

# Gap junction blockade with carbenoxolone differentially affects fictive breathing in larval and adult bullfrogs

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## Abstract

This study examined the role of gap junctional communication in the modulation of respiratory related motor output using *in vitro* brainstem preparations of larval ( $N = 14$ ) and adult ( $N = 14$ ) bullfrogs (*Rana catesbeiana*). Superfusion of the isolated brainstem for at least 1 h with the gap junction blocker carbenoxolone (CBX; 100  $\mu\text{M}$  and 1 mM) dissolved in artificial cerebrospinal fluid (aCSF) elicited significant changes in respiratory-related burst frequency in both larval and adult preparations. In tadpole preparations, both concentrations of CBX significantly decreased gill and lung burst frequency over 20–40 min, with 1 mM CBX producing complete cessation of gill and lung burst activity by 40 min in all preparations. There was little or no change in other burst characteristics such as burst amplitude or duration. By contrast, superfusion of the adult brainstem preparation with CBX significantly increased lung burst frequency over 10–20 min, and caused cessation of lung burst activity with 100  $\mu\text{M}$  CBX (five of seven preparations) and with 1 mM CBX (seven of seven preparations). Adult preparations that ceased activity with 100  $\mu\text{M}$  CBX recovered in control aCSF, but those in 1 mM did not recover, despite up to 3 h superfusion with control aCSF. In two additional adult preparations, 1 h exposure to hypercapnic aCSF (7–10%  $\text{CO}_2$ ) following the cessation of fictive breathing with 1 mM CBX failed to evoke respiratory activity. The inhibition of fictive breathing in tadpoles suggests that gap junctional communication may be important for respiratory rhythmogenesis prior to the development of central  $\text{CO}_2$  chemosensitivity. Following metamorphosis to the terrestrial adult, however, gap junctional communication may contribute to regulation of respiratory frequency and possibly the transduction of central  $\text{CO}_2$  chemosensitivity.

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**Keywords:** Amphibian, bullfrog (*Rana catesbeiana*); Control of breathing, respiratory motor output; Development, tadpole; Gap junctions, connexins, respiratory motor output; Pharmacological agents, carbenoxolone

## 1. Introduction

Gap junctions are intercellular channels that provide a pathway for direct cell-to-cell exchange of ions, second messengers, and small metabolites thereby allowing for electrical and metabolic

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coupling of adjacent cells (Bruzzone and Resson, 1997).

Electrical coupling via gap junctions is important in the formation of neuronal circuits (Kandler and Katz, 1995) and has been proposed to play a role in the synchronization of neural activity and in the generation of oscillatory activity from neuronal networks (Christie et al., 1989; Draguhn et al., 1998; Traub et al., 1999; Lewis and Rinzel, 2000).

A number of recent studies have examined the possible role of electrotonic coupling via gap junctions in the generation and modulation of respiratory rhythm and central CO<sub>2</sub> chemoreception in mammals (for reviews, see Solomon and Dean, 2002; Dean et al., 2002). Evidence for the possibility of gap junctional communication in respiratory rhythm generation includes the expression of connexins (Cx), the proteins that comprise gap junctions, in neurons and glia of the neonatal and adult pre-Bötzinger complex (PBC), a region of the medulla widely accepted as a site of respiratory rhythm generation in mammals (Parenti et al., 2000; Solomon et al., 2001; Solomon and Dean, 2002). Furthermore, bi-directional electrical coupling has been demonstrated between rhythmogenic respiratory neurons in the PBC and between pairs of hypoglossal motoneurons of neonatal mice (Rekling et al., 2000). A functional role for gap junctions in respiratory rhythmogenesis has been demonstrated in mammals in recent studies examining the effects of pharmacological blockade of gap junctions on the frequency and synchrony of respiratory related motor output (Rekling et al., 2000; Bou-Flores and Berger, 2001; Solomon et al., 2003). Bath application of gap junction blockers, including carbenoxolone (CBX), 18 $\alpha$ -glycyrrhetic acid (18 $\alpha$ -GA), 18 $\beta$ -glycyrrhetic acid (18 $\beta$ -GA), halothane, heptanol and octanol reversibly reduced or suppressed respiratory frequency and increased the degree of synchronization within inspiratory bursts in the in vitro medullary slice and en bloc brainstem spinal cord preparation of neonatal mice (Rekling et al., 2000; Bou-Flores and Berger, 2001). In contrast, pharmacological blockade of gap junctions in the arterially perfused adult rat increased the frequency of respiratory related motor output re-

corded from the phrenic nerve and reduced burst synchronization (Solomon et al., 2003). Recently, gap junctions have been implicated in the transduction of CO<sub>2</sub>/H<sup>+</sup> chemoreception in a number of brainstem locations (Huang et al., 1997; Oyama et al., 1999; Dean et al., 2001).

Though most investigations into the mechanisms underlying respiratory rhythmogenesis have been done in mammals (Rekling and Feldman, 1998; Smith et al., 1990), recent studies of amphibians indicate the conservation of basic features of respiratory control systems and rhythm generation (Hedrick et al., 2001; Broch et al., 2002; Winnill and Hedrick, 2003). Dye and electrotonic coupling as well as Cx expression, indicate the existence of gap junctional coupling in the central nervous system (CNS) of larval and adult amphibians (Sonnhof et al., 1977; Brenowitz et al., 1983; van der Heyden et al., 2001; Bácskai and Matesz, 2002), though evidence for the functional significance of gap junctions in the amphibian CNS is lacking. In the present study we used an in vitro brainstem spinal cord preparation from adult and larval bullfrogs to examine the role of gap junctions in respiratory rhythm generation during development. Using the gap junction blocker CBX, we tested the hypothesis that gap junctional communication plays a role the generation of respiratory related motor output in bullfrogs.

