fig. 3A). Lung burst activity in pre-metamorphic tadpoles (Fig. 3B) showed a similar response to hypoxia-IAA superfusion compared with gill ventilation. Only three of six preparations exhibited any lung burst activity after 50 min exposure, and the one preparation that had gill activity after 60 min also exhibited lung burst activity (Fig. 3B). All lung burst activity was abolished by 80 min superfusion with hypoxia-IAA. Upon reoxygenation with control aCSF, five of six preparations recovered and produced fictive lung bursts at

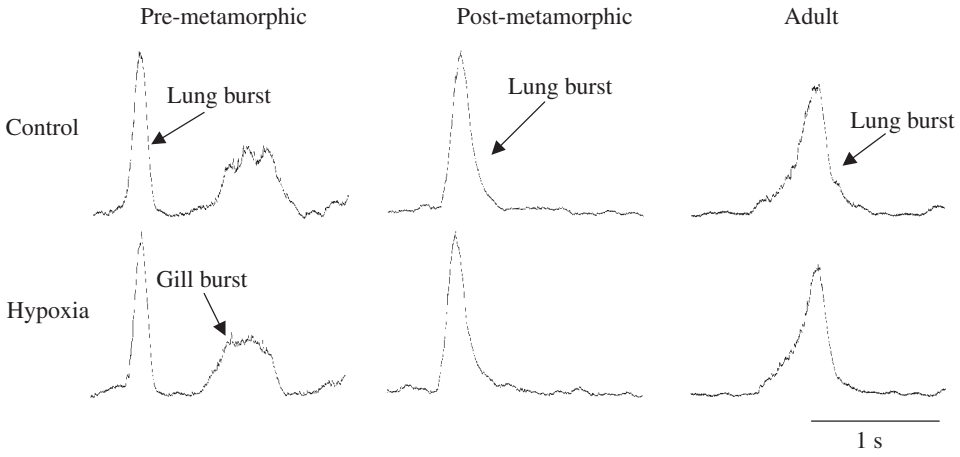

Fig. 4. An expanded trace illustrating typical respiratory-related bursts in the pre-metamorphic tadpole, post-metamorphic tadpole and adult during control conditions and hypoxia. Note there is no change in individual burst characteristics, including amplitude, duration or rise time at any developmental stage.

a frequency that was not significantly different from control recordings (Fig. 3B; $P>0.05$).

Lung ventilation frequency in adult brainstem preparations showed similar responses whether superfused with hypoxic aCSF alone or with hypoxia-IAA (Fig. 3C). In hypoxia-IAA, four of six preparations ceased activity by 30 min and five of six preparations ceased activity by 40 min; by 50 min, all preparations had ceased activity (Fig. 3C). Upon reoxygenation, five of six preparations recovered to control levels (Fig. 3C).

Hypoxia-induced changes in respiratory pattern formation

Fig. 4 illustrates typical gill and lung bursts in the pre-metamorphic preparation and typical lung bursts in the post-metamorphic and adult preparation during control conditions and hypoxia. Hypoxia had no significant effect on respiratory-related burst characteristics including burst duration, amplitude or rise time at any of the developmental stages examined (data not shown). There was no change in rise time between control and hypoxia at any stage, which would indicate a shift from the typical 'bell-shaped' burst profile, to the rapid onset, rapid offset burst characteristic of gasping in mammals (St John and Knuth, 1981).

Lung burst episodes were present with control aCSF in three of six pre-metamorphic tadpole preparations. During control superfusion, lung episodes occurred at a frequency of 1.4 ± 0.6 min^{-1} and with a mean of 3.0 ± 0.4 bursts per episode (Fig. 5A,B). There was no significant change in the frequency of episodes or the number of individual bursts per episode during hypoxic exposure in the pre-metamorphic tadpole preparation (Fig. 5A,B).

During the initial exposure to hypoxia (5–15 min), and prior to the cessation of respiratory activity, fictive breathing in post-metamorphic tadpole and adult preparations became more episodic, characterized by an increase in both the frequency of lung episodes and the number of individual bursts per episode in the adult preparations (Fig. 5A,B). Lung episodes were present during control conditions in three of five post-metamorphic tadpoles and two of seven adults. During the initial period of hypoxia, all five post-metamorphic tadpole preparations and all seven adult preparations exhibited lung episodes. In the post-metamorphic tadpoles, episode frequency was 0.6 ± 0.5 min^{-1} with control superfusion and increased to 3.1 ± 0.7 min^{-1} during the initial period of hypoxia (Fig. 5A; $P<0.05$). The number of individual bursts per episode in the post-metamorphic tadpoles increased from 2.0 ± 0.04 bursts per