## 2. Methods

### 2.1. Animals

Experiments were performed on 14 adult (body mass 208–387 g) and 14 premetamorphic larval (body mass 4.2–6.9 g) North American Bullfrogs (*Rana catesbeiana*). Tadpoles were classified according to the staging criteria of Taylor and Kollros (1946) (T–K). Premetamorphic tadpoles from paddle stages (VI–X) with undifferentiated limbs and foot stage tadpoles (XI–XVII) were used. The tadpoles in this study were between T–K stages VI–XVI. Animals were purchased from a commercial supplier (Charles D. Sullivan Co., Inc.; Nashville, TN). Adults were maintained in plastic tanks with continuous access to water;

tadpoles were kept in fiberglass aquaria with aerated, dechlorinated tapwater. All animals were maintained at room temperature (20–23 °C). All experimental procedures were approved by the CSUH Institutional Animal Care and Use Committee.

## 2.2. *In vitro* brainstem preparation

Prior to surgery, tadpoles were anesthetized by submersion in an aqueous solution of ethyl-*m*-aminobenzoate (MS-222, Sigma Chemical Co., St. Louis, MO; 0.5 g L<sup>-1</sup>) buffered to pH 7.8 with sodium bicarbonate. Adults were anesthetized with 1 ml halothane applied to the skin on a piece of gauze in a sealed plastic chamber (1.5 L) (Hedrick and Winnill, 2003). When breathing movements ceased and the withdrawal and corneal reflexes were abolished (adults: 10–20 min; tadpoles: 2–5 min), animals were removed from anesthesia. Tadpoles were placed in ice water for 1 h to slow metabolism and maintain anesthesia for subsequent dissection.

A small opening was then made in the cranium with a dental drill, allowing for the transection and removal of the forebrain rostral to the optic lobes. During decerebration and subsequent dissection, the brainstem was constantly perfused with cold (5–10 °C) artificial cerebrospinal fluid (aCSF) with the following composition: (in mM) adult-NaCl, 75.0; KCl, 4.0; MgCl<sub>2</sub>, 1.0; NaH<sub>2</sub>PO<sub>4</sub>, 1.0; NaHCO<sub>3</sub>, 40.0; CaCl<sub>2</sub>, 2.5; glucose, 5.0; tadpole-NaCl, 104.0; KCl, 4.0; MgCO<sub>2</sub>, 1.4; NaHCO<sub>3</sub>, 25.0, CaCl<sub>2</sub>, 2.4; glucose, 10.0 (adapted from Kinkead et al., 1994; Torgerson et al., 2001), and equilibrated with 98% O<sub>2</sub>/2% CO<sub>2</sub>. The spinal cord was transected caudal to the brachial nerves and cranial nerve (CN) roots were severed at their exit from the cranium. The entire dissection required approximately 30 min to complete.

The isolated brainstem was pinned ventral side up in a sylgard-lined (Dow Corning) recording chamber (7 ml) and 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<sup>-1</sup>.

Suction electrodes, fabricated from thin-walled capillary glass and held in micromanipulators (Narashige), were attached to the nerve roots of CNs V (trigeminal), X (vagus) and XII (hypoglossal) in the adult preparation and CN V, VII (facial) and XII in the tadpole. Nerve activity was amplified 10 000 times with a differential AC amplifier (A-M systems model 1700; Everett, WA), filtered (100 Hz–5 kHz) and recorded on a computer that interfaced with a data acquisition system sampling at 2 kHz (Powerlab 8/S; AD Instruments, Milford, MA).

## 2.3. *Experimental protocol*

CBX (3-hydroxy-11-oxoolean-12-en-30-oic acid; Sigma) was used as an uncoupler of gap junctions in the amphibian brainstem preparation. CBX was dissolved in aCSF to achieve a final concentration of 100 μM or 1 mM. A single brainstem preparation was used once for each concentration of CBX (100 μM or 1 mM).

The brainstem preparation was superfused with aCSF for 1 h, or until the signal was stable, before a 10-min control recording was obtained. After the initial control recording was taken, each brainstem was superfused continuously with either 100 μM or 1 mM CBX for up to 75 min. Respiratory related motor output was recorded throughout the CBX superfusion and used for analysis. Fictive respiratory-related neural discharges were classified based on previously described criteria, obtained from comparison of the *in vitro* motor output to fictive breathing in the less reduced decerebrate, paralyzed, unidirectionally-ventilated *in situ* preparation (Kogo et al., 1994; Gdovin et al., 1998). Fictive lung ventilation was defined as high amplitude, low frequency bursts occurring simultaneously in CN V, CN X and CN XII in the adult (CN V, CN VII and CN XII in the tadpole), having an incrementing–decrementing pattern and a duration <1 sec (Reid and Milsom, 1998). Fictive gill activity in the tadpole and buccal activity in the adult are characterized by low amplitude, high frequency oscillations. Discharges of motor output not meeting these criteria were assumed to be associated with events other than normal fictive breathing and were excluded from

analysis (Reid and Milsom, 1998; Hedrick and Winnill, 2003). In the adult bullfrog brainstem, non-respiratory bursts, which are characteristically long duration, high amplitude bursts, account for approximately 10% of all lung burst activity (Hedrick and Winnill, 2003). The addition of CBX did not affect the number of non-respiratory bursts in this preparation. For each experiment, activity from either CN V or CN X in the adult and CN V or CN VII in the tadpole, was used for analysis; there was no difference in results from the two nerves analyzed in each preparation. Burst frequency is defined as neural bursts per minute. Burst duration was measured from the onset of deviation from the baseline to the return to baseline in the integrated neural trace. Burst amplitude was measured in arbitrary units and analyzed as a percentage of control from the integrated neural trace.