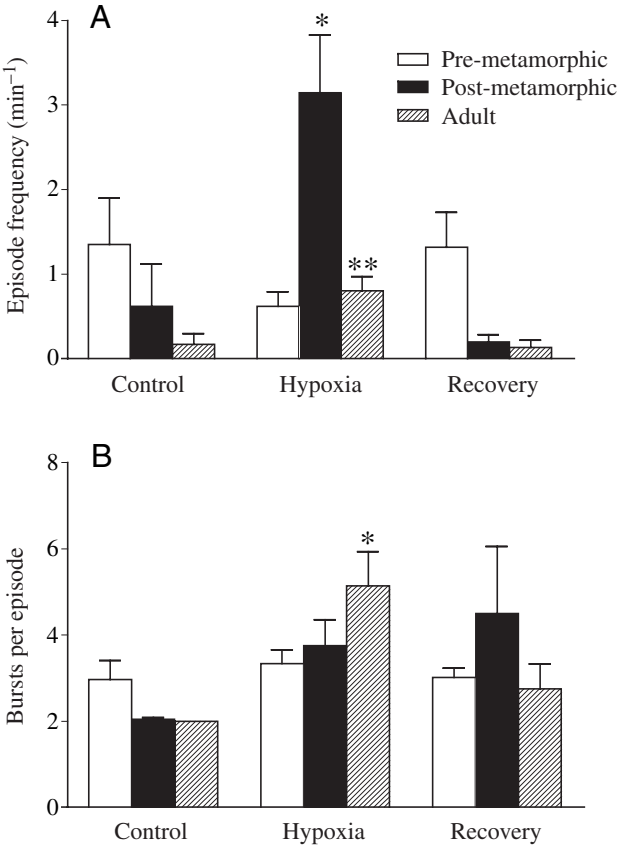

Fig. 5. Summary of effects of hypoxia on (A) lung burst episode frequency and (B) number of lung bursts per episode in pre-metamorphic tadpole (white bars), post-metamorphic tadpole (black bars) and adult (hatched bars) brainstems. Data for post-metamorphic tadpole and adult preparations were obtained during the initial 5–15 min exposure to hypoxia, prior to the respiratory cessation that occurred in these preparations (see text). *$P<0.05$ compared with control values.

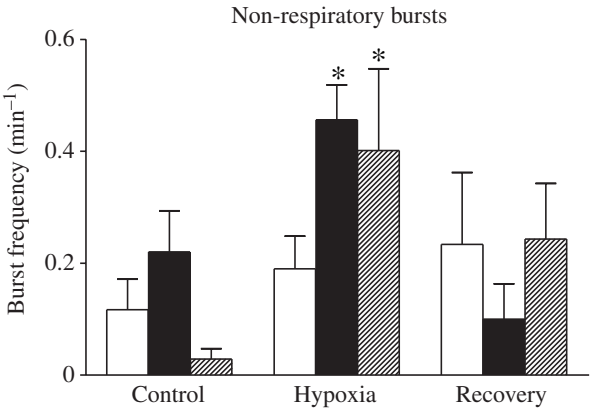

Fig. 6. Summary of effects of hypoxia on frequency of non-respiratory bursts in pre-metamorphic (white bars), post-metamorphic (black bars) and adult (hatched bars) brainstems. Non-respiratory bursts in post-metamorphic and adult preparations were obtained during the initial 5–15 min of hypoxia. *P<0.05 compared with control values.

episode to 3.8±0.6 bursts per episode during hypoxia, but this was not significant (Fig. 5B). In adult preparations, episode frequency increased significantly from 0.2±0.2 min^{-1} to 0.8±0.2 min^{-1} during hypoxia (Fig. 5A; P<0.01). The number of individual bursts per episode also increased significantly during hypoxia in the adult preparation, from a control level of 2.0±0.2 bursts per episode to 5.1±0.8 bursts per episode in hypoxia (Fig. 5B; P<0.05). In both post-metamorphic tadpole and adult preparations, hypoxia-induced changes in episode frequency and the number of individual bursts per episode were fully reversible when preparations were again superfused with oxygenated aCSF, as indicated by the lack of significant difference between control and recovery values (Fig. 5).