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 drug administration compared with the control. Statistical significance was assumed if  $P < 0.05$ . All statistical analyses were carried out using a commercial software package (GRAPHPAD PRISM, v. 4.0 (PC version), San Diego, CA).

### 3. Results

#### 3.1. Effects of CBX on respiratory frequency

The response to CBX superfusion differed dramatically between tadpole and adult brainstem preparations. In tadpoles, 100  $\mu\text{M}$  or 1 mM CBX produced a significant slowing of lung and gill burst frequency (Fig. 1), whereas in adult preparations, CBX produced a significant increase in lung burst frequency (Fig. 2). In both tadpoles and adults, the effects of CBX (inhibition or excitation) were followed by cessation of respiratory activity in some or all preparations, depending on the concentration of CBX.

There was a dose-dependent effect of CBX on fictive breathing in tadpoles and adults, with 1 mM CBX producing faster, more pronounced

effects than 100  $\mu\text{M}$  CBX. In the tadpole, fictive lung burst frequency in the tadpole averaged  $3.6 \pm 0.6 \text{ min}^{-1}$  ( $N = 5$ ) for the 100  $\mu\text{M}$  CBX group and  $5.0 \pm 1.3 \text{ min}^{-1}$  ( $N = 9$ ) for the 1 mM CBX group under control conditions (Fig. 3A). CBX significantly slowed lung burst frequency to  $1.2 \pm 0.3 \text{ min}^{-1}$  after 30 min superfusion with 100  $\mu\text{M}$  CBX (Fig. 3A; ANOVA;  $P < 0.05$ ) and decreased to  $0.9 \pm 0.3 \text{ min}^{-1}$  after 20 min with 1 mM CBX (Fig. 3A; ANOVA;  $P < 0.01$ ). Lung burst frequency was eliminated in one of five preparations superfused with 100  $\mu\text{M}$  CBX and in nine of nine preparations superfused with 1 mM CBX (Fig. 3A). Aside from one preparation at 1 mM CBX, lung burst activity fully recovered after reperfusion with control aCSF, with no significant difference between recovery and control values (Fig. 3A).

Gill burst frequency was more sensitive to the effects of CBX than lung burst frequency. Control gill burst frequency was  $53.4 \pm 3.0$  and  $49.3 \pm 3.4 \text{ min}^{-1}$  at 100  $\mu\text{M}$  CBX and 1 mM CBX, respectively, and decreased significantly (ANOVA;  $P < 0.01$ ) after 10 min exposure to CBX (Fig. 3B). Gill burst activity was completely eliminated with 1 mM CBX by 30 min, but activity fully recovered with both concentrations of CBX (Fig. 3B).

In adult preparations, both 100  $\mu\text{M}$  and 1 mM CBX caused a significant increase in lung burst frequency after 10 min (Fig. 3C). Prolonged exposure to 100  $\mu\text{M}$  CBX caused respiratory cessation in six of eight preparations, and seven of seven preparations exposed to 1 mM CBX (Fig. 3C). Although all of the preparations exposed to 100  $\mu\text{M}$  CBX recovered, and there was no significant difference in frequency compared with control (Fig. 3C), none of the preparations exposed to 1 mM CBX recovered (Fig. 3C), despite superfusion with control aCSF for up to 3 h.

Buccal activity, present in about half of the preparations, which ranged between  $46 \pm 7 \text{ min}^{-1}$  (1 mM CBX) and  $51 \pm 9 \text{ min}^{-1}$  (100  $\mu\text{M}$  CBX), showed a trend toward decreasing frequency with CBX, but none of the changes were significantly different from control values (Fig. 3D).

## Tadpole

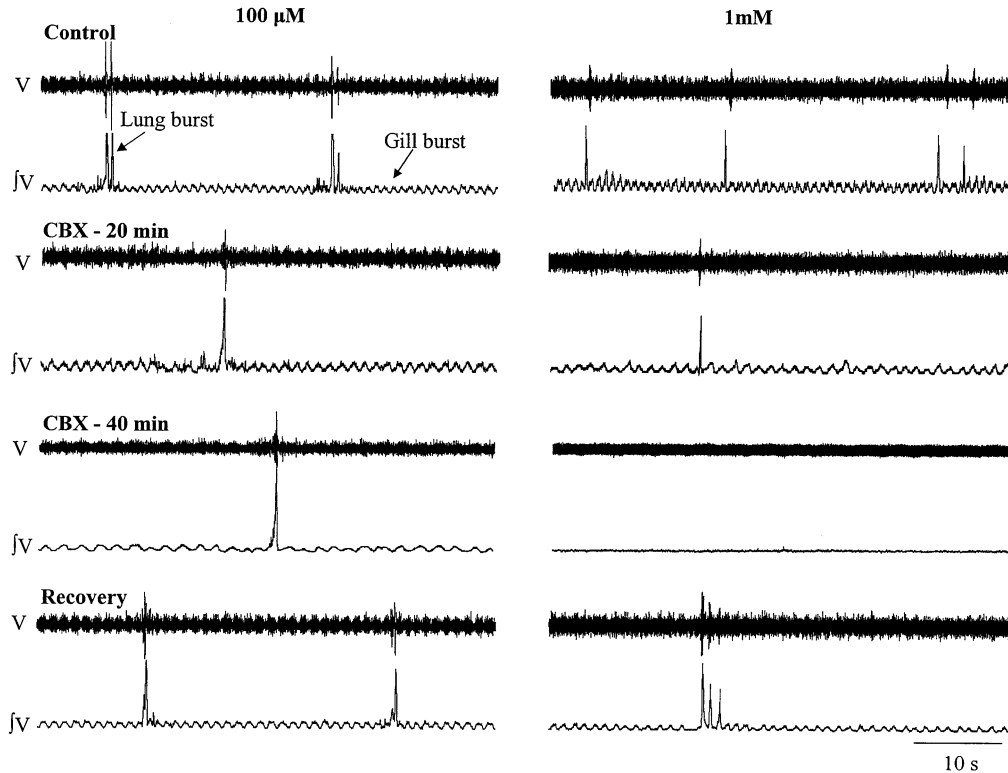


Fig. 1. Respiratory-related neural activity recorded from tadpole brainstem preparations. The effects of 100  $\mu\text{M}$  CBX (left panel) and 1 mM CBX (right panel) are shown as raw and integrated activity from CN V in two different tadpole preparations. Gill and lung burst activity are indicated in control (top), CBX superfusion after 20 and 40 min (middle), and after recovery with control aCSF (bottom). Respiratory activity initially slowed and stopped completely with 1 mM CBX, but both preparations recovered after 60–90 min washout with control aCSF.

### 3.2. Effects of CBX on burst amplitude and duration

In tadpole preparations, CBX at either concentration had no effect on the amplitude or duration of lung bursts (Fig. 4A and B). Gill burst amplitude was similarly unaffected by CBX (Fig. 4C), but gill burst duration significantly increased with 100  $\mu\text{M}$  CBX after 30 min exposure, and returned to control levels during recovery (Fig. 4D).

In adult bullfrog preparations, amplitude and duration of lung bursts were not affected by

exposure to CBX ( $P > 0.05$ ; Fig. 5A and B). High frequency, low amplitude fictive buccal bursts were present in four of seven preparations (see Fig. 3D). Buccal burst amplitude and duration were unchanged during exposure to CBX (Fig. 5C and D;  $P > 0.05$ ) and buccal activity ceased between 4 and 15 min of CBX exposure.

### 3.3. Effects of hypercapnia in the presence of CBX

Because 1 mM CBX in adult preparations resulted in an irreversible cessation of respiratory activity (see Figs. 2 and 3C), we tested the effects