We analyzed the frequency of non-respiratory bursts before, during and following the hypoxia challenge (Fig. 6). An example of a non-respiratory lung burst is provided in Fig. 1 for a post-metamorphic tadpole (see also Hedrick and Winmill, 2003). Non-respiratory bursts are those that do not conform to the criteria established for normal lung bursts (see Materials and methods) and are typically long duration, high amplitude bursts and make up about 10% of the total bursts (Hedrick and Winmill, 2003). In this study, pre-metamorphic tadpoles had a non-respiratory burst rate of 0.2±0.05 min^{-1} and this did not change significantly during hypoxia or recovery (Fig. 6). Non-respiratory burst frequency under control conditions was 0.2±0.1 min^{-1} for post-metamorphic brainstems and 0.03±0.02 min^{-1} for adult brainstems. Both groups showed a significant increase in non-respiratory burst frequency in hypoxia, increasing to 0.5±0.1 min^{-1} in post-metamorphic tadpoles and to 0.4±0.1 min^{-1} in adult preparations (P<0.05; Fig. 6). The effect of hypoxia on non-respiratory bursts was fully reversible upon reoxygenation during recovery in control aCSF (Fig. 6).

Discussion

There are two important findings from this study. First, there is a clear developmental change in the central respiratory response to hypoxia. Pre-metamorphic brainstem preparations had little or no respiratory response to hypoxia for up to 3 h, whereas post-metamorphic and adult preparations initially increased lung burst episodes and reversibly abolished respiratory-related activity. Second, the ability of pre-metamorphic tadpoles to sustain activity in severe hypoxia appears to rely on a significant contribution from anaerobic metabolism. However, glycolysis does not appear to play any appreciable role in sustaining respiratory activity during hypoxia in the adult brainstem. These data argue strongly for a developmental 'switch' in the central respiratory response to hypoxia that coincides with the onset of metamorphosis in bullfrogs.

Developmental changes in the respiratory response to hypoxia

The pre-metamorphic tadpole brainstem appears to be very tolerant to severe hypoxia. This remarkable degree of hypoxia tolerance appears to be due to the ability of the pre-metamorphic tadpole brain to use anaerobic metabolism to maintain neural activity. However, these data differ from the *in vivo* hypoxic response of tadpoles where hypoxia induces significant increases in gill and lung frequency at moderate levels of hypoxia [P_{O_2} ~100 Torr (100 mmHg)], but is suppressed when P_{O_2} reaches about 20 mmHg (West and Burggren, 1982). These responses are likely to be mediated by oxygen-sensitive chemoreceptors in the gills of tadpoles (Straus et al., 2001). However, the present study used very severe hypoxia (near 0 kPa), rather than moderate levels of hypoxia used with freely behaving animals. We are unaware of any studies that have examined the effects of severe hypoxia/anoxia on respiratory or behavioral activity in tadpoles.

By contrast, the *in vitro* post-metamorphic tadpole and adult bullfrog brainstem preparations respond to prolonged hypoxia with a complete and reversible cessation of all respiratory-related motor activity, occurring within about 25 min. Fictive apnea in these preparations persists during prolonged hypoxia of up to 2 h and respiratory rhythm is restored when the preparations are returned to oxygenated aCSF. The adult brainstem does not appear to use glycolysis to maintain respiratory activity because the cessation of respiratory activity was identical in hypoxia with or without inhibition of anaerobic metabolism (Fig. 3C). Most adult brainstems exposed to hypoxia with IAA recovered upon reoxygenation, but this differs from the response *in vivo* where *Rana pipiens* given IAA died after 20 min exposure to anoxia (Rose and Drotman, 1967). This difference may be due to IAA affecting other organ systems (e.g. heart and circulation) in the intact animal. Overall, these data are consistent with natural history observations indicating that *Rana* tadpoles are more hypoxia tolerant than adult animals (Bradford, 1983).

The mechanisms underlying the cessation of respiratory activity in the post-metamorphic and adult brainstem in hypoxia are unclear. Respiratory cessation occurs long

before there are measurable changes in extracellular K^+ (Knickerbocker and Lutz, 2001) or excitatory neurotransmitters (Lutz and Reiners, 1997) indicative of acute energy failure in the frog brain. Synaptic depression is a common feature of the mammalian brain that occurs prior to a loss of ion homeostasis (Hansen, 1985), which could account for the respiratory depression in this study. In the anoxic turtle brain, reductions in ion channel conductance lead to elevations of action potential thresholds, thus depressing electrical activity through 'spike arrest' (Sick et al., 1993). Spike arrest may be possible in the frog brain because anoxia results in a decreased rate of K^+ leakage into the extracellular space (Knickerbocker and Lutz, 2001). Channel arrest is a characteristic feature of anoxia-tolerant animals such as turtles (Hochachka and Lutz, 2001), but there is little evidence for such extreme downregulation of ion channels in the frog brain (Lutz and Nilsson, 2004).

Other possible mechanisms that explain the respiratory cessation in our study are the production of lactate in the hypoxic brainstem and/or the activation of ATP-sensitive K^+ (K_{ATP}) channels. Blood lactate increases significantly in toads (*Bufo marinus*) when inspired P_{O_2} drops below 10 mmHg (D'Eon et al., 1978) and in brain tissue of frogs (*Rana temporaria*) within 10 min exposure to anoxia (Wegner and Krause, 1993). This should cause a significant drop in brain tissue pH and potentially affect breathing. However, decreased pH increases in fictive breathing in the adult bullfrog brainstem (Kinkead et al., 1994; Morales and Hedrick, 2002), thus making changes in lactate and pH an unlikely mechanism for the respiratory cessation during hypoxia. Neuronal K_{ATP} channels play a protective role in brain hypoxia in mammals by increasing K^+ channel conductance when ATP concentration decreases during severe brain hypoxia (Ballanyi, 2004). Brain ATP levels decrease significantly in *Rana temporaria* after 20 min anoxia (Wegner and Krause, 1993) and drop to approximately 50% of normoxic levels in the first 30 min of anoxia in *Rana pipiens* (Lutz and Reiners, 1997; Knickerbocker and Lutz, 2001). In preliminary experiments using adult bullfrog brainstems, we examined the potential role of K_{ATP} channels in mediating this response with the K_{ATP} channel blocker glipizide. In some experiments glipizide blocked the respiratory cessation in hypoxia, consistent with a role for K_{ATP} channels, but glipizide had no effect in other experiments. This would suggest that the respiratory cessation that occurs in the hypoxic bullfrog brainstem is not entirely due to reductions in ATP gating the K_{ATP} channel.

Because IAA fails to inhibit breathing with a faster time course than with hypoxia alone, these data suggest the presence of an oxygen 'sensor' in the mature amphibian brainstem linked to the reversible cessation of respiratory activity. There is substantial evidence for the presence of an oxygen 'sensor' in the mammalian brainstem from *in vivo* (Solomon et al., 2000) and *in vitro* (Telgkamp and Ramirez, 1999) preparations. The oxygen sensing ability of mammalian neurons may use a variety of cellular mechanisms, including the gating of several different ion channels (see Acker and Acker, 2004). The pre-Bötzinger Complex (PBC), a proposed site for respiratory rhythm generation in the mammalian brainstem, has been shown to function as an oxygen sensor that can regulate breathing *in vivo* (Solomon et al., 2000).

The timing of developmental changes in the respiratory response to hypoxia may have important ecological implications. Bullfrog tadpoles are facultative air breathers until T-K stage XXI (Crowder et al., 1998). All stages of bullfrog tadpoles breathe air, but the frequency is low in normoxic water and increases in hypoxic water (West and Burggren, 1982; Burggren and Doyle, 1987; Crowder et al., 1998). Following the loss of the gills between T-K stages XXII-XXIII (Crowder et al., 1998), the post-metamorphic tadpole becomes an obligate air breather. Lacking alternative means of meeting oxygen demands in a hypoxic environment, the post-metamorphic tadpole and adult amphibian may tend to shut down metabolic processes, including respiratory activity, in order to conserve cellular energy. Evidence for this decrease in metabolic costs in the face of hypoxia has been observed *in vivo*. For example, adult *Rana pipiens* exposed to anoxia demonstrate a rapid decrease in pulmonary respiratory movements, with respiratory cessation occurring after approximately 30 min of anoxic exposure (Rose and Drotman, 1967). Interestingly, this time course *in vivo* is nearly identical with the time course of respiratory cessation for post-metamorphic tadpoles and adult bullfrog brainstems in this study (Fig. 2B). We suggest that the reversible cessation of respiratory activity, by an unknown mechanism, during hypoxia in post-metamorphic tadpoles and adults may be an adaptive, energy-saving response to periods of severe hypoxia.

The present data are entirely consistent with the general view that neonatal mammals survive hypoxic conditions that result in severe neuronal damage in adult mammals (e.g. Duffy et al., 1975). The increased hypoxia tolerance of neonatal mammals involves increased ability to generate ATP anaerobically and to downregulate ion channels to preserve ion gradients that decrease ATP demand (Bickler and Hansen, 1998; Bickler et al., 2003). It is clear from our study that pre-metamorphic tadpole are capable of generating respiratory neural activity longer in hypoxia and part of this ability involves increased reliance on anaerobic metabolism compared with post-metamorphic animals. Taken together, these data point to some common mechanisms in vertebrates with respect to the development of hypoxia tolerance.