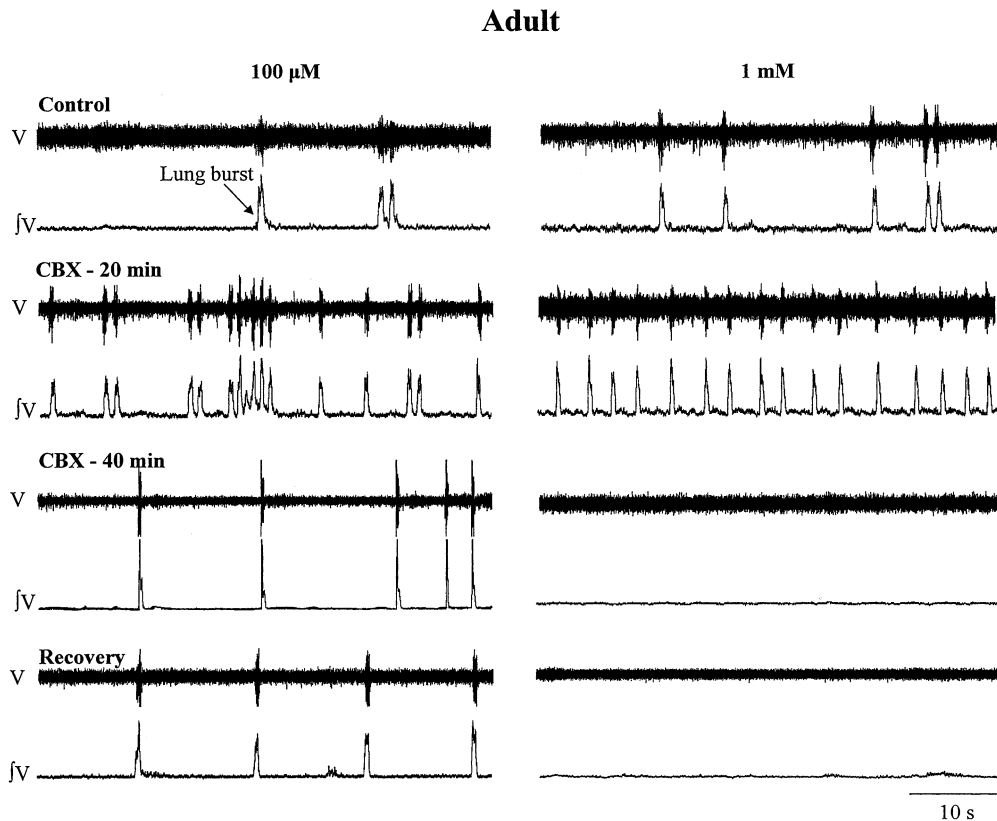


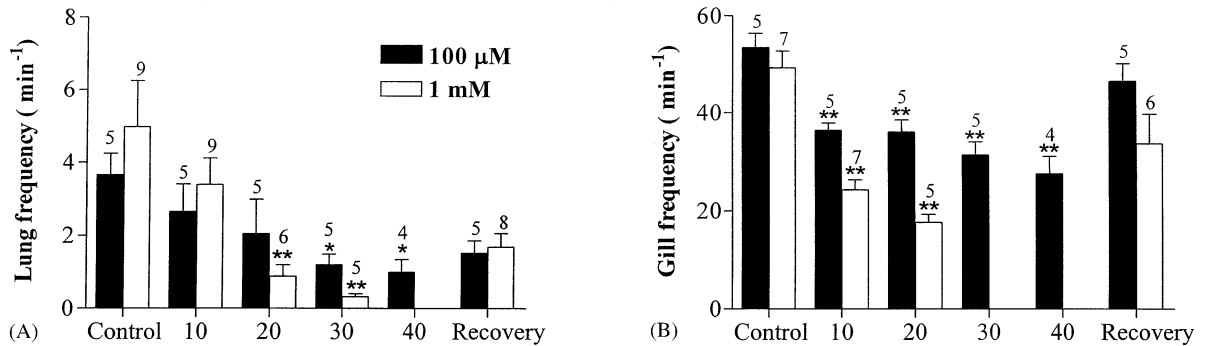
Fig. 2. Respiratory-related neural activity recorded from adult brainstem preparations. The effects of 100  $\mu\text{M}$  CBX (left panel) and 1 mM CBX (right panel) are shown as raw and integrated activity from CN V in two different adult preparations. There was a clear excitation of lung burst activity after 20 min exposure to CBX in both preparations and a subsequent cessation of activity after 40 min with 1 mM CBX. The effects of 100  $\mu\text{M}$  CBX were fully reversible in control aCSF (60 min washout), but 1 mM CBX produced an irreversible cessation of fictive breathing (recorded at 45 min) following 3 h washout.

of hypercapnia (7–10%  $\text{CO}_2$ ; pH 7.47–7.24) on two additional preparations after neural activity had ceased following the application of 1 mM CBX (data not shown). We superfused the preparation with aCSF, but raised the  $\text{CO}_2$  to 7% (bal.  $\text{O}_2$ ) for 20–30 min following the 1 mM CBX treatment. When respiratory activity failed to appear,  $\text{CO}_2$  was raised to 9–10% for an additional 30–40 min. In both experiments, respiratory activity did not resume with hypercapnia. This experiment was not done in tadpole preparations because isolated brainstem preparations from early-mid stage tadpole do not show any significant response to hypercapnia (Torgerson et al., 1997).

#### 4. Discussion

In the present study we observed significant effects on the generation of respiratory related motor output from the isolated bullfrog brainstem in response to pharmacological blockade of gap junctions. Bath application of the gap junction blocker CBX initially stimulated respiratory burst frequency in the adult while it inhibited fictive breathing in the tadpole and prolonged exposure to CBX stopped all motor output in both preparations. These results suggest that gap junctional communication is important in the generation of respiratory related motor output in bullfrogs *in vitro*, and indicate that the functional

## Tadpole



## Adult

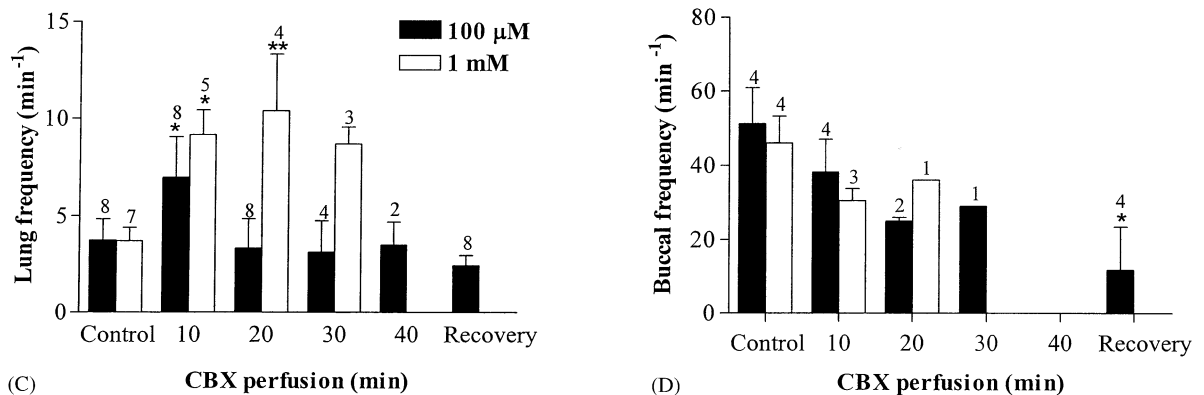


Fig. 3. Summary of effects of 100  $\mu\text{M}$  CBX (filled bars) and 1 mM CBX (unfilled bars) on respiratory-related burst frequency ( $\text{min}^{-1}$ ) in tadpole and adult brainstem preparations. (A) Tadpole lung bursts, (B) tadpole gill bursts, (C) adult lung bursts, and (D) adult buccal bursts. Lung and gill burst frequency in tadpoles significantly decreased with both concentrations of CBX, whereas lung burst frequency in adults significantly increased with CBX. Buccal frequency was unaffected by CBX. The numeral above each bar represents the number of preparations with measurable respiratory-related activity. The absence of a bar indicates no respiratory activity is present. \*  $P < 0.05$ ; \*\*  $P < 0.01$  compared with control (Dunnett's test).

role for gap junctions in respiratory rhythmogenesis changes during development.