Hypoxia-induced changes in respiratory pattern generation

The episodic breathing pattern observed during normocarbia in amphibians and reptiles is clearly an endogenous property of the central nervous system. In unidirectionally ventilated toads (West et al., 1987; Smatresk and Smits, 1991) and bullfrogs (Kinkead and Milsom, 1994), where the natural oscillations of blood gases associated with periods of ventilation and apnea were experimentally prevented, breathing is still episodic. Furthermore, the *in vitro* bullfrog (Kinkead et al., 1994; Reid and Milsom, 1998; Hedrick and Winmill, 2003) and turtle (Douse and Mitchell, 1990)

brainstem preparations are capable of generating fictive lung episodes similar to that seen in the whole animal, despite removal of all peripheral inputs.

Although episodic breathing is an endogenous feature of the amphibian brainstem respiratory network, it is not clear if the increase in episodic breathing during hypoxia (Kruhøffer et al., 1987; Smatresk and Smits, 1991; Kinkead and Milsom, 1994) results from stimulation of peripheral oxygen chemoreceptors or from stimulation of a central oxygen chemosensor. Chemosensory feedback has been suggested to play a role in the modulation of the episodic breathing pattern in the decerebrate, unidirectionally ventilated toad *Bufo marinus*, since bilateral vagotomy prevents hypercapnia-induced increases in breathing episodes (Reid et al., 2000). Because our preparation was devoid of feedback from peripheral oxygen-sensitive chemoreceptors, the data in this study argue that brainstem hypoxia is capable of stimulating episodic breathing in the bullfrog brainstem.

Severe hypoxia in mammals transforms the breathing pattern from eupnea to gasping, a form of widespread respiratory excitation (St John and Knuth, 1981; Solomon, 2000). This transformation is characterized by a shift in the pattern of phrenic nerve activity from the slowly augmenting, rapidly decrementing eupneic burst, to a rapid onset, slowly decrementing burst characteristic of gasping (St John and Knuth, 1981). The *in vitro* medullary slice preparation from neonatal mice has been demonstrated to be capable of producing multiple respiratory-related burst patterns resembling eupnea, sighs and gasping and hypoxia changes the pattern of neural activity recorded from neurons of the ventral respiratory group (VRG) from eupneic to gasp-like bursts (Lieske et al., 2000).

Gasp-like activity does not appear to be a feature of the isolated amphibian brainstem in hypoxia. During superfusion with hypoxic aCSF, there was no significant change in burst characteristics, including burst amplitude, duration, or rise time, which would indicate a change to fictive gasping. These burst characteristics did not change during hypoxic exposure at any of the developmental stages examined; thus, the primary responses to hypoxia in this preparation are associated with changes in respiratory frequency and clustering of breaths into episodes in post-metamorphic tadpoles and adults.

Post-metamorphic tadpoles and adults exhibited a significant increase in the frequency of non-respiratory bursts in hypoxia (Fig. 5). Non-respiratory bursts have been characterized in previous studies with amphibians (Reid and Milsom, 1998; Hedrick and Winmill, 2003) and lampreys (Thompson, 1985), but the function of these bursts remains unclear. In the isolated lamprey brainstem, non-respiratory bursts have been characterized as 'arousal' breathing (Thompson, 1985) and are similar to the non-respiratory bursts in the present study. In adult amphibians, non-respiratory bursts are typically large amplitude, long duration bursts that are more resistant to the inhibitory effects of MS-222 anesthesia than typical respiratory bursts (Hedrick and Winmill, 2003). Because non-respiratory bursts increased significantly with hypoxia only in post-metamorphic and adult brainstem preparations, these breaths may play a role similar to gasping in adult mammals; that is, a form of 'arousal' that is a last-ditch effort by a brainstem motor network to re-start breathing when normal respiratory efforts fail (Solomon, 2000). Another similarity amphibian non-respiratory bursts share with gasping in mammals is that they are both readily reversible upon reoxygenation (Solomon, 2000). Thus, non-respiratory bursts may be important for generating a widespread neural activity to resuscitate breathing during severe hypoxia and may be functionally equivalent to gasping in mammals.

Conclusions

This study demonstrates a developmental change in the central mechanisms modulating the respiratory response to hypoxia in bullfrogs. The pre-metamorphic tadpole respiratory CPG appears to be far more tolerant to hypoxia, primarily owing to the ability to use anaerobic metabolism, while respiratory activity in the post-metamorphic tadpole and adult is reversibly abolished shortly after the onset of hypoxia and does not appear to use glycolysis to maintain respiratory activity. We suggest that the reversible cessation of respiratory activity in the hypoxic post-metamorphic bullfrog brain is an adaptive, energy-saving response that may be mediated by a brainstem oxygen sensor.

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