#### 4.1. Critique of methods

In the present study we used CBX to block gap junctional communication in the isolated brainstem. Glycyrrhetic acid derivatives and related compounds (including  $18\alpha\text{-GA}$ ,  $18\beta\text{-GA}$ , and CBX) have been shown to significantly decrease gap junctional coupling and are less cytotoxic than other classes of gap junction inhibitors (Davidson,

et al., 1986; Rozental et al., 2001). CBX was selected for use in this investigation because it has been shown to be the most potent blocker of gap junction permeability among the glycyrrhetic acid derivatives and higher-order alcohol gap junction blockers (Rozental et al., 2001). Previous studies of the effects of gap junction blockers on respiratory rhythmogenesis in mammals have found CBX to be the most effective and consistent of several gap junction uncoupling agents (including glycyrrhetic acid derivatives and higher-order alcohol gap junction inhibitors) in eliciting

## Tadpole

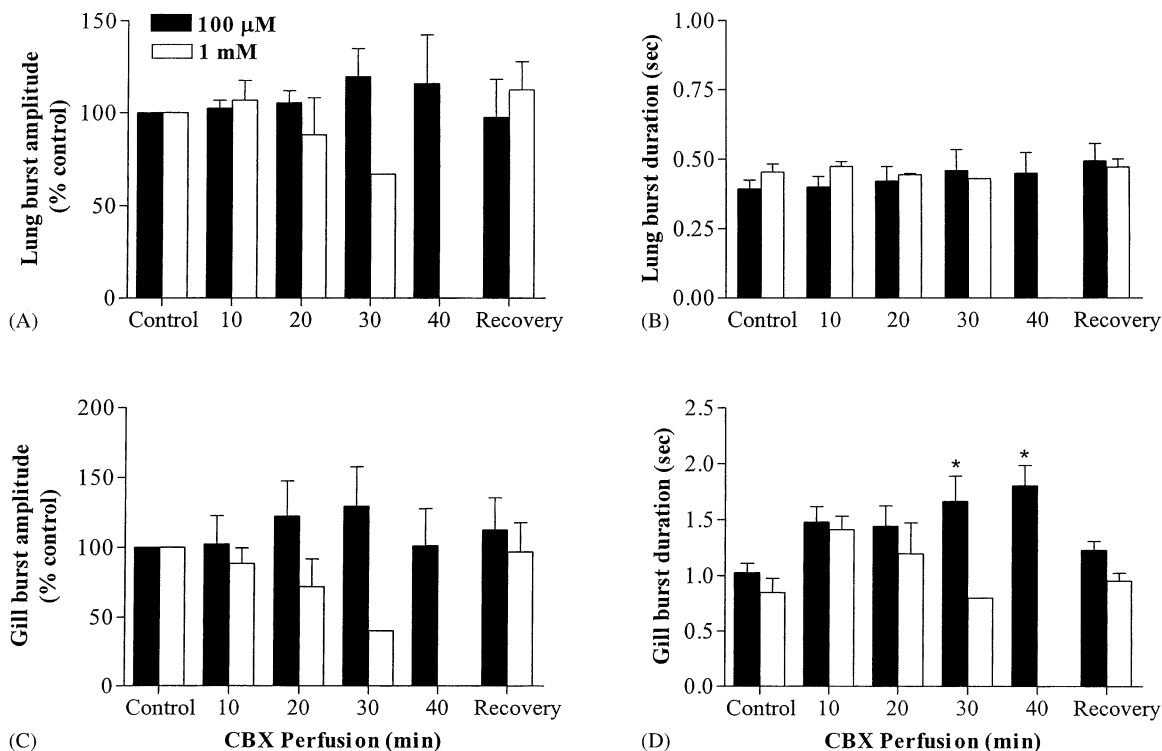


Fig. 4. Summary of effects of 100  $\mu\text{M}$  CBX (filled bars) and 1 mM CBX (unfilled bars) on burst amplitude (% control) and burst duration (sec) for tadpole preparations. (A) Lung burst amplitude, (B) lung burst duration, (C) gill burst amplitude, and (D) gill burst duration. CBX had no effect on burst amplitude or duration on gill/lung activity in tadpoles except for a reversible increase in gill burst duration after 30–40 min with 100  $\mu\text{M}$  CBX. \*  $P < 0.05$ , compared with control.

effects, and these effects were reversible even in exposures up to 45 min (Bou-Flores and Berger, 2001; Solomon et al., 2003). Furthermore, other glycyrrhetic acid derivatives must be dissolved in ethanol or dimethylsulfoxide (DMSO), which is known to have excitatory effects on respiratory motor output in the isolated amphibian brainstem (Hedrick and Morales, 1999). Thus, the use of CBX, which is soluble in aCSF, eliminates the possibility that observed effects can be attributed to the vehicle.

Investigations of the effects of CBX, both junctional and non-junctional, have shown that exposure to 100  $\mu\text{M}$  CBX does not alter resting conductance, action potential waveform or spontaneous firing in neurons of the locus coeruleus in neonatal and adult rats (Travagli et al., 1995;

Ishimatsu and Williams, 1996). However, in another recent study, Rekling et al. (2000) found that local perfusion with 100  $\mu\text{M}$  CBX lasting  $\geq 20$ –30 min, reduced input resistance and excitability of inspiratory type-1 PBC neurons in the *in vitro* neonatal rat medullary slice preparation. When dual whole cell voltage clamp is used to measure junctional conductance, 100  $\mu\text{M}$  CBX does not completely inhibit electrical coupling, but decreases it by about 4-fold (Goldberg et al., 1996; Rozental et al., 2001). In the present study we used concentrations of CBX similar to those of recent mammalian studies (Bou-Flores and Berger, 2001; Rekling et al., 2000; Solomon et al., 2003) as well as a higher concentration (1 mM). Although the direct effects of CBX on electrical coupling, input resistance and excitability have not been measured

### Adult

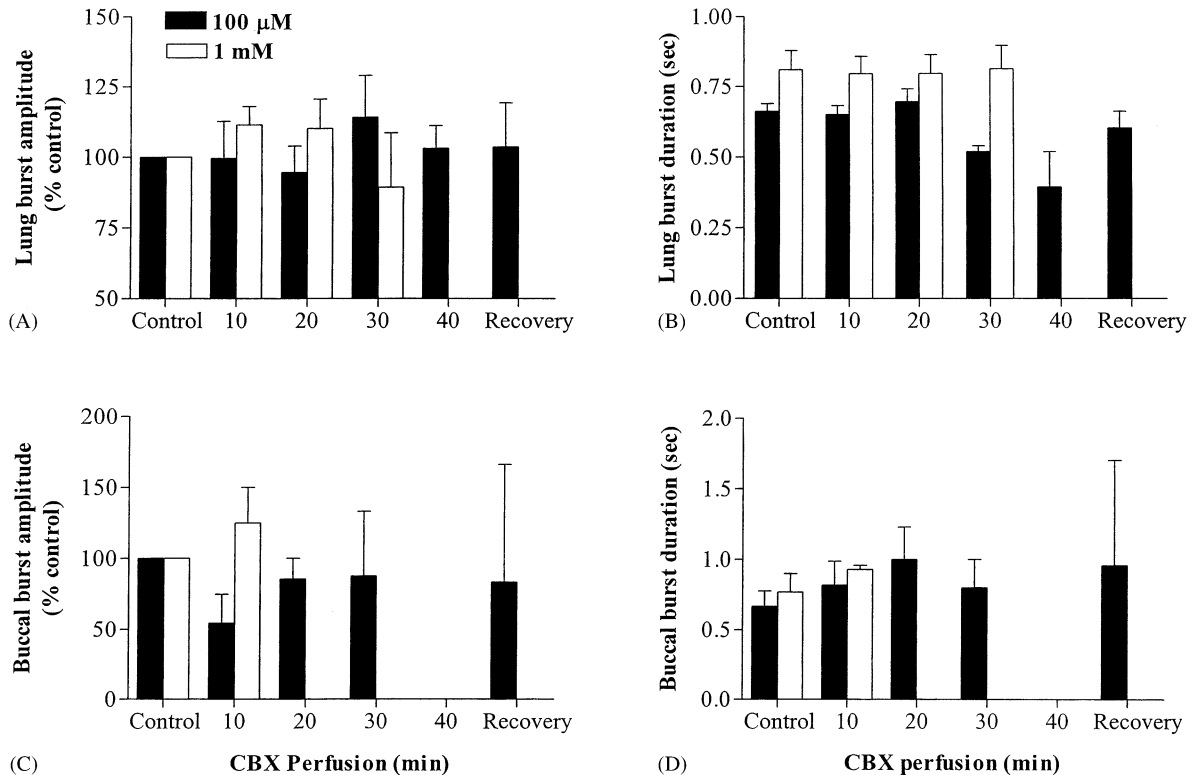


Fig. 5. Summary of effects of 100  $\mu\text{M}$  CBX (filled bars) and 1 mM CBX (unfilled bars) on burst amplitude (% control) and burst duration (sec) for adult preparations. (A) Lung burst amplitude, (B) lung burst duration, (C) buccal burst amplitude, and (D) buccal burst duration.

in amphibian neurons, our results suggest that gap junctions modulate respiratory frequency differently in tadpole and adult brainstem preparations.

The possibility that the results of the present study can be attributed to CBX-induced changes in neuronal excitability and/or cytotoxicity cannot be completely ruled out. High concentrations of glycyrrhetic acid derivatives are known to elicit cytotoxic effects (Davidson et al., 1986; Rozental et al., 2001). Although respiratory-related activity did not return following CBX superfusion in the adult with 1 mM CBX, tadpole preparations fully recovered from the same concentration of CBX, suggesting that the cytotoxic effects of CBX probably did not account for these differences, unless cytotoxicity is specific to adult preparations. Superfusion with a lower concentration of CBX

(100  $\mu\text{M}$ ) also caused cessation of respiratory activity in most adult preparations, but these preparations fully recovered from the effects of CBX. We believe that the observed effects on respiratory frequency were mediated by blockade of gap junctional communication based on the consistency of our findings with mammalian studies (see below).

#### 4.2. Role of gap junctional coupling in respiratory rhythmogenesis

Dye-coupling, electrical-coupling and freeze-fracture techniques have revealed the presence of gap junctions in motoneurons and primary afferent fibers of the amphibian spinal cord (Sonnhof et al., 1977; Brenowitz et al., 1983; Bácskai and

Matesz, 2002). Gap junctional coupling among motoneurons is thought play a role in the synchronization of firing (Bou-Flores and Berger, 2001; Tresch and Kiehn, 2002). In *Xenopus* tadpoles, gap junctional coupling has been shown to contribute significantly to the phasic excitation of motoneurons of the spinal locomotor system controlling swimming (Perrins and Roberts, 1995). We do not believe that the effects of CBX on respiratory related motor output observed in the present study involve the blockade of gap junctions between motoneurons. Because rhythm alone was altered, without changes in other burst pattern characteristics, such as amplitude and duration, suggests that CBX affected gap junctions within respiratory rhythm-generating networks.

In tadpoles, blockade of gap junctions by exposure to CBX significantly decreased lung and gill burst frequency and ultimately resulted in a reversible cessation of all respiratory related motor output with 1 mM CBX (Fig. 1). These results suggest that gap junctions may be important for enhancing respiratory rhythmogenesis in the larval bullfrog brainstem. This is consistent with the view that gap junctions are important for maturation of neural networks (Kandler and Katz, 1995). In the adult preparation, perfusion with CBX produced an increase of fictive lung burst frequency before activity ceased in most preparations. Owing to these disparate results in larval and adult preparations, we suggest there is a developmental change in the role of gap junctions in rhythm-generating networks in the bullfrog brainstem.

A developmental change in the role of gap junctions in respiratory rhythmogenesis, as indicated by our findings, is consistent with recent mammalian studies. In the neonatal mouse medullary slice preparation, Bou-Flores and Berger (2001) found that bath application of gap junction blockers decreased respiratory frequency while increasing synchronous activity within individual bursts, similar to the effects of CBX we have observed in tadpoles. By contrast, blockade of gap junctional coupling in the *in vitro* arterially perfused adult rat resulted in an increase in the frequency of bursts recorded from the phrenic nerve and reduced synchronization within bursts

(Solomon et al., 2003). Anatomical studies indicate the presence of gap junctions within the PBC of neonatal and adult rodents, which is the likely substrate for the effects of gap junctional blockade on respiratory rhythm observed in these studies (Parenti et al., 2000; Rekling et al., 2000; Solomon and Dean, 2002). Taken together, findings from the present study and recent mammalian studies indicate that modulation of gap junctional coupling may contribute to respiratory frequency control and suggest a developmental change in the functional role of gap junctions in respiratory rhythmogenesis in vertebrates.

Modeling studies have shown that the strength of electrical coupling can alter the frequency of an oscillating neuronal network. Kepler et al. (1990) demonstrated that changes in coupling strength between two coupled oscillators can either increase or decrease the frequency of bursting. Experimental evidence and mathematical models of the medullary pacemaker nucleus, controlling the electric organ in the weakly electric fish, indicate that gap junctions modulate frequency and phase-locking of oscillatory network activity, with inhibition of gap junctional coupling resulting in a decrease in burst frequency (Moortgat et al., 2000). Thus, it is possible that the developmental changes in the functional role of gap junctional communication in respiratory rhythmogenesis observed in the present study and mammalian studies may be mediated by maturational changes in the strength and/or distribution of gap junctional coupling within the rhythmogenic network. The observed increase in respiratory burst frequency with CBX in the adult bullfrog brainstem (this study) and in the adult rat (Solomon et al., 2003), may be explained by changes in coupling strength mediated by gap junctions in medullary respiratory networks.

#### 4.3. Role of gap junctions in central CO<sub>2</sub> chemoreception

There is growing evidence that gap junctions are involved in central CO<sub>2</sub> chemoreception in mammals (see Dean et al., 2002; Solomon and Dean, 2002). Central CO<sub>2</sub> chemoreceptors in the mammalian brainstem appear to be widespread and

these chemoreceptive areas correlate with Cx distribution (Solomon et al., 2001; Solomon and Dean, 2002). Furthermore, direct cell–cell coupling has been demonstrated for neurons with CO<sub>2</sub> chemosensitivity in the locus coeruleus and the solitary complex in mammals (Huang et al., 1997; Oyamada et al., 1999). Although blockade of gap junctions with CBX in neonatal rats reduces respiratory-related activity, this is not due to a loss of chemosensitivity since hypercapnia in the presence of CBX is capable of restoring fictive breathing (Dean et al., 2002).

Central CO<sub>2</sub> chemosensitivity changes with development in amphibians, with increased responsiveness to CO<sub>2</sub> as development proceeds from gill-breathing aquatic tadpoles to lung-breathing terrestrial adults (Torgerson et al., 1997; Taylor et al., 2003). Early to mid-stage premetamorphic tadpoles, as used in this study, show little or no absolute response to CO<sub>2</sub> in vitro (Torgerson et al., 1997; Taylor et al., 2003). Adult brainstem preparations in vitro respond weakly to CO<sub>2</sub> (Morales and Hedrick, 2002), or not at all (Reid and Milsom, 1998); however, the CO<sub>2</sub> response can be enhanced by stimulation of vagal afferent inputs to the CNS (Kinkead et al., 1994), suggesting that peripheral feedback is important for expression of central chemosensitivity in amphibians. In the current study, 1 mM CBX in adult preparations produced an irreversible cessation of fictive breathing; moreover, hypercapnic (7–10% CO<sub>2</sub>) aCSF applied to two adult preparations following respiratory cessation with 1 mM CBX failed to evoke fictive breathing. Although this result is not conclusive, it may suggest that gap junctions in the adult brainstem are important for full expression of central CO<sub>2</sub> responsiveness in the absence of afferent feedback. Another possibility is that CO<sub>2</sub> chemosensitivity and rhythm generating networks in the amphibian brainstem are more closely coupled than in the mammalian brainstem. Although cytotoxicity of CBX at the higher concentration cannot be ruled out, this seems unlikely given that tadpole preparations fully recovered from the same concentration of CBX. The apparent greater sensitivity to CBX in adult preparations compared with tadpole preparations in this study may suggest an increased role for gap

junctions in respiratory rhythm and chemoreceptive networks during development.

In conclusion, CBX has direct effects on the generation of respiratory related motor output in the larval and adult bullfrog in vitro. CBX inhibits fictive breathing in the tadpole, while stimulating lung frequency in the adult before ultimately stopping activity in both preparations. We hypothesize that the observed effects are mediated by the blockade of gap junctions within the respiratory rhythm-generating network and developmental changes in gap junctional coupling within this network underlie ontogenetic changes in control of respiratory frequency.

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